PL-1
MAGNETIC RESONANCE TECHNIQUES FOR IMAGING HUMAN THOUGHT

Robert Turner

Developments of MRI methods now enable brain scientists and clinicians to monitor non-invasively the perceptual and cognitive activity of the human brain, with a temporal resolution of better than 1 second and spatial resolution of 2-3 mm. The preferred technique, BOLD fMRI (blood oxygenation level dependent functional magnetic resonance imaging), uses the very fast MRI technique of echo-planar imaging (EPI). Contrast indicating brain activity is provided by naturally-occurring changes in the level of paramagnetic vascular deoxyhaemoglobin.

Hundreds of brain image volumes are normally acquired at repeat times of 1-6 seconds, while brain tasks are performed. These data are generally analyzed using robust multilinear regression techniques such as Statistical Parametric Mapping (SPM). The steps of analysis include: removal of head movement effects, spatial smoothing, and statistical inference, which includes temporal smoothing and removal by fitting of temporal variations slower than the experimental paradigm. Activation maps are generated with great flexibility and statistical power, giving probability estimates for activated brain regions based on intensity or spatial extent, or both combined. Robustness tests of BOLD fMRI have been performed, and sources of variability are becoming understood.

New combinations of brain imaging methods have recently been perfected. The simultaneous use of transcranial magnetic stimulation and fMRI, and continuous monitoring of the EEG signal during MRI scanning, will be described.

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PL-2
SENSORS, AMPLIFIERS, MOTORS AND CLOCKS IN BACTERIA

Stanislas Leibler, U. Alon, N. Barkai, P. Cluzel, M. Elowitz, M. Surette

In recent years, it has become clear that biology is facing a new challenge: to move from the description of individual components, and their mutual interactions, toward a more global, system-level analysis. I will present several examples of relatively simple collective phenomena taking place in cells. In particular, I will concentrate on detailed studies of the best known prototype systems, such as chemotaxis in bacteria, or cellular clocks that begin to uncover the underlying general "design principles".

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PL-3
EXOCYTOSIS AND ENDOCYTOSIS AT SYNAPSES AND NEUROENDOCRINE CELLS

Guillermo Alvarez de Toledo

Release of neurotransmitters and hormones by neurons and endocrine cells is a key event in cellular communication and for whole body homeostasis. The release process occurs by exocytosis, consisting in the formation of a fusion pore between the secretory vesicle membrane and the plasma membrane. For many years, exocytosis has been considered an all-or-none and irreversible event triggered by a cellular signal, which in most cases is an increase in cytosolic calcium through the opening of voltage dependent Ca^{2+} channels.

We have been applying time-resolved (milliseconds) electrophysiological techniques to monitor exocytosis of single secretory vesicles to unravel the dynamics of neurotransmitters as they exit secretory cells. Transmitter release occurs at least in two stages: 1) leak of transmitter through the nascent fusion pore, 2) bulk release when the pore has fully dilated. In addition, the fusion pore is not just a passive structure but it also can be modulated by cytosolic factors. These phases might be under cellular control, and can be aborted at any time, reversing the course of exocytosis (flicker or transient fusion). This mechanism represents the fastest and most selective way of endocytosis, helping to maintain cell integrity and rapid reuse of secretory vesicles. Depending on the phase at which exocytosis is reversed; the amount of transmitter release to the extracellular medium might be different. We propose that exocytosis can be controlled after fusion has occurred.

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PL-4
CONFORMATIONAL DYNAMICS OF NATIVE PROTEIN

Nobuhiro Go

Conformational dynamics is essential for protein molecules to carry out their function. I want to cover the following three aspects of it in my talk. (1) Its experimental detection, (2) theoretical models to describe the dynamics, and (3) mechanism of protein function based on the dynamics.

At first a series of three theoretical models, a) normal mode model, b) principal mode model, and c) JAM (jumping-among-minima) model, will be discussed. The first model emphasizes the harmonic aspect of the conformational dynamics, which is responsible for a broad range of physical properties of native proteins. However, for some other properties anharmonic aspects are conspicuous. The second model is an extention of the first model to include the effects of the anharmonicities. The third model provides further integration of the coexisting harmonic and anharmonic aspects.

Based on the principal mode model, a new method of protein X-ray crystallographic refinement has been developed. This method enables us to determine anisotropic temperature factors as a sum of internal (i.e., shape changing) and external (i.e., rigid body) contributions. By applying this refinement method to cryo-X ray data on myoglobin, a new picture about the glass transition has been obtained.

As an example of understanding protein function from its dynamics, I will discuss the case of electron transfer reaction mediated by a protein molecule.

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PL-5
DNA-PROTEIN INTERACTIONS AT THE SINGLE MOLECULE LEVEL

David Bensimon

The development of techniques to manipulate single molecules has opened a new vista on the study of DNA/protein interactions at the single molecule level. I shall review our studies of the replication of DNA by a single DNA polymerase and the relaxation of supercoiling by a single topoisomerase. Single molecule experiment allow one to recover bulk data and study with greater details some of the reaction steps in the enzymatic cycle, in particular the one involving movement of the enzyme which can be hampered by the load. This might allow one to relate structure and function with unprecedented details on the kinetics of these enzymatic machines.

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PL-6
MECHANICAL PROPERTIES OF SINGLE MYOSIN MOLECULES

J.E. Molloy, C. Veigel, F. Wang, A.E. Knight, G.I. Mashanov, L.M. Coluccio, J.R. Sellers

Many types of cellular motility, including muscle contraction, are driven by the cyclical interaction of myosin with actin, coupled to the breakdown of ATP. The best-studied family of myosins are the two-headed, filament forming, myosin-IIs found in muscles. However, there are now known to be 16 families of myosin, which share a highly conserved, N-terminal, "head" domain but have highly divergent, C-terminal, "tails". We have used an optical tweezers based transducer to measure the force, movement and stiffness produced by different myosin isoforms interacting with F-actin. One of the goals of such experiments is to observe the separate mechanical phases taking place during a single interaction event. This idea is similar in principle to identifying the separate steps of a biochemical pathway. Unfortunately, the mechanical kinetics of skeletal muscle myosin-II make this a difficult task. We hope to exploit the natural diversity of function offered by other, unconventional, myosins in order to elucidate the mechanical pathway at the level of a single interaction. For example, we used our optical tweezers to slowly distort a single myosin I cross-bridge in order to measure its force-extension property and found that the cross-bridge can be made to "slip" between two stably attached conformations, separated in position by about 5 nm.
Supported by Royal Society, BBSRC, Wellcome Trust.

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PL-7
A LONG WAY TOWARDS AN UNDERSTANDING OF A PROTON PUMP

Georg Büldt

30 years of research on the light driven proton pump bacteriorhodopsin has made this membrane protein to a model system for energy conversion and the transport of ions and signals across membranes. Concomitantly it has pushed the development of biophysical methods. The reason for this is manifested in the intrinsic properties of this protein. The time structure of the photocycle ranging from femto- to milliseconds, with consecutive intermediates separated by one order of magnitude in time, made it possible to study different processes from electronic excitation to conformational alterations of the tertiary structure. The organisation of bacteriorhodopsin into two-dimensional lattices in the natural so-called purple membrane gave rise to the pioneering work on cryo-electron microscopy on membrane proteins. Time-resolved infrared spectroscopy yielded information of the proton pathway through the molecule. The difficulties in crystallizing this protein has inspired research to find new ways to crystallize membrane proteins. A very unconventional method, the crystallization in the lipidic cubic phase, has very recently provided high resolution crystal structures of the ground state and the first intermediates K, L, and M. Our work on the late M state structure of wild-type bacteriorhodopsin is discussed in more detail in comparison to the other intermediates and a mechanism for proton pumping is proposed. It will be shown that proton transfer in the extracellular part is quite different from translocation steps across the more hydrophobic cytoplasmic domain.

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SYM-I-1
SPECTROSCOPY OF INDIVIDUAL PHOTOSYNTHETIC PIGMENT-PROTEIN COMPLEXES

J. Köhler, A.M. Van Oijen, M. Ketelaars, T.J. Aartsma, J. Schmidt

The initial steps of photosynthesis involve the transfer of energy of absorbed light to the photochemical reaction centre, where a charge separation takes place and the excitation energy becomes available in the form of chemical energy. The great difficulty to determine the various parameters that play a role in the description of the electronic structure of light-harvesting complexes is the fact that the optical absorption lines are inhomogeneously broadened as a result of heterogeneity in the ensemble of absorbing pigments.
We have circumvented this problem by studying individual LH2 complexes from *Rhodopseudomonas acidophila*. The results show that the properties of the electronically excited states of the two rings of BChl a molecules, absorbing at 800 nm and 850 nm, differ strongly. In contrast to the B800 BChl a molecules, for which the optical absorption results in electronically excited states that are localized on a few BChl a molecules, the same process leads for the B850 BChl a molecules to electronically excited states that are coherently delocalized over the eighteen BChl a molecules.

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SYM-I-2
SINGLE-MOLECULE STUDIES OF FLUORESCENT PROTEINS AND MOLECULAR MOTORS

W.E. Moerner[1], S. Brasselet[1], B. Lounis[1], E.J.G. Peterman[1], H. Sosa[2], L.S.B. Goldstein[2]

Single-molecule optical techniques have now begun to find applications far beyond the early studies in solids at cryogenic temperatures. In the case of biomolecules at room temperature, enhanced versions of optical microscopy such as total internal reflection, epifluorescence, and confocal microscopy all allow observation of single fluorophores in aqueous environments. We utilize these methods to explore the behavior of several proteins and enzymes, in particular, a protein construct based on mutants of the green fluorescent protein (GFP) and single fluorescently-labeled kinesin molecular motors. For a dual-GFP construct designed to sense local calcium concentrations by fluorescence resonant energy transfer ("cameleon", A. Miyawaki and R.Y. Tsien), we observe a variety of fluctuations which may be ascribed to calcium ion binding as well as to orientation changes of the fluorophores. For the molecular motors, single-molecule polarization studies are facilitated by bifunctional attachment of the fluorophore to the protein. These studies provide new information about the conformations of the system in various nucleotide binding states. The possible future applications of single-molecule methods to biological problems may explore issues such as heterogeneity, kinetics, and conformational changes in a variety of situations.

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SYM-I-3
ACTIVITY FLUCTUATIONS, ENERGY LANDSCAPES, MEMORY AND CATALYSIS IN SINGLE ENZYME MOLECULES

Rudolf Rigler, Zeno Földes Papp, Lars Edman

Combining confocal single molecule detection and fluorescence correlation spectroscopy we were able to show that the turnover of non fluorescent substrate molecules into a fluorescent product by individual horseradish peroxydase molecules involves a spectrum of conformational transitions to reach the catalytically competent state (1). This pathway can be envisaged as a trajectory through a multidimensional landscape of activation barriers leading to catalysis. Experiments in which catalysis of single enzyme molecules is followed in different stages of unfolding/folding indicate that the energy landscape is beeing dependent on the state of folding/unfolding.It is proposed that the scenario of conformational substates is an equilibrium distribution of conformers populated during the folding process. From the analysis of higher order correlation functions we have found evidence for the existence of non Markovian states which relax into a regime whith no memory of its past history(2). Their relation to conformational transitions and catalysis will be discussed.
(1) Edman, L., Földes Papp, Z., Wennmalm, S. and Rigler R. (1999) Chem Phys.247,11-22
(2) Edman, L. and Rigler, R. (2000) Proc. Natl. Acad. Sci. US in press

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SYM-I-4
SINGLE-MOLECULE MICROSCOPY OF MEMBRANE PROTEINS

Thomas Schmidt

The lateral and rotational mobility of proteins is believed to play a key role in cellular signal transduction and might have influence on electrophysiological response. However, the time-scales and modes at which such mobility occurs remains unknown, except for a small number of cases. By single-molecule imaging, single-molecule anisotropy, and single-molecule correlation spectroscopy, we are now able to unveil new information about the stoichiometry, the lateral and the rotational mobility of fluorescence-labeled fusion-proteins (including GFP labeled lipids) in membranes under physiological conditions.

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SYM-II-1
SINGLE MOLECULE MECHANICS, SMALL CANTILEVERS AND ATOMIC FORCE MICROSCOPY

Paul Hansma

Small cantilevers, with dimensions of order $12 \times 5 \times 0.1$ microns have spring constants of 60 pN/nm resonant frequencies of order 200 kHz, force noise of 1.3 pN, position noise of 0.02 nm for a 3 kHz bandwidth in aqueous solutions. This resonant frequency is about 25 times higher, the force noise and position noise are both about a factor of 5 lower than the noise in a commercial cantilever with comparable spring constant.
They can monitor a process, association and dissociation of complexes of GROES with GROEL, that operates, using ATP as fuel, with a power consumption of order 7×10^{-20} Watts. Histograms of the complex lifetimes do not follow a simple exponential decay, but peak near 5 seconds.
Pullling experiments with the AFM reveal the secret that accounts for the remarkable fracture toughness of abalone shells: 3,000 times the fracture toughness of pure calcium carbonate single crystals, which make up 97% of the shell's mass.

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SYM-II-2
DYNAMIC FORCE MICROSCOPY AND SPECTROSCOPY FOR HIGH RESOLUTION BIOMOLECULAR STUDIES

Mervyn J. Miles, Massimo Antognozzi, Terence McMaster, Javier Tamayo

Low force interactions between the AFM probe and the specimen are particularly important in imaging delicate biomolecular structures. In conventional AFM, tapping mode has reduced the shear force applied by the probe during scanning, and imaging in liquid environments has not only reduced the normal capillary force but also allowed the observation of processes brought about by the introduction of active molecules into the liquid environment. However, a serious disadvantage of operating tapping mode in liquid is the loss of force and phase sensitivity due to damping of the cantilever oscillations by the surrounding liquid. Recent developments in enhancing the quality factor of the cantilever have overcome this problem and the resulting higher resolution images from biomolecules to cells will be presented. The same technique has been used to improve the sensitivity of single molecule force spectroscopy. An alternative low-force technique for both imaging and spectroscopy is transverse dynamic force microscopy, which can operate in true non-contact, resulting in extremely low force interactions, ideal for imaging soft biological systems. This technique has also several significant advantages for force spectroscopy studies.

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SYM-II-3
CONFORMATIONAL CHANGES, FLEXIBILITIES AND INTRAMOLECULAR FORCES OBSERVED ON INDIVIDUAL PROTEINS

Daniel J. Müller, Filipp Oesterhelt, Norbert Dencher, Holger Seelert, Henning Stahlberg, Hermann Gaub, Andreas Engel

Atomic force microscopy (AFM) allows the surfaces of individual proteins to be imaged in aqueous solution with a lateral resolution < 0.5 nm and a vertical resolution < 0.1 nm which is sufficient to directly observe both, tertiary structural arrangement and secondary structural elements of native proteins. Conformational changes of individual proteins were observed by time-lapse AFM and multivariate statistical classification unraveled major conformations of their variable structural elements. We further report on the possibility to image protein complexes before and after the removal of individual subunits using the AFM tip as a nanotool. Based on these results, single molecule imaging and single molecule force-spectroscopy capabilities of the AFM have been combined providing novel insights into the inter- and intramolecular interactions of proteins. Applied to membrane proteins, this technique allows to measure forces required to unfold secondary structural elements and to record unfolding pathways of individual proteins.
D.J. Müller, D. Fotiadis, S. Scheuring, S.A. Müller, A. Engel *Biophys. J.* (1999) 76:1101.
H. Seelert, A. Poetsch, N. Dencher, A. Engel, H. Stahlberg, D.J. Müller *Nature* (2000) in press.
D.J. Müller, W. Baumeister, A. Engel *Proc. Natl. Acad. Sci. USA* (1999) 96:13170.
F. Oesterhelt, D. Oesterhelt, M. Pfeiffer, A. Engel, H. Gaub, D.J. Müller *Science* (2000) 288:143.
M.E. Müller-Institute, Biozentrum, University of Basel, 4056 Basel, Switzerland

SYM-II-4
NANOSCALE SPECTROSCOPIC CONTRAST IN NEAR-FIELD MICROSCOPY

Fritz Keilmann

Microscopy is traditionally restricted to the visible spectrum, because the resolution deteriorates with longer wavelength. On the other hand, compound-specific spectroscopic contrast such as "fingerprint" vibrational absorption - well established as the FTIR analytical tool - is restricted to long-wavelength infrared. Here we demonstrate that microscopy and spectroscopy can indeed be married.
Scattering from a sharp probe tip is a new way of performing SNOM (scanning optical near-field microscopy). The tip and its shaft can be regarded as an optical antenna. The spatial resolution of such a (scattering) s-SNOM is given by the curvature radius of the tip apex, and therefore independent of the wavelength.
Practical experimental configurations use standard tapping-mode AFM with metallized tip, and a superimposed optical channel consisting of a focussed visible or infrared beam and a detector for the scattered radiation. The signal is demodulated at the tapping frequency or its harmonic [1] to suppress background. Imaging in the mid-infrared has demonstrated contrast due to vibrational absorption, with below 100 nm resolution and SERS-like absorption enhancement [2], and contrast due to semiconductor electrons with 30 nm resolution. Imaging in the visible has been done with an interferometric detection system allowing nanoscale images simultaneously in amplitue and phase contrast [3], and has resolved the detailed optical response near individual 30 nm Au colloids.
1 B. Knoll, F. Keilmann, subm.,
2 B. Knoll, F. Keilmann, Nature 399, 134 (1999),
3 R. Hillenbrand, F. Keilmann, subm.

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SYM-III-1
PROTEIN FOLDING, EVOLUTION AND DISEASE
Christopher M. Dobson

There has recently been considerable progress in understanding the fundamental principles that govern protein folding both *in vitro* and *in vivo*. Of particular importance for this progress has been the concerted application of a wide range of experimental techniques each able to describe aspects of the structural changes taking place during the folding process. Recently, research has also focussed on the question of what happens if proteins do not fold correctly, or if they find themselves in an environment where at least partial unfolding takes place. We have been investigating in particular the nature of protein fibrils of the type associated with amyloidogenic diseases including those fomed from c-type lysozymes. These proteins have been important model systems for studying fundamental aspects of folding, and the discovery that clinical cases of amyloidosis are connected with single point mutations in the human lysozyme gene has enabled us to explore the molecular basis of this disease in a well defined system. This work has recently been extended by the discovery that many proteins not associated with clinical manifestations of disease can form amyloid fibrils in the laboratory under specific conditions. This has enabled the fundamental nature of the structure and mechanism of formation of these fibrils to be explored in detail. This talk will report recent results from our laboratory and the significance of these for understanding protein folding and its links with human disease and biological evolution.

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SYM-III-2
MECHANISMS OF PROTEIN FOLDING ASSISTED AND UNASSISTED BY CHAPERONINS
Sheena E. Radford

To understand how a protein folds information is needed about the structure and stability of intermediate partially folded states (if they exist) and the transition states that separate them, and the kinetics of the various steps involved. Current models of folding have arisen from detailed biophysical studies of the folding of a number of model proteins. Important questions that remain include (1) whether intermediate partially folded states are important species that guide folding molecules to the native state or are misfolded states that prevent or slow folding to the native state; and (2) the relevance of folding studies performed *in vitro* to the 'real' situation of protein folding in the cell. In this lecture I will discuss these issues using lysozyme (a two domain α/β protein) and the bacterial immunity proteins (which are small helical bundle proteins). In addition, we have used the techniques commonly applied to protein folding studies *in vitro*, to investigate the relationship between protein folding mechanisms assisted and unassisted by GroEL. Finally, using peptide models we have addressed the ephemeral question of the nature of the molecular recognition event between the GroEL apical domains and their protein substrates. Taken together, a view for GroEL-assisted folding involving binding of partially folded states, reorganisation of non-native interactions by conformational rearrangements of elements of secondary structure, but not by global unfolding, and facilitation of the rate limiting steps in folding that involve the formation of the correct tertiary interactions that define the close packed native state.

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SYM-III-3
STRUCTURES AND MECHANISMS OF MOLECULAR CHAPERONES
Johannes Buchner

Protein folding is known to be a spontaneous reaction. In the cell a complex machinery exists which assists the folding process. These proteins, collectively called molecular chaperones, are present at high concentrations under physiological conditions. As members of the heat shock protein (Hsp) family their levels are significantly increased after exposure to stress. Some chaperones exhibit a complicated architecture such as the GroE double ring or the hollow sphere of small Hsps. Using in vitro protein folding assays we have analysed the interaction of different classes of molecular with nonnative proteins. Small Hsps are the most efficient chaperones in this context. However, their function seems to be restricted to binding thus preventing further irreversible folding reactions. This is especially important under heat shock conditions where large numbers of polypeptides unfold. Consistent with this idea, we found that the yeast small Hsp Hsp26 is activated by dissociation under these conditions. In other chaperone systems, further processing of the bound protein requires ATP-induced conformational changes and, in many cases, the association with cofactors. For the GroE system these reactions lead to the formation of a cavity in which a single protein molecule can fold. However, our results show that this encapsulation does not guarantee that the native state is reached. Defining the folding trajectories a protein can take with the help of chaperones will be important to understand both molecular chaperones and protein folding.

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SYM-III-4
MOLECULAR CHAPERONES IN PROTEIN FOLDING IN THE CELL
Sarah Teter, Walid A. Houry, F. Ulrich Hartl

Although the folded structure of a protein is determined by the information contained in its amino acid sequence, efficient realization of this information *in vivo* may require the assistance by molecular chaperones, at least for a significant fraction of newly-synthesized polypeptides. *De novo* folding in the cytosol generally depends on chaperones of the Hsp70 family and on the cylindrical chaperonins. According to our current model, Hsp70 binds to nascent polypeptides on ribosomes, preventing misfolding until all the information required for productive folding is available. In *E. coli*, Hsp70 (DnaK) cooperates with the ribosome-bound chaperone and prolylisomerase trigger factor (TF) in protecting nascent chains. The combined deletion of DnaK and TF is lethal, indicating that these components provide an essential function. While most proteins appear to fold rapidly upon release from this first set of chaperones, a subset of polypeptides must subsequently be transferred to a chaperonin for folding to the native state. The chaperonins are also essential for cell growth. They form large cylindrical complexes that bind unfolded polypeptides, up to ~60 KDa in size, in their central cavity. As shown for the *E. coli* chaperonin GroEL, the dome-shaped co-factor GroES caps the opening of the cylinder, resulting in the displacement of bound polypeptide into an enclosed folding cage, a mechanism that effectively prevents protein aggregation during folding.

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SYM-IV-1

DISTANCE DETERMINATION BY CW AND PULSED EPR OF NATURAL AND/OR CONSTRUCTED RADICAL SITES AS A TOOL TO INVESTIGATE PROTEIN RELAXATION AND PROTEIN DYNAMICS ASSOCIATED WITH FUNCTION

Arnold J. Hoff[1], Sergei A. Dzuba[2], Igor V. Borovykh[1], P. Gast[1]

In the past few years a number of groups, including ourselves, have developed EPR techniques to determine distance with great accuray (better than 0.3 Å up to distances of 50 Å) between narurally ocurring and artificially constructed radical sites for a number of proteins, including but not limited to, electron transport proteins and proton pumps. These techniques offer a window on structural changes in the protein induced by, or occurring concomitant with, its functioning. For example, it has been demonstrated that in protein dynamics a tier of hierarchies exist that become active on raising the temperature, suggesting a landscape of self-similar potential energy barriers (S.A. Dzuba, P. Gast and A.J. Hoff, Chem. Phys. Lett. 268, 427–433). Other applications include investigations of light-induced structural changes in a bacterial photosynthetic reaction center (I.V. Borovykh, S.A. Dzuba, I.I. Proskuryakov, P. Gast and A.J. Hoff, Biochim. Biophys. Acta 1363 (1998) 182–186; Zech, S.G., Bittl, R., Gardiner, A.T. and Lubitz, W. Appl. Magn. Reson. 13 (198) 517–529), and work on nitroxide-labelled bacteriorhodopsin (primarily by the groups of Hubbell and Steinhoff). We will review these and other applications, and discuss exciting novel possibilities opened up by applying high-frequency EPR techniques.

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SYM-IV-2

LIGAND BINDING AND CONFORMATIONAL MOTIONS IN MYOGLOBIN

G. Ulrich Nienhaus*‡, Andreas Ostermann†, Robert Waschipky*, Fritz G. Parak†

Proteins are complex physical systems. Within their properly folded, native states they can assume a huge number of slightly different structures (conformational substates), nearly isoenergetic minima in a rough energy landscape with a typical roughness of 10 kJ/mol. At ambient temperature, proteins fluctuate continuously among substates; these fluctuations are essential for carrying out functional processes. Myoglobin, a small globular heme protein that reversibly binds small ligands such as O_2, and CO at the heme iron, has been studied extensively as a model system for the interplay among structure, dynamics and function in proteins. We have used cryocrystallography in combination with laser excitation to compare the structural changes that occur upon ligand dissociation in a carbonmonoxy-myoglobin (MbCO) crystal below and above the dynamical transition around 180 K. Moreover, using time-resolved infrared spectroscopy, we have correlated the observed structural changes with kinetic processes. Upon photodissociation below 180 K, CO is shown to rebind from multiple sites within the heme pocket in an essentially immobilized, frozen protein. Above 180 K, the protein structure acquires flexibility, it fluctuates and relaxes. Concomitantly, CO binding slows significantly, as the ligands escape from the heme pocket into other internal cavities and the surrounding solvent.

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SYM-IV-3

BIOMOLECULE-SOLVENT INTERACTIONS AND DYNAMICS STUDIED BY MAGNETIC RELAXATION DISPERSION

Bertil Halle, Vladimir P. Denisov, Michael Gottschalk, Kristofer Modig, Silke Wiesner

This lecture describes multinuclear (^1H, ^2H, ^{17}O, ^{23}Na) magnetic relaxation dispersion (MRD) studies of the interaction of solvent species (water, organic co-solvents, ions) with proteins and oligonucleotides. After an introduction to the MRD methodology, several recent studies performed in our laboratory will be summarized. Topics to be discussed include hydration of denatured and molten globule proteins, hydration of fatty acid binding proteins and of bacteriorhodopsin, and ion binding in the minor groove of B-form DNA.

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SYM-IV-4

CONFORMATIONAL CHANGES IN BACTERIORHODOPSIN REQUIRED FOR PROTON PUMPING

Pal Ormos+, Loránd Kelemen+, Hans Jürgen Sass*, Dominic Hehn*, Dirk Neff*, Joel Berendzen§, Georg Büldt*

Motions of the light absorber retinal and the protein are considered crucial for the proton transport in *Bacteriorhodopsin* (bR). With spectroscopic techniques (FTIR and VIS) we have yielded important information about these motions. However, the complete atomic structure of the protein in different functional states is necessary for an understanding of the proton translocation process. Single crystals of bR were grown in the bicontinuous lipidic phase enabling X-ray crystallography at high resolution. By FTIR spectroscopy it was shown that bR in these crystals is fully active, a prerequisite for structure determination of intermediate states. During the pumping cycle a characteristic conformational alteration is essential for switching the accessibility of the retinal Schiff base between the two sides of the membrane. We have determined for wild-type bR the protein structure in the late M intermediate, which follows this key conformational switch. Structures before and after the switch show major changes: 1. A hydrogen bond network connecting the Schiff base to the extracellular side is disrupted. 2. Following the motion of helices F and G cavities open up in the cytoplasmic domain. Reprotonation of the Schiff base occurs through one of these cavities, involving water molecules connecting amino acid residues Asp-96 and Ala-215 and a fluctuating water molecule.

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SYM-V-1
MODULATION OF K^+ CHANNEL GATING BY EXTERNAL K^+ AND STABILISATION OF THE VOLTAGE SENSOR

José López-Barneo, Patricia Ortega-Sáenz, Antonio Castellano

Gating of voltage-dependent K^+ channels is influenced by the permeating ions. Extracellular K^+ determines the occupation of sites in the channels where the cation interferes with the motion of the gates. When external [K^+] decreases, some K^+ channels open too briefly to allow the conduction of measurable current. We have shown that residues T449 and D447 in the pore region of *Shaker* K^+ channels regulate channel activity and occupation by external cations. Given that extracellular K^+ is normally low, we have also studied if negatively charged amino acids in the extracellular loops of *Shaker* K^+ channels contribute to increase the local [K^+]. Surprisingly, neutralisation of the charge of most acidic residues has minor effects on gating. However, a glutamate residue (E418) in the membrane spanning segment S5 is absolutely required for keeping channels active at the normal external [K^+]. E418 is conserved in all families of voltage-dependent K^+ channels. Although the channel mutant E418Q has kinetic properties resembling those produced by removal of K^+ from the pore, it seems that E418 is not simply concentrating cations near the channel mouth but it has a direct and critical role in gating. E418 appears to stabilise the conductiving vs. closed and conductiving vs. C-inactivated states of the channels thus preventing the collapse of the K^+ conductance. E418 seems to contribute to link channel permeation and activation and inactivation gating.

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SYM-V-2
VOLTAGE-GATED P/Q-TYPE CALCIUM CHANNELS: SINGLE CHANNEL BIOPHYSICAL PROPERTIES AND MIGRAINE

Daniela Pietrobon

Mutations in the human gene encoding α_{1A}, the pore-forming subunit of neuronal voltage-gated P/Q-type calcium channels, are linked to several human diseases including familial hemiplegic migraine (FHM), episodic ataxia type-2 (EA-2) and spinocerebellar ataxia type 6 (SCA6). By combining single-channel and whole-cell patch-clamp recordings, we studied the effect of four missense mutations linked to FHM on the biophysical properties of human recombinant P/Q-type calcium channels expressed in HEK293 cells. Two of the mutations located in the pore region, T666M and V714A, decrease the unitary conductance, and T666M affects both the selectivity and the pH dependence of the channel. Strikingly, the reduction in single channel conductance was not observed in some patches or periods of activity, suggesting that the abnormal channel may switch on and off. Mutations V714A and I1811L in the pore region and R192Q in IS4 increase the channel open probability, po. Human P/Q channels show three modes of gating with different open/closed time distributions, different voltage-dependence of po, different kinetics and voltage-dependence of inactivation. Mutation I1811L increases po by affecting the equilibrium between gating modes. All four mutations affect the density of functional channels, which is decreased by the three mutations in the pore, but increased by mutation R192Q. According to our data the FHM mutations can lead to both gain- and loss-of-function of human P/Q-type calcium channels.

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SYM-V-3
DOUBLE-BARRELED STRUCTURE OF THE MUSCLE CL^-CHANNEL, CLC-1

Michael Pusch

The prototype CLC-0 *Torpedo* Cl^- channel is a dimer with each subunit forming a pore that can gate independently from the other pore. A common slower gate acts on both protopores. Finding such a "double-barreled" structure for other ClC-channel has been hindered by a small single channel conductance and complex gating. We demonstrated a double-barreled appearance for the muscle channel CLC-1 using single-channel recording. Bursts of the two protopores are, however, less well defined in CLC-1 compared to CLC-0 because the slow gate is only about 3-fold slower than the fast gate. We also developed envelope protocols to study the two gates of ClC-1 over a wide voltage range and found a fast gate time constant of ~15 μs at 200 mV and a "slow" one of ~1 ms. Both time constants have exponential voltage dependence for $V > -50$ mV reaching a plateau in the tens of ms range at negative voltages. Both processes were affected in a qualitatively similar manner by several experimental manipulations suggesting that they are strongly coupled. The S(-) enantiomer of clofibric acid (CPP(-)) is known to strongly block in a stereoselective manner the muscle Cl^- conductance. We found that CPP(-) produces a voltage-dependent block of CLC-1 with a maximal K_D of ~40 μM at −80 mV. The effects CPP(-) on CLC-0 and a mutant of it indicate that CPP(-) acts mainly on the fast gate of single protochannels. Together, these results strongly suggest that CLC-0 and CLC-1 share a dimeric double-barreled structure.

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SYM-V-4
PACEMAKER ION CHANNELS: CHARACTERIZATION OF A NEW ION CHANNEL FAMILY

R. Seifert, R. Gauß, A. Scholten, W. Boenigk, F. Müller, U. B. Kaupp

Pacemaker currents control rhythmic activity of heart cells and of many neurons as they provide a depolarizing inward Na^+ current upon hyperpolarization of the cell membrane. These currents have been studied in great detail by physiologists who named them "funny currents" (If) or "queer currents" (Iq) because of their unusual electrophysiological properties.

Recently the genes for the underlying "pacemaker" channels have been cloned from vertebrates and invertebrates. At least four different genes exist in mammals. The channels have been termed HCN channels which stands for Hyperpolarization – activated and Cyclic Nucleotide – gated channels.

The members of this multigene family differ in their activation kinetics and in the voltage range of activation. Here we report the characterization of different HCN isoforms with respect to their activation and ion conduction properties. Large differences exist especially in the regulation of channel activity by cyclic nucleotides. While vertebrate channels mainly shift their activation curve to more positive potentials upon binding of cAMP the HCN channel from sea urchin greatly increases its maximal open probability when cAMP is bound.

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SYM-VI-1
CRYSTAL STRUCTURE OF CALCIUM PUMP OF SARCOPLASMIC RETICULUM

Chikashi Toyoshima, Masayoshi Nakasako, Hiromi Nomura, Haruo Ogawa

Calcium ATPase is a representative member of P-type ATPases that transport ions across the membrane against concentration gradient. The crystal structure of the calcium ATPase of sarcoplasmic reticulum was solved by X-ray crystallography at 2.6 Å resolution with two calcium ions bound in the transmembrane domain consisting of 10 α-helices. The two calcium ions are located side by side surrounded by 4 transmembrane helices, two of which are unwound to realise efficient co-ordination geometry. The cytoplasmic region consists of 3 well-separated domains, with the phosphorylation site in the central catalytic domain and the adenosine binding site on another. The phosphorylation domain has the fold of L-2 haloacid dehalogenase. The atomic model was fitted to an 8 Å-resolution density map of the enzyme in the absence of Ca2+ and the presence of vanadate (Zhang et al., *Nature* 392, 835-839 (1998)). The density map was very well explained by large domain motions.

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SYM-VI-2
DISSECTION OF THE FUNCTIONAL DOMAINS OF THE SR CA^{2+}-ATPASE BY SITE-DIRECTED MUTAGENESIS

Jens Peter Andersen

The sarcoplasmic reticulum Ca^{2+}-ATPase has been expressed in COS cells to levels higher than 200 pmol ATPase/mg total membrane protein, permitting extensive structure-function analysis by site-directed mutagenesis. The mutant Ca^{2+}-ATPases have been examined by a panel of assays comprising Ca^{2+}-transport, Ca^{2+}-occlusion, ATP-binding and -hydrolysis, phosphoenzyme formation from ATP and P_i, dephosphorylation kinetics, and sensitivity to the inhibitors vanadate, thapsigargin, CPA, and BHQ. Thereby, mutational effects on the partial reaction steps of the enzyme cycle have been determined. Recently, we have applied rapid kinetic analysis on a millisecond time scale to study conformational transitions and the rate of Ca^{2+} dissociation at 25 °C. While the residues involved directly in ligation of ATP and Ca^{2+} are located in the cytoplasmic head and the transmembrane sector, respectively, the stalk sectors S3, S4, and S5 play crucial roles in the intramolecular communication between the ATP- and Ca^{2+}-sites. Our findings point to a mechanism in which S5 is instrumental in conveying information about the occupancy of the ion binding sites to the phosphorylation domain, controlling phosphorylation and dephosphorylation, whereas S4 is involved in transmission of ATP-derived energy required in the membrane domain for Ca^{2+} translocation. Residues at the M3S3 boundary are important for the rates of binding and dissociation of Ca^{2+}, suggsting a role in gating of the pathway for ion movement.

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SYM-VI-3
INTRAMOLECULAR SIGNAL TRANSDUCTION WITHIN BETP, AN BACTERIAL OSMOSENSOR AND OSMOREGULATOR

Reinhard Krämer, Susanne Morbach

BetP is the major uptake system for compatible solutes (i.e. osmoprotectant substances) in the Gram-positive *Corynebacterium glutamicum*. It catalyzes membrane potential-driven cotransport of glycine betaine with two sodium ions. BetP has a molecular mass of 64.2 kDa, the membrane part consists of 12 transmembrane domains and two hydrophilic extensions, a negatively charged N-terminal and a positively charged C-terminal domain. The transport activity of BetP is strictly dependend on osmotic stress. The carrier is inactive in the absence of osmotic challenge and becomes activated within a ms time scale in response to an osmotic upshift. We are interested in the molecular basis of osmosensing of this membrane embedded carrier protein, as well as in the intramolecular signal transduction from sensory domains to the catalytic part of the transporter. By measuring structural and functional properties of wild type and recombinant forms of BetP, we were able to identify signal input pathways leading to modulation of transport activity. On the one hand, by using spectroscopic and molecular techniques the N- and C-terminal domains of BetP have been shown as being involved in the signal input from the two hydrophilic phases during osmosensing. On the other hand, manipulation of the physical state of the hydrophobic surrounding, e.g. by addtition of local anesthetics to the phospholipid bilayer, also confers signal input. Structural and functional aspects of conformational events during osmosensing and osmoregulation by the glycine betain carrier BetP will be discussed.

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SYM-VI-4
AFFINITY, SPECIFITIY, DIVERSITY – FUNCTION OF THE ABC-TRANSPORTER TAP IN CELLULAR IMMUNE RECOGNITION AND VIRUS ESCAPE STRATEGIES

Robert Tampé

Cytotoxic T-cells distinguish between self and non-self by monitoring peptides presented in association with MHC class I molecules on the cell surface. These peptides are generated in the proteasomal degradation pathway and have to cross the membrane of the endoplasmic reticulum (ER) for chaperone-assisted loading onto class I molecules. The ABC- (ATP Binding Cassette) transporter associated with antigen processing (TAP) performs this task. By using various biochemical and biophysical approaches the translocation mechanism of TAP was dissected into ATP-independent substrate binding steps, which synchronize ATP hydrolysis and substrate translocation. By using combinatorial peptide libraries, the recognition principle of TAP has been deciphered. Interestingly, several herpes viruses escape immune surveillance by blocking TAP function.

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SYM-VII-1
PROTON SOLVATION AND PROTON MOBILITY

Noam Agmon

Experimental evidence for proton solvation and proton mobility in water is analyzed and the results are compared with recent simulations.
Differences in proton solvation energies may be understood by considering the strength of first-shell hydrogen-bonds solvating the protonated cluster, which may be estimated from their length using experimental attributes. In dilute acidic aqueous solutions the protonated water monomer is just slightly more stable than the protonated dimer (by ca. 0.6 kcal/mol).
Thus isomerization between these two structures is a key step in proton mobility. The rate limiting step is postulated to involve cleavage of a second shell hydrogen-bond. This appears to be supported by quantal simulations. Hydroxide mobility may occur by a similar mechanism, but the proton in the deprotonated dimer is not equally shared by the two water molecules, and this contributes to increase the barrier for hydroxide mobility.

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SYM-VII-2
H-BONDED NETWORK PROTON TRANSFER VIA INTERNAL WATER MOLECULES IN MEMBRANE PROTEINS AS REVEALED BY TIME RESOLVED FTIR DIFFERENCE SPECTROSCOPY

Klaus Gerwert

The light driven proton transfer in bacteriorhodopsin is investigated by time resolved FTIR difference specroscopy. The internal asp 85 and 96 are identified as catalytic proton binding sites on the release and uptake pathway (1). Further proton transfer takes place via an H-bonded network in an Grothuis like proton transfer mechanism (2,3). This is revealed by changes of an IR continuum absorption during proton transfer. The continuum changes are no longer observed, when the proton transfer is disturbed by mutations. Similar mechanisms are proposed for the bacterial photosynthetic reaction center (4) and the redox driven proton pump cyochrom-c-oxydase (5).
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SYM-VII-3
PATHWAY FOR PROTON TRANSFER IN BACTERIAL REACTION CENTERS

Melvin Okamura, Mark Paddock, George Feher

The bacterial reaction center (RC) is the protein from photosynthetic bacteria that couples light induced electron transfer to proton pumping across the membrane. This process is mediated by a quinone, Q_B that takes up two protons upon two electron reduction, $Q_B + 2H^+ + 2e^- \rightarrow Q_BH_2$. The pathway for proton transfer to Q_B through the RC protein was elucidated by blocking proton transfer to Q_B, either using site directed mutagenesis of protonatable residues or by binding metal ions at specific surface sites. Both protons enter the RC near a group of His and carboxylic acid residues and proceed through a cluster of internal carboxylic acids and water molecules to a region near Q_B where the paths diverge to protonate either carbonyl oxygen of the quinone. The pathway is characterized by a high density of acidic residues and water molecules.

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SYM-VII-4
SIMULATING PROTON TRANSLOCATIONS IN PROTEINS: PROBING PROTON TRANSFER PATHWAYS IN THE RHODOBACTER SPHAETOIDES REACTION CENTER

Arieh Warshel[1], Yuk Y. Sham[1], Ingo Muegge[2]

A general method for simulating proton translocations in proteins and for exploring the role of different proton transfer pathways is developed and examined. The method is demonstrated in a preliminary study of proton translocations process in the reaction center of *Rhodobacter Sphaeroides*. It is found that proton transfer across water chains involves significant activation barriers and that ionized protein residues are probably involved in the proton transfer pathways. The potential of the present method in analyzing mutation experiments is briefly discussed and illustrated. The present study also examines different views of the nature of proton translocations in proteins. It is shown that such processes are mainly controlled by the electrostatic interaction between the proton site and its surroundings [1] rather than by the local bond rearrangements of water molecules that are involved in the proton pathways. [2] Thus, the overall rate of proton transport is frequently controlled by the highest barrier along the given conduction pathway.
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SYM-VIII-1
STRUCTURE-FUNCTION RELATIONS OF THE MYOSIN MOTOR IN MUSCLE

Malcolm Irving

Myosins are involved in a wide range of cell functions, of which the best characterised is the contraction of skeletal muscle. Crystallographic studies have led to an atomic model for the mechanism of the myosin motor, in which the head region of myosin bends between its catalytic (actin- and ATP- binding) and light-chain domains. This model has been tested in isolated muscle fibres by applying rapid length changes during active contraction. Changes in the orientation of the light-chain domain of the myosin head during the length step and the subsequent rapid force recovery were measured by polarised fluorescence from bifunctional rhodamine probes attached to pairs of cysteine residues on the regulatory light chain (Corrie et al. Nature, 400, 425–430, 1999). Further evidence for bending of the head has been provided by measurements of the intensity of the 14.5 nm X-ray reflection following rapid length changes (Dobbie et al., Nature, 396, 383–387, 1998; Irving et al., Nature Structural Biology, 2000, in press). Together, these experiments provide strong support for the tilting head model. They also suggest that myosin heads can be bent by external stress, and that the elasticity of the myosin head couples conformational change to force production.

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SYM-VIII-2
THE MOTOR PROTEIN KINESIN – STRUCTURE, INTERACTIONS WITH MICROTUBULES, AND FUNCTIONS IN INTRACELLULAR TRANSPORT

E. Mandelkow

Kinesin is the protoypic intracellular motor that moves cellular structures along microtubules. The movement is fuelled by the hydrolysis of ATP. There are diverse isoforms of kinesin which are specialized for different tasks. Kinesin motors usually move towards the "plus"-end of microtubules, but there are also reverse kinesins such as Ncd. We have recently determined the X-ray structures of kinesin constructs containing the head and neck domains, both in monomeric and dimeric forms (review, Sack et al., Eur. J. Biochem. 261:1, 1999). They reveal the folding of the polypeptide chain and the nucleotide binding site which shows remarkable similarities with the muscle motor myosin and with G-proteins. The interaction with microtubules was studied by cryo-electron microscopy and image reconstruction (in collaboration with A. Hoenger, EMBL, see Hoenger et al., J. Mol. Biol. 297:1087, 2000). This suggests a model of how kinesin advances along microtubules. Finally, we have studied the interaction between kinesin and microtubule- associated proteins, especially the neuronal tau protein (which aggregates in the brain in Alzheimer's disease). Tau interferes with the plus-end directed transport in cells so that organelles accumulate at the cell center and disappear from cell processes (axons, dendrites), leading to increased vulnerability (Trinczek et al., J. Cell Sci. 112:2355, 1999). This suggests a delicately regulated balance between motors and MAPs on the microtubule surface.

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SYM-VIII-3
THE BACTERIAL FLAGELLAR MOTOR

Richard M. Berry

The bacterial flagellar motor is a rotary molecular engine powered by the flow of ions across bacterial cytoplasmic membranes. Each motor drives a protruding helical filament, and the rotating filaments provide the propulsive force for cells to swim.
Ion flux is driven by an electrochemical gradient, the protonmotive force (pmf) or sodium-motive force (Namf) in motors driven by H^+ and Na^+ respectively. The rotating heart of the motor is a set of rings in the cytoplasmic membrane, about 45 nm in diameter, containing a total of a few hundred molecules of several different proteins. This rotor is surrounded by a ring of 8 to 16 independent torque generators, which are anchored to the cell wall and consist of the proteins MotA and MotB in H^+ motors, PomA and PomB in Na^+ motors.
Much is known about the energetics of H^+ motors. The ion flux is about 1000 ions per motor per revolution. At low speeds up to about 10 Hz, the motor torque is proportional to pmf. The torque varies little with speed up to a "knee" at about 160 Hz, which is approximately the speed of rotating filaments in swimming cells. At higher speeds torque falls linearly, through zero torque at about 300 Hz. When the motor is forced backwards, torque differs little from the stall torque up to at least −100 Hz. Each torque generator has a high duty ratio and takes about 50 steps per revolution.
Na^+ motors contain the auxilliary proteins MotX and MotY, of unknown function, and can rotate as fast as 1700 Hz at high Na^+ concentrations. Otherwise, they appear to be similar to H^+ motors in structure and function.

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SYM-VIII-4
A ROTARY MOLECULAR MOTOR THAT CAN WORK AT NEAR 100% EFFICIENCY

Kazuhiko Kinosita, Jr.

A single molecule of F_1-ATPase, a soluble portion of ATP synthase, has been shown to be a rotary stepper motor in which a central rotor unit rotates against surrounding stator subunits in a unique direction. Mechanical characteristics of this motor have been elucidated in detail under an optical microscope by attaching, to the rotor subunit, tags of different sizes. A μm-sized actin filament showed most clearly that the motor runs in discrete 120° steps, that each step is driven by hydrolysis of one ATP molecule, and that the energy conversion efficiency in making the mechanical steps can reach ~100%. A single fluorophore on the rotor showed that stepping occurs under no load and thus is a genuine characteristic of this motor, and that the rate of ATP binding is independent of the load. An aggregate of submicron particles demonstrated that the maximal speed of the motor derived from a thermophilic bacterium is ~100 revolutions per second at room temperature, that the motor makes clear 120° steps even at this speed, and that each stepping occupies only a fraction of the ATP hydrolysis cycle. If time allows, I will discuss possible mechanism of this rotary molecular motor.

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SYM-IX-1
NOVEL CAPABILITIES OF LIPOSOMES FOR TOPOLOGICAL TRANSFORMATION

Fumimasa Nomura, Kingo Takiguchi, Hirokazu Hotani

To study the dynamic behavior of biomembrane in aqueous solution, the transformation of liposomes caused by surfactants were monitored by direct, real-time observation using dark-field optical microscopy. When Triton X-100 was added to a liposome solution, spherical liposomes became smaller in size with maintaining their spherical shapes. During this process, the shrinking liposome exhibited two phases alternatively, i.e., its membrane fluctuated vigorously or kept still. Some liposomes encapsulated a few smaller liposomes inside. Surprisingly, when the outer liposome was vigorously fluctuating in shape, the encapsulated liposomes escaped out, indicating that Triton X-100 transiently generates a hole on the outer one and thereby excludes inner vesicles directly.
Furthermore, we found various transformation behaviors other than the continuous shrinkage, by change in lipid compositions and/or surfactant types. When both lipid and surfactant possess positive electric charge, liposomes underwent cyclic inside-out transformation. This might be caused from the unbalance between the surface areas of outer and inner leaflets of a liposome bilayer membrane, and the unbalance might result from the removal of lipid molecules only from outer leaflet by the surfactant. In contrast, in the continuous shrinkage described above, the removal of lipid molecules from outer leaflet might be compensated continuously by the molecules of inner leaflet through flip-flop translocation.

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SYM-IX-2
CRITICAL MIXING AND RED BLOOD CELL LIPIDS

Sarah L. Keller

When phospholipids are mixed with cholesterol in a monolayer at an air-water interface, coexisting 2-dimensional liquid phases are observed if the surface pressure is lower than the miscibility critical pressure. The sizes and shapes of the coexisting liquid phases are described by a competition between line tension and electrostatic dipolar repulsion [1]. Although stripe phases are predicted over a wide range of surface pressures, experimentally they only appear near the critical point [2]. This provides a method by which to search for miscibility critical points in complicated mixtures of lipids. Lipids were extracted from red blood cells and used to make monolayers simulating the inner and outer lipid leaflets of the red cell membrane. Stripe phases were observed in these monolayers at high surface pressures corresponding to an area per molecule near that in a red blood cell membrane [3]. This suggests that lipids in a red blood cell membrane are near a miscibility critical point, which could significantly affect the biophysical properties of the red blood cell membrane.
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SYM-IX-3
ELASTIC AND MECHANICAL ASPECTS OF CELL SHAPE

Elisha Moses[1], David Biron[1], Roy Bar-Ziv[5], Tsvi Tlusty[2], Pazit Libros[3], Dror Sagi[1], David Mirelman[3], Samuel A. Safran[2], Alexander Bershadsky[4]

We show how mechanical and elastic properties of the cytoskeleton and membrane combine with flow dynamics in cells to bring about quantitative conclusions regarding cell shape, structure and function.
In the first example, cytoskeletal disruption leads to the nonlinear dynamic instability of pearling in the cell, brought on by a tension whose source is related to adhesion, and opposed by the cytoskeletal rigidity. Quantitative analysis of cell shape and structure is enabled because of the special sequestering properties of the actin-disrupting drug latrunculin.
In a second example, we present scaling laws for the width of the cleavage furrow of a dividing cell, based on the activity of molecular motors during cytokinesis. Various regimes in the division of two types of amoeba are observed, and the appearance of collective behavior as a remedy for defective division is demonstrated.

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SYM-IX-4
LIPID AND DNA AT SOLID SURFACES

Joachim O. Rädler, R. Galneder, V. Kahl, B. Maier, J. Nissen, R. Zantl

There is a wide interest to keep biological molecules functional at inorganic solid surfaces. An interesting biocompatible coating are lipid membranes, which provide a natural fluid interface. Membranes completely wet solid surfaces and form a continuous supported membrane. The kinetics and interface dynamics of spreading is investigated and described by two-dimensional fluid invasion models. Supported membranes are used to demonstrate that DNA bound to oppositely charged, cationic membranes exhibits no loss of lateral mobility. Quantitative fluorescence microscopy of single DNA molecules on cationic supported membranes shows static and dynamic power law scaling as predicted for polymers in two dimensions. In particular the Flory scaling exponent, Rouse dynamics, rotational relaxation times, 2D-reptation are revealed by direct computer-assisted image analysis. Densely decorated surfaces exhibit segregation of chains and nematic ordering on the molecular length scale. The mobility of DNA can be controlled by buffer conditions and the state of the underlying lipid membrane with potential application in microfluidic devices and 2D electrophoresis. In order to follow the adsorption of DNA to cationic membranes, we use lipid coated silica beads captured by an optical trap. A new microelectrophoresis set-up allows to continuously monitor the zeta-potential of the bead. Charge inversion in the case of DNA adsorption to oppositely charged membranes is directly measured.

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SYM-IX-5
DYNAMICS OF BINDING AND UNBINDING OF MEMBRANES AND MOLECULES

Udo Seifert

Binding and unbinding of lipid membranes or of specific receptor/ligand pairs are ubiquitous phenomena in biophysics. Model systems for studying these problems include vesicles interacting with substrates and dynamical force spectroscopy of such receptor/ligand pairs using AFMs or membrane force probes.
In both cases, not only equilibrium quantities like adhesion or binding energy contribute but also dynamical, i.e., non-equilibrium aspects. Specifically, a recent experiment has shown that bound vesicles can undergo a dynamically induced unbinding transition in shear flow. A theoretical analysis of this phenomenon based on low Reynolds number hydrodynamics yields the critical shear rate as a function of the equilibrium adhesion energy and the geometry of the vesicle [1].
For the unbinding of adhesion patches involving several specific molecule pairs, a simple model is introduced. Based on known properties of the unbinding of a single pair, predictions are made how the critical force required to rupture several parallel bonds depends on both the number of bonds initially present and the dynamical loading rate [2].
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SYM-X-1
CELL ADHESION RECEPTOR REGULATION

Anne Pierres, Anne-Marie Benoliel, Pierre Bongrand

Cells have a need to initiate and break adhesions with surrounding cells or surfaces. These processes are mediated by a variety of membrane receptors. The functional capacity of these receptors is highly regulated through multiple mechanisms including the following : *a) control of intercellular forces.* Cell membranes are coated with a carbohydrate-rich layer that may be downregulated in order to increase adhesiveness. *b) expression.* Cells can increase very rapidly the surface density of adhesion receptors through exocytosis of stored organelles. Conversely, adhesion may be terminated by proteolytic cleavage of binding molecules. *c) intrinsic receptor regulation.* Receptors may undergo structural changes resulting in marked alteration of binding properties, including attachment or detachment kinetics, affinity change or even modulation of mechanical strength. These changes may be triggered by exogenous signals as well as intracellular biochemical cascades. *d) regulation of membrane/receptor relationship.* This may involve multiple mechanisms. Thus, cells may concentrate receptors to convex regions (i.e. the tip of surface protrusions) in order to enhance their accessibility. Also, receptors may be aggregated into small clusters of a few molecules, resulting in highly enhanced capacity. The strength of receptor-to-cell attachment may be increased through cytoskeletal reorganization. Also, the lateral mobility of membrane receptors may be altered.

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SYM-X-2
ADHESIVE BIOMEMBRANES: SOFT SWITCHES

Robijn Bruinsma, Erich Sackmann

The talk reviews recent progress in our physical understanding of adhesion between model biomembranes. Optical and mechanical studies model vesicles consisting of membranes, containing both adhesion molecules and repeller molecules, indicate that adhesion of such *multi-component* membranes exhibits a diverse range of unexpected phenomena, in particular *adhesion-induced phase-separation.* This phenomenon leads to highly heterogeneous adhesion surfaces exhibiting spontaneous formation of *focal adhesion sites*, a well-known characteristic of actual biomembranes. Recent experiments also offer a fascinating paradox: the characteristic mechanical force level required to cause unbinding between two membranes containing adhesion molecules is *small* compared to the force levels required to unbind individual pairs of adhesion molecules. The talk will provide a theoretical framework to interpret these phenomena.

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SYM-X-3
UNEXPECTED COMPLEXITY IN THE STRENGTH OF CELL ADHESION BONDS

Evan Evans

Well known in biology, ligand-receptor interactions are the fundament of nanoscale chemistry in recognition and adhesion plus a host of other processes from outside to inside cells. But not well-appreciated is that energy landscapes of cell adhesion bonds are *rugged* terrains with more than one prominent energy barrier. In conventional *test tube* assays, near equilibrium kinetics – where bonds dissociate under zero force – only reveal a single-outer barrier which is the classical paradigm of biological chemistry. However, when cell adhesion bonds are tested by the method of *dynamic force spectroscopy*, hidden-inner barriers are exposed that tie bond strength to unbinding kinetics over shorter spans in time scale. The intriguing question is why did nature structure energy landscapes in cell adhesion bonds to create a hierarchy of time scales for dissociation kinetics accessed by force? Examining this question for a wide variety of specific interactions from biotin-(strept)avidin to carbohydrate-selectin and antigen-antibody bonds is leading to a new perspective of the important connection between force – time – chemistry in cell adhesion.

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SYM-X-4
MOLECULAR DYNAMICS OF CELL-MATRIX ADHESIONS IN CULTURED FIBROBLASTS: A DIGITAL MICROSCOPIC STUDY OF LIVE CELLS

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This study consists of dynamic and quantitative analysis of the formation and molecular dynamics and segregation of cell-matrix adhesions. We demonstrate that attachments of cultured cells to the underlying substrate are highly heterogeneous structures. Quantitative microscopy revealed at least 3 types of adhesions, including classical focal contacts (enriched with paxillin, vinculin, α_v integrin and phosphotyrosine), fibrillar adhesions (enriched with tensin and α_5 integrin) and mosaic adhesions with subdomains of the types. We further show that segregation of these adhesions depend on the rigidity of the matrix and competence of the actomyosin machinery of the cells. Time-lapse microscopy of cells expressing GFP-paxillin to track focal contacts or GFP-tensin for fibrillar adhesions reveal that both types of major adhesions are highly dynamic. Small focal contacts often translocate by centripetal extensions followed by peripheral contractions at a mean rate of 19 μm/h. Fibrillar adhesions arise from the medial ends of stationary focal contacts, contain $\alpha_5\beta_1$ integrin and tensin without other focal contact components, and associate with fibronectin fibrils. Fibrillar adhesions translocate centripetally at a mean rate of 18 μm/h in an actomyosin-dependent manner. We propose a dynamic model for the regulation of matrix adhesions and transitions between focal contacts and fibrillar adhesions, with matrix deformability functioning as a mechanical switch.

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SYM-XI-1
THE CONFORMATIONAL CHANGES IN PHOTOACTIVE YELLOW PROTEIN THAT LEAD SIGNAL GENERATION IN *ECTOTHIORHODOSPIRA HALOPHILA*

K.J. Hellingwerf, W. Crielaard, J. Hendriks, Th. Gensch, M. van der Horst

Photoactive yellow protein (PYP), a blue-light phototaxis receptor from the eubacterium *Ectothiorhodospira halophila*, shows many similarities with archaeal sensory rhodopsins, although PYP contains a 4-hydroxy-cinnamic acid chromophore, and is water-soluble. Activation of PYP proceeds through light-induced *trans/cis* isomerization of the 7,8-vinyl bond of its chromophore, which is followed by a large number of conformational alterations and ultimately leads to a modulation of the motility machinery of *E. halophila*.
The coumaryl chromophore of PYP is present in anionic form in the ground state (pG) of the protein and is buried within its major hydrophobic core, stabilized via a hydrogen-bonding network involving Y42, T50 and (protonated) E46. Photoactivation of PYP initiates a photocycle (Φ = 0.35) with several transient intermediates. At the very short timescale, red-shifted intermediates are formed, which decay into a relatively stable blue-shifted intermediate (pB), in which the chromophore is protonated.
Structural characteristics of transient photocycle intermediates of PYP have been determined. Our experiments suggest that particularly the N-terminal domain of PYP is transiently unfolded in the pB state (the presumed signaling state). In addition, these experiments revealed that the conformation of the pB intermediate is strongly affected by the mesoscopic environment of the protein.

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SYM-XI-2
MOLECULAR MECHANISM FOR VECTORIAL PROTON TRANSLOCATION BY BACTERIORHODOPSIN

Sriram Subramaniam, Richard Henderson

We present an atomic resolution model for protein and retinal structural changes that are involved in the vectorial, light-driven transport of protons by the membrane protein bacteriorhodopsin. The "switch" mechanism which ensures the vectorial nature of pumping is shown to be constructed from two elements: (i) unbending of retinal following the release of a proton from the Schiff base, resulting in displacement of the Schiff base nitrogen towards the cytoplasm, and (ii) a protein conformational change which is mainly localized to the cytoplasmic side. Electron crystallographic determination at 3.2 Å resolution of the structure of a mutant locked in this conformationally altered state reveals major rearrangements of helices F and G which provide an "opening" of protein on the cytoplasmic side of the membrane. The type of helix movements reported here for bacteriorhodopsin are likely to reflect a common structural theme in the activation of other related seven-helix proteins that function as transmembrane ion pumps, sensory transducers and G-protein coupled receptors.

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SYM-XI-3
SENSORY RHODOPSIN/TRANSDUCER INTERACTION AND TAXIS TRANSDUCTION BY HALOARCHAEA

John Spudich

The completed genome sequence of the archaeon *Halobacterium salinarum* reveals 18 transducer proteins that control a phosphorylation pathway modulating the flagellar motors of the organism. Several are membrane proteins sensing stimuli from outside the cell, others are soluble and sense the cytoplasmic environment, and two, HtrI and HtrII, are integral membrane subunits of a phototaxis signaling complex, containing seven-helix receptors, sensory rhodopsins I and II (SRI & SRII), respectively. By deletion analysis, studies with transducer chimeras, and engineered sulfhydryl crosslinking analysis, we have demonstrated that: (i) the membrane-embedded SR subunits interact with dimers of their cognate Htr subunits by lateral transmembrane helix-helix contact; (ii) the packing arrangement of the Htr monomers in the dimer is controlled by SR photoactivation; and (iii) the interacting transmembrane portion of the SR-Htr complex is a modular membrane-embedded photosignaling structure capable of generating photosensory signals to the cytoplasmic portions of heterologous transducers, such as the Tar and Tsr chemotaxis receptors of *E. coli*. Progress will be presented on the electron crystallography of the signaling complex, structural changes from molecular spectroscopy, and analysis of purified functional complexes reconstituted in vitro. In addition newly discovered SR homologs in eukaryotic microbes (directly demonstrated in fungi and implicated also in algae) will be presented.

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SYM-XI-4
PROTEIN INTERACTIONS OF GLUTAMIC ACID-RICH PROTEINS (GARPS) IN PHOTORECEPTORS

U.B. Kaupp, H.G. Körschen

The β subunit of the rod cGMP-gated ion channel features a unique bipartite structure. The membrane-spanning part is homologous to the α subunit, whereas its large cytoplasmic N-terminal domain (GARP-part), except for a few amino acid residues, is identical with two soluble proteins, designated glutamic acid rich proteins (GARP1 and GARP2). GARPs are specifically expressed in the outer segment of rods but not of cones. The common N-terminal region of soluble GARPs and the GARP-part carries four short repeats that are highly conserved among each other and that represent the most conserved structural elements between GARPs from different species. These repeats recruit components of the enzyme cascade, including the phosphodiesterase (PDE) and the guanylyl cyclase (GC), and surprisingly the ATP-binding cassette (ABCR) transporter ("rim" protein). Soluble GARPs are tightly associated with the margin of the disc membrane, probably by binding to one or several of the proteins at the disc rim. They also associate with themselves, suggesting that binding of other proteins competes with homooligomerisation of GARPs. GARPs probably do more than one trick. GARP2, the most abundant form, strongly binds to and thereby inhibits light-activated PDE, whereas the association with inactive PDE is weaker. Thus, two mechanisms of PDE inactivation - GTPase activity and binding of GARPs - may operate in parallel. We propose that GARPs organize a dynamic protein complex within the small annular-shaped space between disc margin and plasma membrane.

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SYM-XII-1
BIOLOGY MEETS MICROLITHOGRAPHY
Robert H. Austin

Silicon micromachining has opened up a new world of sub-micron spaces in which you can study biological objects on a scale commensurate with their size and operational environment. Since silicon micromachining is so highly sophisticated in terms of technology, it is possible to design and construct highly creative structures which can probe specific aspects of a biological object. Such structures can also be very practical and useful in applied areas such as biotechnology.
The manipulation and sorting of biological particles poses unique challenges to microfabrication because of the complex physical properties of biological particles. These properties range from size to rigidity to adhesive properties. Often it is vital to ascertain the uniqueness of the particles, to sort them and find a very rare individual in a population of millions. I will present examples of our attempts to attack these problems and produce microfabricated devices useful in medicine and molecular biology.

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SYM-XII-2
BIOANALYTICS AT THE MICRO- AND NANOMETER SCALE
Horst Vogel

The ability of organisms, or individual cells to sense and to react to different external and internal stimuli (light, hormones, odorants, taste, neurotransmitters) is crucial for their survival. Our recent progress to investigate molecular signal recognition and transduction processes by surface sensitive physical techiques and molecular biological methods will be reported.
Controlled self-assembly of proteins (channel proteins, G protein-coupled receptors) in a functionally form on sensor surfaces is a prerequisite for the investigation of molecular interactions by surface-sensitive techniques. Receptors are reversibly immobilized on solid supports; ligand binding to and concomitant conformational changes of the receptors are probed by optical techniques such as total internal reflection fluorescence and infrared spectroscopy, fluorescence correlation spectroscopy and surface plasmon microscopy. Based on this experience, we have developed a method to detect channel gating of ionotropic receptors in tethered membranes on planar solid surfaces. As an alternative to traditional patch clamp measurements, this approach offers novel possibilities for an efficient screening of receptor function. In addition, G protein-coupled receptors are immobilized in form of micropatterns on solid supports and their activation and interaction with G proteins is investigated by optical spectroscopy.
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SYM-XII-3
OPTO-ELECTRIC BIOCHIPS
Günter R. Fuhr, Christoph Reichle, Thomas Schnelle

The characterisation and handling of individual cells in physiological media are key problems in medicine and molecular biotechnology. Manipulations should be effective and loss-free. Here we demonstrate multi-function biochips combining electric, optic and hydrodynamic forces. The micro tools can handle cells of different origins suspended in physiological solutions. Highly conductive media (up to 1.5 S/m) allow the creation of repelling forces on particles and cells by negative dielectrophoresis while laminar streaming through micro channels with typical sizes in the micrometer range avoids cell-surface contacts.
We give an introduction into the fabrication techniques of micro systems with high optical quality. The peripheral equipment consists of rf-generators, optical equipment (laser and microscope), pumps, injection modules etc. and is computer-controlled. Applications in basic research and biotechnology are explained and discussed. Currently these are cell sorting, cell loading, cell permeation and the induction of defined cell-cell interactions.
The combination of optical and electrical devices can measure pN-forces, e.g. between micro particles, particle-cell systems or cell pairs. We give an example, measuring single particle–cell interactions at a very low level of chemical binding in the well known streptavidin-biotin-system. Such chips should find many applications in physics, chemistry and biology.

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SYM-XIII-1
MORPHOLOGICAL CORRELATES OF SYNAPTIC PLASTICITY

Tobias Bonhoeffer

I will present evidence for brain-derived neurotrophic factor being one crucial important component in synaptic plasticity in the hippocampus. BDNF-knockout animals show severely compromised LTP and long-lasting LTP is even completely abolished. It can be rescued by locally infecting cells with an adenovirus vector containing the BDNF gene, indicating that the BDNF protein is required at the time of LTP-induction. Since it is well known that BDNF can influence the morphology of neurons, it is attractive to speculate that it might provide a link between functional and morphological aspects of synaptic plasticity. Such morphological changes had been difficult to demonstrate, mainly because it has not been easy to pin-point the location of the synapses which are expected to change. We have now tackled this problem by combining two-photon imaging with a local superfusion technique thereby confining the region on the postsynaptic dendrite where the synaptic changes could occur. We were able to show that, indeed, LTP induction reliably leads to the appearance of new spines in this area. These remained stable in shape and position for up to 24 hours. The most attractive explanation for the formation of additional spines is a concurrent emergence of new synapses on these structures. These data indicate that in the mammalian hippocampus not only physiological but also structural changes play an important role when neurons change the efficacy of their connections.

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SYM-XIII-2
TIMING OF SYNAPTIC TRANSMISSION

J.H. Bollmann, B. Sakmann, J.G.G. Borst*

The probability of transmitter release depends critically on presynaptic calcium influx. The brief increase in the presynaptic calcium concentration ($[Ca^{2+}]_i$) during action potentials is reported by a calcium sensor located at or near the vesicles. Its ability to rapidly bind and release calcium helps to preserve temporal information across synapses. Despite the importance of the calcium sensor for synaptic strength and the identification of a number of putative candidates for this protein, detailed knowledge of its calcium-binding properties are still lacking for the mammalian central nervous system. We measured the calcium sensitivity of this sensor in a glutamatergic calyx-type synapse in the medial nucleus of the trapezoid body in the rat auditory brainstem. A homogeneous rise in the presynaptic $[Ca^{2+}]_i$ to 1 μM by laser photolysis of caged calcium resulted in a clearly measurable increase in release. A step to > 30 μM depleted the entire releasable vesicle pool in less than 0.5 ms. We constructed a model of the calcium sensor that could reproduce the time course of the probability of transmitter release triggered by action potentials, voltage steps or $[Ca^{2+}]_i$ steps. The model suggested that a brief elevation of $[Ca^{2-}]_i$ to < 10 μM would be sufficient to reproduce the physiological release pattern following action potentials. Thus, the Ca^{2+} sensitivity of phasic release at this mammalian CNS synapse is high and the distinction between phasic and delayed release is less pronounced than previously assumed.

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SYM-XIII-3
ROLE OF DYNAMIN IN ENDOCYTOSIS

Harvey McMahon

To understand the molecular mechanism of clathrin-mediated endocytosis our laboratory has taken a structural and biochemical approach. One of the proteins currently under investigation is dynamin, the large GTPase that plays an essential role in vesicle scission during endocytosis. Dynamin forms a helical collar around the neck of an invaginating clathrin-coated vesicle where it may regulate, pinch or pop the vesicle from the parent membrane. We have previously published evidence that on GTP hydrolysis dynamin spirals undergo a lengthwise extension- which we believe drives the vesicle away from the membrane causing lipid fission. To address the possibility that dynamin could be a regulator of vesicle scission we have carried out mutagenesis of the protein and show that the GTP hydrolysis activity of dynamin is coupled to a conformational change and that this activity of the protein is essential for vesicle scission.

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SYM-XIII-4
VISUALIZING NEURAL ACTIVITY WITH GENETICALLY ENCODED OPTICAL PROBES

Gero Miesenböck

Information in nervous systems is often carried by neural ensembles – groups of neurons in transient functional linkage – and written in a code that involves the spatial locations of active cells or synapses and the times at which activity occurs. Even in favorable neuroanatomical circumstances, recording ensemble signals presents a serious experimental challenge. Electro-physiological methods are generally limited to a few neurons at a time, while synthetic indicator dyes face problems of access and specificity, particularly in functionally intact systems. Our strategy to overcome these difficulties relies on protein-based sensors that provide direct optical images of neural activity. Since these molecules are encodable in DNA, they can be introduced into intact animals by genetic manipulation, and their expression pattern can be tailored to include – exclusively and at the same time comprehensively – the neurons of interest. Cellular or synaptic correlates of perceptual, motor, or cognitive tasks can thus be analyzed in virtual isolation at successive stages of intact neural pathways.

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SYM-XIV-1
WHAT THE EYE TELLS THE BRAIN: SIGNAL PROCESSING IN THE VERTEBRATE RETINA

Josef Ammermüller

The vertebrate retina is an exquisite filter and encoding network. It transforms the visible part of the electromagnetic spectrum into electrical signals that can be understood by the brain.
This is accomplished by the neuronal interaction of photoreceptors and various types of interneurons. This interaction is the basis for dynamic adaptation, gain control, and filter adjustment. The basic processing steps within the retina, which are mainly "analogue", will be presented. The interneurons feed the pre-processed signals into the output of the retina, the ganglion cells. They transform the analogue signals into trains of electrical pulses (action potentials), which are sent to the brain via app. one million nerve fibres in the optic nerve. This "parallel bus" transmits all relevant information about a visual scene.
We will show that ganglion cells act in concert in order to reliably encode visual features. In addition, the eye is not a passive detector. It actively scans visual scenes by eye movements – even during fixation when microscopic movements have to be present to maintain perception. These microscopic movements, which shift the image on the retina by app. one photoreceptor diameter, synchronize action potentials in ganglion cells. In the experiment, this synchronised activity can be used as a triggering signal for stimulus feature detection with an artificial neural network. We suggest, that the brain uses the same signal characteristics for decoding visual stimulus features from the ganglion cell action potential trains.

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SYM-XIV-2
SYNAPTIC ADAPTATION IN CORTICAL NETWORKS

John Hertz

Two recent theoretical threads in the theory of cortical neurons and their network dynamics have been (1) mechanisms underlying irregular asynchronous firing, and (2) effects of synaptic adaptation. In this talk I will pull these two threads together in a mean field theory for low-rate asynchronous firing states in dilute networks of spiking neurons with synaptic depression or facilitation. Changes in firing rates cause changes in synaptic strengths and vice-versa, potentially leading to instabilities. I will show that depression of synapses between excitatory neurons or between inhibitory ones always tends to stabilize the asynchronous state against such fluctuations, while depression tends to destabilize it. Facilitation has the opposite effect: in synapses between excitatory and inhibitory neurons it is stabilizing, but in synapses between excitatory neurons or between inhibitory ones, it is destabilizing.

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SYM-XIV-3
SOUND LOCALIZATION AND FORMATION OF TEMPORAL-FEATURE MAPS IN THE BARN OWL'S AUDITORY SYSTEM

Richard Kempter, Christian Leibold, Hermann Wagner, J. Leo van Hemmen

Computational maps are of central importance to the neural representation of the outside world. In a map, neighboring neurons respond to similar sensory features. A well studied example is the computed map of interaural time difference (ITD), which is essential in sound localization in a variety of species andallows resolution of ITDs of the order of 10 microseconds. Nevertheless it is unclear how such orderly representations of temporal features arise. We address this problem by modeling the ontogenetic development of the map of ITD in the experimentally well characterized nucleus laminaris of the barn owl. We show how the owl's map of ITD can emerge from a combined action of homosynaptic spike-based Hebbian learning and its propagation along the presynaptic axon. In spike-based Hebbian learning, synaptic strengths are modified depending upon the timing of pre- and postsynaptic spikes. A mathematical analysis suggests that both Hebbian learning and ist presynaptic propagation are necessary for map formation but the latter can be orders of magnitude weaker than the former. This may be a key mechanism to the formation of computational maps, in particular, when time plays a key role.
RK is supported by the DFG, Kl 608/10-1 and Ke 788/1-1. CL holds scholarship of the state of Bavaria.

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SYM-XIV-4
USING RENEWAL NEURONS TO TRANSMIT INFORMATION

C. van Vreeswijk

Information theory is a popular tool in determining the role of cortical processes. It has been used to explain the desirability of V1 receptive fields and to determine the time over which rates are averaged in information processing. Information theory is useful because it says how much signal is can be extracted in a noisy environment. However this depends crucially on the model of the noise. Describing neurons as Poisson processes or Gaussian channels, as is commonly done, yields results that do not generalize to more detailed models. I will discuss information theoretic results for model neurons described by renewal processes. Unlike Poisson processes, these can have a refractory period. The Poisson model has led people to equate rate encoding with a code for which a spike counter is an optimal decoder. This is not true for a generalized renewal process. The precise timing of the spikes yields more information than the total number of spikes alone. For Gaussian channels the output variance is independent of the mean rate, and an extra constraint of sparseness has to be imposed to obtain realistic network properties. Because with renewal neurons the variance depends on the mean, models using renewal neurons automatically yield sparse representations when information is maximized.

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SYM-XV-1
THEORETICAL BIOPHYSICS OF THE COCHLEA
Hendrikus Duifhuis

The cochlea is the biophysical input interface of the mammalian auditory system. It provides the preprocessing, and the stimulation of the fibers of the auditory nerve.
Puzzling properties of this interface have been, and still are, its fast and smooth operation, thereby covering a remarkable amplitude range (dynamic range approx. 120 dB) and a wide frequency range (more than 9 octaves), both with good resolving power.
Apparently the dynamic range is mapped nonlinearly by an instantaneous compressive non-linearity. This nonlinear process involves the intact outer hair cells, but it is as yet not fully understood *how*.
The frequency mapping –or filtering- in the cochlea has been subject to considerable theoretical study, and several 1D-, 2D-, and 3D-models have been proposed. Many of the studies are limited to a linear analysis of the system, assuming that the linear approximation is sufficiently accurate, and because of lack of tools of a proper non-linear analysis.
Moreover, many studies use simplifying boundary conditions that are biophysically untenable. E.g., the acoustical coupling of different points in the cochlea is essential, and so are the middle ear interface, and the apical helicotrema.
Implications of these properties will be demonstrated and discussed within the context of where we are, and what are the urgent issues.

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SYM-XV-2
EXPERIMENTAL BIOPHYSICS OF THE COCHLEA
Anthony W. Gummer

The extraordinarily high frequency selectivity and large dynamic range of the cochlea is achieved by two transduction processes located in the outer hair cells (OHCs). The first is mechanoelectrical in the stereocilia located on the apical cell surface and the other is electromechanical in the basolateral cell wall. The experiments described here have been designed to elucidate the biophysics of these mechanisms.
Mechanoelectrical transduction, by which deflection of the stereocilia is transduced into current, was studied experimentally in isolated OHCs with whole-cell patches. By destroying (5-mM BAPTA or 20-U/mL elastase) the extracellular linking protein between the tips of stereocilia and the sides of the adjacent taller stereocilia, evidence is provided for mechanical gating by an intracellular protein.
Electromechanical transduction, by which a change of membrane potential induces contractile motion of the cell body, was studied experimentally for isolated OHCs as well as OHCs embedded in the organ of Corti. By stimulating electrically and measuring the transduced mechanical force, it is shown that the OHC is able to produce forces that are independent of frequency to well-above their characteristic frequency on the basilar membrane.

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SYM-XV-3
SIGNIFICANCE OF TEMPORAL PATTERNS IN ACOUSTIC SIGNALS FOR DETECTION AND OBJECT FORMATION
Georg M. Klump

Amplitude and frequency modulation are a common feature of acoustic communication signals. These and other environmental sounds are subject to additional amplitude modulation that results from air turbulence on the path of transmission from the sound source to the receiver. Psychophysical studies in humans, other mammals and in a bird will be reviewed demonstrating that amplitude modulation can be exploited to considerably improve signal detection – an effect that has been termed "Comodulation Masking Release" (CMR). Auditory systems of vertebrates will even restore signals in perception that are masked by short periods of high-level background noise resulting in the illusion of continuity of a physically discontinuous signal. On the other hand, temporally coherent modulation of parts of a composite signal make it difficult for auditory systems to separate parts for individual analysis (e.g., as shown in experiments on modulation detection interference). These different psychophysical effects have been interpreted to indicate mechanisms of auditory grouping and object formation. Only few attempts have been made to investigate the underlying neural mechanisms in the auditory system. The results of these neurophysiological studies will be reviewed, compared to the psychophysical evidence and discussed with respect to possible neural codes.

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SYM-XV-4
THEORETICAL BIOPHYSICS AND MODELS OF THE AUDITORY SYSTEM
Birger Kollmeier

This review talk concludes the series of invited lectures that cover the fascinating – and yet not fully understood – functioning of the auditory system by emphasizing both experimental and theoretical aspects of the cochlea and the subsequent neural processing of acoustical information.
To quantitatively understand the function of the auditory system, neurophysiological, psychophysiological and psychophysical data are used to construct auditory models. These models either concentrate on single functional aspects of the auditory system (such as, e.g., localization models) or try to give a more integrative description of several auditory functions by using the same model structure (such as, e.g., perception models to describe detection, discrimination and identification in humans). Moreover, the models explicitely simulate the behaviour of single neurons or neuronal assemblies (such as, e.g., physiological models of pitch perception) or describe the "effective" processing in the auditory system by using physiologically-motivated black boxes as processing units (such as, e.g., the Oldenburg model).
The explanation levels of current auditory models and our current understanding of auditory processing will be reviewed. The talk will then focus on the perception model developed in the authors group. Model predictions (such as, e.g., modulation perception, speech reception in noise, performance of hearing-impaired listeners) and possible applications (i.e., robust front ends for speech recognizers, audio and speech coders and "smart" hearing aids) will be discussed.

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SYM-XVI-1
FUNCTIONAL MAGNETIC RESONANCE IMAGING
Chrit Moonen

Local cerebral oxygen consumption ($CMRO_2$) is increased during brain activation. The associated increase in perfusion is much larger than the increase in $CMRO_2$. Therefore, blood oxygenation is increased upon brain activity. BOLD fMRI (Blood Oxygenation Level Dependent) employs hemoglobin as a source of contrast. The ferrous iron of deoxyhemoglobin is paramagnetic but diamagnetic in oxyhemoglobin. When red blood cells containing deoxyhemoglobin are placed in a strong external magnetic field, the magnetic field surrounding the red blood cell is distorted. The field distortions lead to local variations in Larmor precession frequency. The resulting increase in spin phase incoherence leads to small changes in the MR signal decay constant T2* and can be measured using T2*-weighted fMRI methods.
Stimulation and rest periods, each about 30s, are alternated during continuous fMRI data acquisition. Multi-slice EPI is the most widely used fMRI method because of its high speed (50 ms per slice). Alternative techniques include rapid, true 3D, methods. Motion-correction routines are used to improve signal stability. Statistical tests are used to determine activated voxels. Recently, short stimulation periods are used (single-event fMRI). The hemodynamic response is the crucial element in single-event fMRI necessitating a high temporal MRI resolution and adapted statistical procedures.

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SYM-XVI-2
VISUALIZATION OF REGULATORY MECHANISMS IN TUMOR ANGIOGENESIS BY MAGNETIC RESONANCE IMAGING
Michal Neeman, Rinat Abramovitch, Yael S. Schiffenbauer, Hagit Dafni, Assaf Gilead, Gila Meir

Solid tumor growth is dependent on extensive expansion of the vascular bed. Angiogenesis is the main mechanism for increasing the tumor blood supply, and is complemented by vasculogenesis and vascular co-option. We employed magnetic resonance imaging (MRI) for detection of the kinetics of tumor associated angiogenesis. Detection of blood vessels can be achieved by intrinsic contrast MRI relying on the paramagnetic properties of hemoglobin. Thus changes in blood oxygenation can be used for assessing vascular function. This approach was used for analysis of multiple genetic, hormonal, and growth factor modulators of angiogenesis. The plasticity of the immature vascular bed is associated with the lack of pericytes and smooth muscle cells. This plasticity is manifested for example by extensive vascular remodeling in the tumor periphery during the early stages of tumor implantation, and preceding the initiation of tumor growth. The recruitment of the perivascular pericytes and smooth muscle cells is manifested in vivo as an increase in reactivity of the vessels to vasomodulators. We detected vascular response to hypercapnia as a measure of vascular maturation using intrinsic contrast MRI. The approaches developed here provide a tool for preclinical analysis of response to candidate antiangiogenic therapies, and could potentially be translated to clinical MRI to provide surrogate markers of response to treatment.

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SYM-XVI-3
MR AND TRANSGENIC ANIMAL MODELS
Klaas Nicolay, Marijn J. Kruiskamp, Robin A. de Graaf, Ferdi A. van Dorsten

Magnetic resonance (MR) techniques play a rapidly increasing role in medical diagnostics and in fundamental biophysical, biochemical and biological research. The growing impact of MR is the combined effect of the non-invasiveness of the technique and the wide range of different parameters that it provides. MR technology as applied to living systems (termed *in vivo* MR) consists of two basic modalities, MR imaging (MRI) and MR spectroscopy (MRS). MRI provides high-resolution images of the structure and function of tissues while MRS enables the *in vivo* measurement of the concentrations, distribution and biophysical properties of low-molecular weight metabolites. This presentation will focus on the use of high-field MR techniques for the study of the structure and function of tissues in genetically manipulated (transgenic) mice. Because of its non-invasiveness and versatility, MR is ideally suited for assessing the structural and functional consequences of the loss or gain of protein function. This phenotyping is a tedious and time consuming process and requires sophisticated methodology since altered gene expression often results in subtle functional alterations as a result of adaptation. It is to be expected that high-field MR will play a central role in the science of functional genomics that aims to exploit the wealth of genetic data emerging from the various genome projects, to gain an improved understanding of cell function in health and disease.

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SYM-XVI-4
CARDIOVASCULAR MAGNETIC RESONANCE IMAGING
Albert C. van Rossum

Due to faster image acquisition, better image quality, and high quantitative accuracy, the clinical role of Cardiovascular Magnetic Resonance (CMR) is gradually expanding from assessing anatomy of the heart and great vessels to functional evaluation of valvular and ischemic heart disease. Due to its precision and reproducibility MRI has become a standard for the assessment of ventricular volumes, ejection fraction, and myocardial mass. Detailed non-invasive information regarding regional myocardial function may be obtained by analysing intramural deformation with use of tagging techniques.
MR coronary angiography is currently performed using 3-dimensional techniques. To reduce respiratory motion, acquisitions are either made within a breath-hold or by use of respiratory gating. Trade-offs exist between resolution, acquisition time, spatial coverage, and SNR. New intravascular contrast agents are being developed to improve signal to noise. Intracoronary flow can be measured at rest and during intravenous administration of vasodilating agents, allowing a non-invasive determination of coronary flow reserve.
Imaging of myocardial perfusion has two applications. One is the detection of stress-induced myocardial perfusion deficits using first-pass techniques, the other is discriminating viable from non-viable tissue through delayed contrast enhancement of the myocardium. The high resolution allowing differentiating between subendocardium and subepicardium, combined with the detailed information on myocardial function, may become a challenge to routinely applied nuclear techniques.

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1A-1
DAPI INTERACTS WITH UNUSUAL GC STRUCTURES: A FLUORESCENCE CORRELATION SPECTROSCOPY STUDY

Maria Luisa Barcellona, Yan Chen, Joachim D. Müller, Enrico Gratton

The binding of DAPI to double stranded GC polymers either in alternating or in homopolymer sequence was investigated using fluorescence technique. We employed fluctuation correlation spectroscopy, which measures the diffusion coefficient of fluorescent particles, to demonstrate that the fluorescence was originating from relatively slowly diffusing entities. These entities display a large heterogeneity of diffusing coefficients indicating that molecular aggregation is extensive. and also a long fluorescence lifetime.
At very low GC polymer-dye coverage we observed a relatively bright fluorescent component with a lifetime value of approximately 4 ns. The stoichiometry of binding of this bright species was such that it can only arise from rare structures, either unusual loops or large molecular aggregates. The amount and characteristics of this bright fluorescent component were different between the homo- and the alternating polymer, indicating that the difference in sequence is responsible for the different aggregates.
At large GC polymer coverage, we observed a relatively wide distribution of fluorescent species with short lifetime values, between 0.12 and 0.2 ns. Given the stoichiometry of binding of this component, we concluded that it could arise either from intercalative and/or non-specific binding to the DNA molecules. We comment on the origin of the rare, but brightly fluorescent binding sites and discuss the potential to detect such unusual DNA structures.

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1A-2
DIFFERENCES IN THE LOCALISATION OF CHROMOSOMES, CENTROMERES AND GENES IN THE NUCLEI OF HUMAN GRANULOCYTES

Eva Bártová, Pavla Jirsová, Stanislav Kozubek, Michal Kozubek, Emilie Lukášová, Magdalena Skalníková, Katerina Buchnícková, Alena Cafourková

White blood cells, so called leukocytes, were studied in our experiments. Particularly granulocytes and the cells of the granulocytic pathway of differentiation were in the scope of our interest.
We have studied the nuclear topography of human granulocytes with a characteristic lobular shape of nuclei. The nuclear positioning of all chromosomes, the nuclear location of centromeric heterochromatin and the topography of genes like ABL, BCR, C-MYC and p53 were detected using fluorescence *in situ* hybridisation (FISH). High order chromatin structure was studied in human granulocytes (differentiation *in vivo*) and compared with the location of the genetic structures in human leukemic promyelocytes HL-60 undergoing granulocytic differentiation *in vitro* that was induced using retinoic acid. The ABL, BCR and p53 genes were located more centrally than the C-MYC genes. In comparison with undifferentiated HL-60 cells, the same peripheral location was detected in the centromeric regions of chromosomes 2, 7, 8, 9, 17 and X. All human chromosomes were determined in the specific nuclear layers.
Our studies contribute to the knowledge of the high order chromatin structure of segmented granulocytes whose chromatin arrangement can play an important role in their immunity function.

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1A-3
3UNZIPPING DNA WITH HIGH SEQUENCE RESOLUTION

Ulrich Bockelmann, Philippe Thomen, François Heslot

Employing techniques of molecular biology and surface chemistry, we separately attach the two strands on one end of a single DNA molecule to a glass slide and a microscopic silica bead. The bead is held by an optical trap and the surface is laterally displaced. This induces a progressive mechanical unzipping of the double helix. Currently we are using an optical trapping interferometer with fast data acquisition combined with a computer controlled system for the sample displacement which allows to measure rapid force variations with sub-pN resolution at high measurement stiffness. A sequence specific force signal is obtained, with details reflecting the genomic sequence. We report on results obtained on a molecular construction which has been optimised for high stiffness and high stability of the attachment to the solid surfaces. A comparison of experimental and theoretical results indicates that the present measurements on DNA unzipping provide very local information on the sequence: the detection of single base pair mutations appears to be in reach.

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1A-4
INVESTIGATION OF THE INFLUENCE OF DIMETHYLSULPHOXIDE ON THE DNA INTERACTION WITH PLATINUM COMPOUNDS IN SOLUTION

Nina Kasyanenko, Alexei Bogdanov, Yulia Kosmotynskaia

The role of dimethylsulphoxide (DMSO) during the *cis*- and *trans*-DDP interaction with DNA molecule in a solution was studied. It was shown, that an optical anisotropy, effective volume and spectral properties of macromolecule change variously at DNA interaction with *cis* and *trans* – DDP in a solution. The DNA parameters in complexes prepared by the consecutive addition of DMSO, *cis*- and *trans*-DDP after necessary time of exposure were determined. The complexes formed after addition of platinum compounds to DNA solution in a various sequence, and also at simultaneous addition of drugs were compared The experience has shown, that the DNA interaction with *cis* – DDP is realised irrespective of, whether it is added to a DNA + *trans*-DDP complex or to pure DNA. The similar result was obtained for trans-DDP. The study of DNA spectral properties indicates the competition between *cis*- and *trans* – DDP for the binding position on DNA. On the contrary, hydrodynamic experiments and the definition of the DNA optical anisotropy have shown, that *trans* – DDP binding causes strong conformation changes in DNA. These changes were unaffected by the following *cis*-DDP binding to DNA. The influence of DMSO on the competition between *cis*- and *trans*-DDP for the binding position on DNA was studied.

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1A-5
DYNAMIC LIGHT SCATTERING OF CONGESTED AQUEOUS SOLUTIONS OF DNA

I. Drevensek-Olenik, M. Copic, V. Arrigler, R. Podgornik

Depolarized dynamic light scattering (DLS) studies of the long ($M_w > 10^8$) DNA segments dissolved in 0.5 M NaCl were performed for sample concentrations ranging from c = 1 mg/ml fto c = 200 mg/ml. This range of concentrations coincides with the range of weakly birefringent structures, which were observed with optical polarization microscopy as intermediate phases between the isotropic (I) and the cholesteric (Ch) lyomesogenic phase. In the most diluted samples (c = 1 mg/ml) only one diffusive mode with the apparent diffusion coefficient $D \approx 3 \cdot 10^{-7}$ cm^2/s was detected. This mode is assigned to the translational diffusion of the DNA molecules and is in agreement with the data reported in the literature (A. W. Fulmer at al., 1981, K. S. Schmitz at al., 1984). The corresponding hydrodynamic radius of the scatterers is about 10 nm. In more congested samples the autocorrelation function $g^1(\tau)$ of the scattered light profoundly deviates from the single-exponential decay, displaying an additional slow dynamic process (time scale of $\approx$1 s) with approximately logarithmic temporal behaviour. With increasing concentration this process becomes strongly predominant over the translational diffusion.

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1A-6
METAL IONS EFFECTS ON PHASE EQUILIBRIUM IN POLYA·POLYU AND POLYA·2POLYU

Galina Gladchenko, Victor Sorokin, Vladimir Valeev, Marina Degtyar, Yurii Blagoi

Mg^{2+}, Ni^{2+}, Cu^{2+}, Cd^{2+} and Zn^{2+} interactions with single-stranded (1S) polyA and polyU, double(2H) – and triple-helical(3H) structures formed by these homopolynucleotides in buffer solutions (pH 6-7) with different Na^+ ion contents (10^{-2}–10^{-1}M) were studied by the method of difference UV spectroscopy. Diagrams of the phase equilibrium between 1S, 2H and 3H structures were built for the concentration range 10^{-5}–10^{-3}M of divalent ions. State diagrams for (polyA + polyU) + Mg^{2+}, Ni^{2+} are qualitatively similar to that of polyA + polyU + Na^+ [1]. Mg^{2+} binding to phosphates stabilizes 1-,2- and 3-helical structures and preferentially MH of higher hierarchy. At [Mt] > $3 \cdot 10^{-5}$M (a triple point at the phase diagram) Ni^{2+} stabilizes 2H more effectively than Mg^{2+}: temperatures of transitions 2H→3H exceed those for Mg^{2+}. t_m values for transitions 3H→1S in polyA·2polyU in the presence of Mg^{2+} and Ni^{2+} coincide.

Mg^{2+} and Ni^{2+} ions restore the helical structure on their interaction with phosphate groups of polyA·polyU melted parts. Ni^{2+} binding to N7A occurs at $4 \cdot 10^{-2}$M.

Zn^{2+} stabilizes both 2H and 3H. Cd^{2+} weakly affects 3H→2H and 2H→1S transitions while Cu^{2+} destabilizes triplexes. The Me^{2+} effect on t_m 3H→2H and t_m 2H→1S obeys to the Mg > Ni > Zn > Cd > Cu raw. High concentrations of transition metal ions ([Cu^{2+}] > 10^{-4}, [Cd^{2+}] > $2 \cdot 10^{-4}$, [Zn^{2+}] > 10^{-3}) induce aggregation of 1S.

[1] Klump, H.H., *Can. J. Chem.* 1988, 66, 804

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1A-7
COPPER IONS EFFECT ON DNA STRUCTURAL TRANSITIONS IN AQUEOUS AND MIXED SOLUTIONS

Elene V. Hackl*, Yuri P.Blagoi

Using methods of IR spectroscopy and light scattering DNA structural transitions under the action of Cu^{2+} ions in aqueous and mixed solutions containing ethanol and 1,2-propanediol (0 ÷ 20 vol. %) at temperatures 29 and 45 ^{0}C are studied. It was shown that on its interaction with Cu^{2+} ions in aqueous and mixed solutions DNA transits into compact state. The measure of sizes of compact particles is obtained and the compactisation model is proposed. The results obtained permit to suppose that the mechanism of DNA condensation under Cu^{2+} ion action is not completely electrostatic and depends on Cu^{2+} ions interaction with DNA bases. Probably, partial DNA destabilization taken place as a result of such interaction facilitates the DNA compactisation under divalent copper ion action in aqueous solution.

DNA compactisation dependencies on the counterions valence, temperature and content of solvents are studied. It is stated that in mixed solutions DNA compactisation depends not only on the solution dielectric permeability but on the solution structure.

The results obtained are being discussed in the frameworks of the theories of counterion condensation (CCT) and equilibrium binding. Unlike CCT, the theory of equilibrium binding shown to describe satisfactorily the DNA compactisation process under the Mt^{2+} ion action.

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1A-8
THERMODYNAMIC PROPERTIES OF DNA CONTAINING INTERSTRAND CROSS-LINK OF ANTICANCER CISPLATIN

C. Hofr, V. Brabec

Cisplatin. [*cis*-diamminedichloroplatinum(II)] is an effective chemotherapeutic agent for the treatment of testicular cancer and is used in combination regiments for a variety of other tumors. The anticancer activity of cisplatin arises from its ability to damage DNA, with adducts formed being various types of cross-links. The *trans* isomer of cisplatin (transplatin) also covalently binds to DNA, but is clinically ineffective. Short oligodeoxyribonucleotide duplexes (20 base pairs) were purified containing single, site-specific interstrand cross-link of either isomer at the central d(TGCT)/d(AGCA) sequence. These duplexes were analysed by differential scanning calorimetry. Thermodynamic data acquired during thermal transition of the modified DNA were used to characterize the effect of the cross-link on the duplex stability. Inspection of measured thermograms revealed significant differences between thermal stability of DNA cross-linked by *cis* or *trans* isomer. The interstrand cross-link of cisplatin increased thermal stability of the duplex noticeably more than that of transplatin. On the other hand the cross-link of cisplatin caused a smaller increase of the overall thermodynamic stability of the modified duplex than that of transplatin. Energetic properties of DNA containing the interstrand cross-link of cisplatin or transplatin were related to available structural data and to the distinctly different clinical efficacy of both isomers.

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1A-9
OPTICAL ANISOTROPY OF CELL NUCLEI DEPENDENCE ON TISSUE HISTOLOGICAL EMBEDDING METHODS

Tatjana Ivanova, Talivaldis Freivalds, Indulis Buikis, Liga Harju

We have shown, that cell nuclei optical anisotropy has been observed in two cases: 1) in the stained nuclei with anisotropic dyes such as toluidine blue, etc. (1st type birefringence); 2) mainly in paraffin tissue sections in unstained cell nuclei (2nd type birefringence). In the first case cell nuclei anisotropy depends from orientation of dye molecules on DNA filaments and stacking between them as well as orientation of DNA filaments themselves in chromatin. In the second case anisotropy was observed mainly in the cell nuclei in paraffin sections. Cell nucleus protein composition and organisation has been associated with anisotropy in this case. The purpose of this work was to estimate tissue paraffin embedding process influence on cell nuclei optical anisotropy. Two organ tissues of white rats were used in experiments: spleen and liver. Cell nuclei birefringence had been compared in specimens, which were fixed with the Cornoy's mixture and embedded in paraffin by two different ways: by classical way and by longer, more gently one – to allow molecules to have some orientation. Polarisation and phase contrast microscopes were used for anisotropy observing and counting and interference microscope was used for birefringence measuring.
The spleen and liver birefringent cell nuclei number was larger using more gentle embedding procedure (5.6 ± 0.6)% versus (3.1 ± 0.6) % and (11.7 ± 0.5) % versus (7.3 ± 0.6) % respectively.
The results shows that paraffin embedding process influence the cell nuclei optical anisotropy.

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1A-10
THERMODYNAMICS OF FOLDING TRANSITIONS IN THE HAIRPIN RIBOZYME: A TR-FRET STUDY

Dagmar Klostermeier, Nils G. Walter, John M. Burke, David P. Millar

The hairpin ribozyme in tobacco ringspot virus satellite RNA consists of two essential helix-loop-helix elements in a four-way helical junction. The catalytically active form of the ribozyme is a "docked" conformation with loop A, which comprises the cleavage site, and loop B on a different helical arm in close proximity. The open and docked conformations can be distingiushed in time-resolved fluorescence resonance energy transfer (tr-FRET) experiments: In ribozymes that are labeled with fluorescein (donor) and tetramethylrhodamine (acceptor) on the ends of the helical arms that carry the loops docking significantly increases the fluorescence energy transfer efficiency.
Compared to a two-way and three-way junction the four-way junction significantly promotes docking of the ribozyme. This may be due to favourable interactions between the helical arms in the docked conformation. Alternatively, the four-way junction may reduce the entropic cost of docking in comparison with the more flexible two-way junction ribozyme. To test this hypothesis, the temperature dependence of the docking-undocking equilibrium as measured by tr-FRET was analysed with a coupled equilibrium model that accounts for overlapping secondary and tertiary structure transitions to yield the respective ΔH and ΔS values. These results will provide a deeper understanding of the factors governing the folding and stability of RNA molecules.

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1A-11
THE HIGHER-ORDER ARRANGEMENT OF CHROMATIN IN CELL NUCLEI

S. Kozubek, E. Lukášová, E. Bártová, M. Kozubek, M. Skalníková, P. Jirsová, I. Koutná, A. Cafourková

Genes, centromeres and chromosomes are located in specific subregions of cell nuclei which form distinct layers at defined centre-of-nucleus to locus distances. Inside these layers, the genetic loci are distributed randomly. Some chromosomes are polarised with genes found in the inner parts of the nucleus and centromeres located on the nuclear periphery; the polar organisation was not found for some other chromosomes. The arrangement of genetic loci into layers as well as the polar and non-polar organisation of chromosomes are basically conserved in different cell types and in various stages of the cell cycle. Some features of the nuclear structure are conserved even in differentiated cells and during cellular repair after irradiation, although shifted positioning of genetic loci was systematically observed during these processes. The nuclear higher-order structure is related to the gene expression. Transcriptionally active regions are located in the inner parts of cell nuclei and the transition of some genetic loci to the nuclear periphery can be accompanied by the down-regulation of the loci. The structure of the cell nucleus is an important factor in the induction of chromosome aberrations. Exchange aberrations between two genetic loci are obviously less probable if the genetic loci are well separated from each other in the nucleus; if two genetic loci are superimposed in a small nuclear volume, the probability of exchange aberrations will be markedly increased.

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1A-12
IMPROVED ELASTIC ROD MODEL OF 3D RNA STRUCTURE

Ekaterina E. Kozyreva, Eugeny I. Kugushev, Aleksey V. Maikov, Eugeny L. Starostin

A problem of reconstruction of approximate large-scale 3D structure of an RNA molecule from its secondary structure and latest experimental and theoretical data on thermodynamic stability of its structural elements is considered. Both a new mathematical model and its computer implementation are presented. An RNA molecule is treated as a set of basic structural elements (dangling ends, stems and loops of various types) modeled by elastic rods with different elastic parameters depending on the primary structure of the corresponding structural element. A numerical procedure is developed for computation of shapes of the RNA elements and for assembling the whole molecule. The comparison of the available X-ray diffraction analysis results (yeast phenylalanine tRNA) with the proposed rod model reveals a good correspondence of the overall tracing of the polynucleotide chain.

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1A-13
DNA DELIVERY BY PHAGE: A STRATEGY TO FORM TOROIDAL CONDENSATES OF ARBITRARY SIZE INTO LIPOSOMES

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We report a new strategy for encapsulating and condensing DNA. When T5 phage binds to its *E. coli* membrane protein receptor, FhuA, its double stranded DNA (120 000 bp) is progressively released base pair after base pair in the surrounding medium. Using cryo-electron microscopy we have vizualized the structures formed after T5 phage DNA is released into neutral unilamellar proteoliposomes reconstituted with the receptor FhuA. In the absence of spermine, the transferred DNA appeared to occupy uniformely all the internal space of the liposome. In the presence of spermine, toroidal condensates of circumferentially wrapped DNA were formed. The sizes of these toroids were shown to vary, from 90 to 200 nm in their outer diameters, depending upon the number of DNA stands transferred. We have also analyzed T5 DNA release in bulk solution containing the detergent-solubilized FhuA receptor. After DNA release in a spermine containing solution, huge DNA condensates with a diameter of about 300 nm were formed containing the DNA's from as many as 10–20 capsids. At alkaline pH, the condensates appeared as large hollow cylinders with a diameter of 200 nm and a height of 100–200 nm. These condensates were formed rapidly and have been obeserved 15 minutes after the mixing of FhuA and T5 phages Due to the progressive release of DNA from the phage capsid, the mechanism of toroid formation leads to toroids of arbitrary and controllable size.

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1A-14
ELASTICITY OF DNA – INSIGHTS FROM MOLECULAR DYNAMIC SIMULATIONS

Filip Lankas, Jiri Sponer, Pavel Hobza, Joerg Langowski

Harmonic elastic constants of 3-11 base-pair DNA oligonucleotides containing adenine, guanine, inosine (I) and 2-aminoadenine (D), were evaluated using seven 5ns unrestrained molecular dynamics simulation trajectories of 17-bp duplexes with explicit inclusion of water and counterions. All simulations were carried out with the Cornell *et al.* force field and particle mesh Ewald method for long-range electrostatic interactions. The elastic constants including anisotropic bending and all coupling terms were derived by analysing the correlations of fluctuations of structural properties along the trajectories. The following sequences have been considered: homopolymers d(ApA)n, d(GpG)n, d(IpI)n, d(DpD)n, and alternating d(ApT)n, d(ApI)n and d(GpC)n. The calculated values of elastic constants are in very good overall agreement with experimental values for random sequences. The atomic-resolution molecular dynamics approach, however, reveals a pronounced sequence-dependence of the stretching and torsional rigidity, while sequence-dependence of the bending rigidity is smaller for the sequences considered. The present work shows that large-scale, all-atom molecular dynamics simulations represent a unique source of data to study DNA elasticity. This work is a substantial extension of our recent results (Lankas et al., J. Mol. Biol. (2000) 299 (3), 695–709).

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1A-15
MATHEMATICAL MODELING OF RNA SECONDARY STRUCTURE FORMATION

E.E. Kozyreva, E.I. Kugushev, A.V. Maikov, E.L. Starostin

A universal method for speeding up calculations in the solution of problems on mathematical simulation of the process of biological macromolecules (nucleic acids, proteins) structurization is described. The ration of the molecular chain lengthening speed to the speed of formation and breaking of structural bounds determines the development of structure formation process. The computational complexity of the simulation is estimated as N5, where N – is the whole length of the molecular chain. The structure formation process can be led to the multiple calculations of locally optimal path on an oriented, dynamically changing graph describing transitions admitted inter-structurally. The set of such transitions for each graph vertex grows monotonously with the molecular chain growth. This property allows reducing the computational expenditures up to N4. The method has been used in mathematical modeling and studding of secondary structure formation of wide range of ribonucleic acids (RNAs) of different types. The possible options of the methodology are analyzed.

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1A-16
PHYSICAL PROPERTIES OF BOUND WATER IN HYDRATE SURROUNDINGS OF DNA

Vladimir Y. Maleev, Anatoly I. Gasan, Konstantin M. Virnik, Michael A. Semenov

One of the most important factors determining the secondary structure of DNA and its complexes with biologically active ligands is hydration surroundings. In this paper results of experimental studies of physical properties of bound water in hydrate surroundings of DNA with various nucleotide compositions are presented. The results of investigation of DNA-water system obtained by calorimetry, IR spectroscopy and gravimetry techniques show that in the hydrate surroundings one can distinguish three subsystems with different energetic properties of bound water molecules in the dependence on AT/GC content. A close relationship of energetic and dynamic properties of bound water allows us to calculate the values of dynamic parameters characterizing mobility of water in the DNA hydrate surroundings as dielectric relaxation time of the water dipole, "free volume" occupied by the water molecules in the hydrate shell. It has been found that dynamic mobility of the water in the DNA hydrate surroundings decreases as the GC-pair relative content increases in the sample. A lattice model of bound water diffusion along a DNA surface with taking into account the difference between DNA fractal dimensions for A- and B-form has been proposed. On the base of the model we have estimated values of the water diffusion coefficients in the hydrate shell of A- and B-DNA.

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1A-17
INVESTIGATION OF THE DYNAMICS OF SPIN LABELED SINGLE AND DOUBLE STRAND DNA FRAGMENTS BY EPR SPECTROSCOPY

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DNA in solution is a flexible macromolecule and shows a complex set of dynamic modes. The DNA fragments which have been investigated are 40-mers in B-conformation labeled with a nitroxy radical spin label in order to make possible EPR-measurements. Both, single and double DNA strands have been investigated, the latter ones with one and with two labels situated at each of the strands. EPR experiments have been performed at low temperatures and at room temperature. The spin label shows a significant hyperfine splitting. Dynamic effects like globel tumbling and internal motions have to be taken into account for the interpretation of the spectral line shapes at room temperature. Spectra recorded at temperatures below 200 K do not show these strong motional effects. Experiments with double labeled double strand DNA fragments have been performed in order to investigate possible magnetic interaction between the labels. This interaction causes an additional line broadening at low and at high temperatures.

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1A-18
AB INITIO CALCULATIONS OF EXCITED STATES ON ADENINE AND ITS COMPLEXES WITH METAL IONS

Alexandra Yu. Rubina, Viktor A. Sorokin, Yuri V. Rubin, Manoj K. Shukla, Jerzy Leszczynski

The molecular geometry optimization and transition energy calculations were performed for adenine (A) and its complexes with ions of few transition metals using Gaussian98. Ground geometry optimization was carried out the HF/6-31 + G** and MP2/6-31G** levels of theory, while transition energies were calculated using the configuration interaction involving singly excited configurations (CIS method).
It has been found that the most stable form of $A\cdots Cu^+$ complex is the N7H tautomer of A with Cu^+ bonded at the N9 site of the molecule. The binding of Zn^+ ion is also found to take place at the N9 site of adenine. The observed spectral shift has been discussed in terms of the computed transition energies of these complexes.

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1A-19
HYDRATION OF DIFFERENT STRUCTURAL FORMS OF POLY*RC*: A MONTE CARLO SIMULATION

A.V. Shestopalova, D.B. Anischenko, V.Ya. Maleev

The polyribocytidylic acid (poly*r*C) is known to form various structures in aqueous solutions in the dependence on pH: an ordered single-strand helix, a helical double-strand complex and disordered chain. The aim of this work is to elucidate the role of water in the formation of different structures of poly*r*C.
In the investigation each system represented a cluster of 400 water molecules with molecule placed at its center. The simulation were performed at T = 298 K. Interactions between the molecules were taken into account by means of semiempirical atom-atom potential functions. Cytosine protonation was taken into account by means of partial atom charges recieved by the method MP2. As the objects to be studied neutral and protonated cytosine, their H-bonded pairs and stacked dimers, cytidine monophosphate, tetranucleotude in single-strand and it duplex in double-strand forms of poly*r*C have been choosen.
The analysis of energetic and structural characteristics considering systems allowed us to make such conclusions. The main factor of structure stabilization for helical systems is the water molecules formed bridges and networks around poly*r*C. The stability of a double-strand poly*r*C is connected both with interaction between opposite chains and formation of specific water structure around them. In conclusion a model of the hydration of poly*r*C is proposed.
Partial support for this work from the INTAS'97 grant 31753 is gratefully acknowledged.

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1A-20
SELF-AGGREGATION OF 2'-DEOXYGUANOSINE 5'-MONOPHOSPHATE IN ISOTROPIC AQUEOUS SOLUTIONS

Lea Spindler, Irena Drevensek-Olenik, Martin Copic, Paolo Mariani

Guanosine molecules in aqueous solutions self-aggregate into stacked arrays of planar hydrogen-bonded guanosine tetramers. Congested solutions of guanosine exhibit lyotropic liquid crystalline polymorphism, forming the cholesteric and the hexagonal phase.
Isotropic aqueous solutions of the ammonium 2'-deoxyguanosine 5'-monophosphate d(pG) in the concentration range 0.1–10 wt% were investigated by dynamic light scattering. In diluted solutions up to 5 wt% only one slow diffusive mode with a diffusion coefficient in the interval of $0.4–9.0 \cdot 10^{-9}$ cm^2/s is observed. This mode is assigned to the translational motion of spherical aggregates, similar to those observed for DNA solutions. The hydrodynamic radius of the aggregates increases with increasing concentration from 0.24 μm to 5.4 μm. The existence of such aggregates was confirmed by AFM and optical microscopy. In the concentration range from 5 to 10 wt%, in addition to the slow mode, also a faster diffusive mode is observed. The diffusion coefficient of the fast mode is in the interval of 2.0–3.1 10^{-7} cm^2/s and increases with increasing concentration. The appearance of this mode is correlated with the appearance of a secondary resonance line in the ^{31}P NMR spectra and corresponds to a cooperative motion of the guanosine tetrameric stacks. This shows that the stacks are extensively formed only at concentrations close to the isotropic-cholesteric phase transition.

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1A-21
DOES L-METHIONINE INCREASE THE RATE OF REACTION OF PLATINUM ANTITUMOR DRUGS WITH DNA?

Oldrich Vrána, Viktor Brabec

DNA platination is thought to be a key event in the mechanism of action of platinum anticancer drugs. A significant portion of the administered platinum drug binds to thiols containing compounds such as cysteine, glutathione and methionine. The formation of the Pt(II)–sulfur adducts is generally believed to terminate anticancer activity of Pt cytostatics and induce severe nephrotoxicity and tumor resistance to treatment with platinum drugs. Quite recently it has been shoved that cisplatin and carboplatin readily undergo interactions with methionine. Suprisingly, monodentate S-bound methionine can be displaced by guanosine monophosphate residues. It was suggested that methionine residues in peptides and proteins could play a role in the transfer of Pt drugs onto DNA. To investigate this possibility we have further studied the interaction of single- and double-stranded DNAs with Pt-methionine complex by HPLC and FAAS. The results indicate that in contrast to the reaction of monomeric GMP Pt-methionine complex does not coordinate to high-molecular-mass DNA. Thus, the results of this work support the view that L-methionine inhibits reaction of polymeric DNA with antitumor cisplatin.

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1A-22
FRET FLUCTUATION SPECTROSCOPY: UNRAVELLING THE CONFORMATIONAL MOTIONS OF A DNA HAIRPIN-LOOP

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The motions of a dye-labelled DNA hairpin-loop (Cy5-5′-GGGTT-$(A)_{30}$-AACCC-3′-TMR) have been investigated through the fluctuations in proximity ratio from fluorescence resonance energy transfer (FRET). We examine three solution conditions: 1) MilliQ water, 2) Tris-EDTA buffer, 3) Tris-EDTA buffer plus excess of DNA complementary to the loop sequence, $(T)_{30}$. Correlations in proximity ratio show sub-millisecond dynamics, consistent with other measurements of hairpin-loop kinetics. Static heterogeneity is revealed from the distribution of proximity ratio amplitudes. The observed stretched exponential kinetics are consistent with a model based on the transition between two states over a complex energy landscape. Using this multiple-pathway, two-state model, the single-molecule proximity ratio distribution can be simulated and comparisons were made to experiment.

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1B-1
IN-SITU OBSERVATION OF THE SYNTHESIS OF FLUORESCENT-RNA BY SINGLE RNA-POLYMERASE

Christophe Place, Zoher Gueroui, Eric Freyssingeas, Bruno Berge

We present experiments about the in-situ polymerization of a RNA strand by T7-polymerase transcribing DNA. The synthesis is observed through the appearance of fluorescent spots under the microscope, by using fluorescent-UTP. T7 Phage DNAs strands are immobilized on a hydrophobic surface at a sufficiently low concentration, allowing to observe each DNA well separate from neighbors on a given picture. We demonstrate experimentally that this DNA is in a state which allows transcription. We show the co-localization of appearing fluorescent RNA spots with the DNA spots (observed at the end of the experiment through yoyo staining). This transcription is directed by T7 promoters present on the T7 DNA, since a control experiment using λ-phage DNA, which has no promoter sequences for the T7-RNA polymerase, shows no RNA synthesis. To some extend transcription termination is also observed, through the release of fluorescence in the bulk solution when exposing the system to non-fluo-NTP's.

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1B-2
DNA INTERACTION WITH SYNTHETIC POLYCATIONS IN A SOLUTION

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The DNA conformation parameters in the complexes with different synthetic polymers were determined. The calf thymus DNA samples of different molecular weight have been utilized. The interaction of DNA with different synthetic polymers (polycations with the different charge density) under various ionic conditions was explored.

Is was shown, that the dependence of the DNA effective volume on polycation concentration has a complicated character. The changes in the DNA UV-absorption and circular dichroism spectra are observed during the DNA interaction with some synthetic polycations. The increase in the DNA optical anisotropy is indicated also with the increase of polycation concentration in a solution. The analysis of the experimental data allows to conclude that in some cases the contact of polymers with the DNA nitrogen bases in the major groove is realized during the interaction. In this case the DNA packing with the increase of the polycation concentration in a solution is accompanied by the local destabilization of the DNA secondary structure. The analysis of polyions interaction at different pH values was carried out.

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1B-3
DIRECT OBSERVATION OF SLIDING OF RESTRICTION ENDONUCLEASE *ECO*RI ON A SINGLE DNA MOLECULE

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In recent years, a fluorescence microscopy technique has been used to image the dynamics of individual DNA and protein molecules. For the advanced investigation of the molecular mechanism in DNA-protein interactions such as sliding of restiction endonucleases on DNA molecules, direct observation of a single protein molecule will be significant. To observe dynamics of individual proteins under a fluorescence microscopy on real-time, it requires labeling proteins with a fluorescent dye. In this study, therefore, we developed fluorescent labeling system for a restriction endonuclease *Eco*RI as a model to label DNA binding proteins. *Eco*RI bound on DNA molecules was treated with amine-reactive dye Oregon-Green500. Consequently, we found that restriction endonuclease activity of labeled *Eco*RI was retained, even though *Eco*RI was flurescently labeled. Moreover, when DNA-staining dye YOYO-1 concentration was YOYO-1 : nucleotide pair = 1 : 100 in molar ratio, *Eco*RI digested the DNA molecules as unstained DNA. Finally, we observed that fluorescent labeled *Eco*RI slid on stained DNA straightening on 3-APTES-treated cover glass in the absence of Mg^{2+} using a fluorescence microscopy.

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1B-4
THE INTERACTION OF HUMAN PAPILLOMAVIRUS ONCOPROTEIN E6 WITH CRUCIFORM DNA: A BIACORE STUDY

Yves Nominé, Laurence Choulier, Danièle Altschuh, Gilles Travé

E6 is an oncoprotein implicated in cervical cancers, produced by high-risk human papillomaviruses. We found recently that E6 interacts with cruciform four-way DNA junctions. This was demonstrated by using gel-retardation electrophoretic methods; however, this technique left an uncertainty about the effect of magnesium ion on the interaction. This point is important since this ion is present *in vivo*. Here, we have studied the characteristics of the interaction by BIAcore. We managed to demonstrate that the interaction happens in presence of magnesium, indeed with a better affinity than in the absence of this ion. Moreover, we developed a new model to analyze the BIAcore data. This analysis reveals two distinct association rate constants for E6 binding to DNA junction. The highest rate constant probably corresponds to the interaction between E6 and its structure-dependant site on the DNA, whereas the lowest rate constant corresponds to low-specificity binding to regular double-stranded DNA. This is the first BIAcore analysis of a structure-dependant protein/DNA interaction. The analysis model that we have developed can be adapted for future BIAcore studies of DNA-protein interactions.

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1B-5
THE MODE OF HMG1/DNA INTERACTION CHANGES WITH THE INCREASE OF PROTEIN/DNA RATIO

Alexander Polyanichko, Elena Chkhirzhina, Svetlana Davydenko, Vladimir Vorob'ev

It is known that the most abundant non-histone chromatin protein HMG1 exhibit the ability to package DNA into complexes which can penetrate into cells with subsequent expression of the reporter gene. The aim of this work was to study the interaction of the protein and DNA (both vector pCMV Luci {6011 bps}, and calf thymus). The rat recombinant HMG1 was obtained with an expression system, based on methylotrophic yeast *Pichia pastoris*. The complexes with input protein/DNA ratio **r** (w/w) in the range of 0 ÷ 9 were studied in solutions with different ionic strengths by the methods of analytical ultracentrifugation, gel retardation and circular dichroism.
It has been shown that the HMG1-DNA interaction is strongly dependent on the DNA conformation and protein concentration. The manner of HMG1 interaction has changed in the vicinity of $\mathbf{r} = 5$. It has been confirmed by CD analysis that the direct protein-DNA interaction has occurred at the values of $\mathbf{r} < 4$. We've estimated HMG1 binding site on the DNA at this stage as ~15 bp. At higher protein concentration some protein-protein interactions have been involved in the complex formation. We suppose that the transfection active complexes between HMG1 and plasmid DNA can be formed at this stage. Also it has been shown that the complexes obtained at high protein content were stable in solutions with extremely high ionic strength, though the formation of the complexes depends on salt concentration.

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1B-6
SIMULTANEOUS BINDING OF TWO DNA DUPLEXES TO THE NTRC-ENHANCER COMPLEX STUDIED BY TWO-COLOR FLUORESCENCE CROSS-CORRELATION SPECTROSCOPY

Karsten Rippe

The transcription activator protein NtrC (nitrogen regulatory protein C) can catalyze the transition of *E. coli* RNA polymerase complexed with the sigma 54 factor ($RNAP{\cdot}\sigma^{54}$) from the closed complex ($RNAP{\cdot}\sigma^{54}$ bound at the promoter) to the open complex (melting of the promoter DNA). This process requires phosphorylation of NtrC (NtrC-P), assembly of an octameric NtrC-P complex at the enhancer DNA sequence, interaction of this complex with promoter bound $RNAP{\cdot}\sigma^{54}$ via DNA looping, and hydrolysis of ATP. The DNA binding of NtrC and NtrC-P was studied by two-color fluorescence auto- and cross-correlation spectroscopy (FCS/FCCS) measurements of 6-carboxy-fluorescein and 6-carboxy-X-rhodamine labeled DNA oligonucleotide duplexes. The experiments revealed that the NtrC-P complex can bind two DNA duplexes simultaneously. This activity was not present with the unphosphorylated NtrC protein. In addition, a method to derive equilibrium binding parameters from the FCS/FCCS data was developed that allows a quantitative analysis of the stability of the different NtrC-P-DNA complexes. The results suggest a model for the conformation of the looped intermediate that is formed between NtrC-P and $RNAP{\cdot}\sigma^{54}$ at the *glnA*p2 promoter during the transcription activation process.

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1B-7
GENERATION OF HYDROXYL RADICALS (OH•) PULSES IN SOLUTION BY SYNCHROTRON RADIATION FOR TIME RESOLVED OH• RADICAL FOOTPRINTING

Manfred Roessle, Evgeny Zaychikov, Bianca Scalvi, Malcolm Buckle, Michael Wulff, Friedrich Schotte, Hermann Heumann

The basic principle of the OH footprinting technique is the study of the accessibility of the DNA by OH radicals in the presence of bound protein. OH radicals cleave DNA regularly at each base position. These regions of the DNA that interact with the protein are precluded from cleavage and appear in a gel electrophoresis pattern as windows in the otherwise regular cutting pattern. The rate limiting step of the cleavage reaction is the production of the OH radicals. While chemical generation of radicals limits the time interval to 20s, and is therefore to slow for time resolved investigation, the production of OH radicals using the high flux of ionizing radiation is a fast process and shift the time interval in the msec region. For this purpose the white beam of the X-ray synchrotron radiation spectrum at ESRF's Laue beamline was used to generate short pulses of OH radicals in the msec time range. Within this short time interval the binding and the movements of the DNA-dependent RNA-polymerase during the transcription of DNA into mRNA were traced. The fast mixing of the components was achieved by a continuos flow device, which controlled the flow as well as the reaction time.

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1B-8
DETECTION OF SPECIFIC PROTEIN BINDING BY MECHANICALLY OPENING DNA

Philippe Thomen, Lucile Loiseau, Ulrich Bockelmann, François Heslot

We have chosen the model system of the endonuclease EcoRV acting on λ DNA under in vitro conditions known to allow site specific binding but prevent cutting. We study the binding by recording the separation force, while unzipping DNA in a single molecule configuration. The bound protein acts as a "road block" opposing transiently to the strand separation. Sequence specific force variations are measured with an optical trapping interferometer. Superposed on the average value of about 15 pN corresponding to the strand separation, we observe distinct localized events where the force rises sizeably. We show that this corresponds to the binding of EcoRV at its cleavage site, followed by a force induced ejection of the protein. The amplitude of the force rises shows a statistical variation in the range of 5–25 pN.

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1B-9
DISTANCE MEASUREMENTS ON NUCLEOSOMALER LINKER DNA BY FRET

Katalin Tóth, Nathalie Brun, Jörg Langowski

While the structure of the nucleosome core is known in atomic detail, theprecise geometry of the DNA beyond the core particle is still unknown.
We have used fluorescence resonance energy transfer (FRET) for determining the end-to-end distance of DNA fragments assembled with histones into nucleosomes. The DNA of a length of 150 to 220 bp was labeled with rhodamine-X on one end and fluoresceine or Alexa 488 on the other.
Assembling nucleosomes on these DNA fragments leads to a measurable energy transfer. The end-to-end distance computed from the FRET increases from 65 ± 5 Å at 150 bp to 85 ± 5 Å at 180 bp without measurable change above of it.
These distances are compatible with different geometries of the linker DNA all having in common that no crossing can be observed up to 220 bp.
Addition of H1 histone leads to an increase in energy transfer, indicating a compaction of the linker DNA towards the nucleosome.

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1C-1
THE MECHANISM OF INTRAPROTEIN RADICAL TRANSFER DURING PHOTOACTIVATION OF DNA PHOTOLYASE

Corinne Aubert*, Marten H. Vos†, Paul Mathis*, André P.M. Eker‡, Klaus Brettel*

DNA photolyase catalyses the repair of major UV-induced DNA lesions (pyrimidine dimers), using the energy of near-UV light. This enzyme is found in a variety of organisms ranging from bacteria to multicellular eukaryotes. It binds a flavin adenine dinucleotide (FAD) as the essential catalytic cofactor, and it only functions when the FAD is fully reduced ($FADH^-$). The catalytically inactive semi-reduced radical state $FADH^•$ can be reduced to FADH by illumination with visible light in the presence of an exogenous electron donor, a process named photoactivation.
We studied the mechanism of photoactivation of *E. coli* DNA photolyase using time-resolved absorption spectroscopy. We show that excited $FADH^•$ abstracts an electron from a nearby tryptophan in 30 ps. After subsequent electron transfer along a chain of three tryptophans, the most remote tryptophan (as cation radical) releases a proton to the solvent in about 300 ns. In contrast to charge-neutral H-atom transfer, suggested by others to be the prevailing mechanism of long-range radical transfer in proteins, our results demonstrate the feasibility of sequential electron transfer generating amino acid cation radicals before charge neutralisation by deprotonation.

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1C-2
IS THERE ANY ROLE OF DNA DAMAGES IN THE PHOTOINDUCED INACTIVATION OF NON-ENVELOPED VIRUSES SENSITIZED BY PORPHYRIN DERIVATIVES?

Gabriella Csík, Marianna Egyeki, Philippe Maillard, Katalin Tóth

The photo-chemical treatment is a new and very promising approach in the virus inactivation and consequent disinfection of biological fluids and blood products. Due to the permanent risk of the transmission of infectious diseases, this field is kept under intensive study. Although photo-treatment is already in use, the mechanism of virus-photosensitizer interaction and viral photoinactivation is still unknown. The scope of present study was the investigation of the primary target of photodynamic action in the nucleoprotein complex and the effect of molecular structure of sensitizer on dye – nucleoprotein/nucleic acid binding. We selected T7 phage as a model of non-enveloped viruses holding double-stranded DNA. Glycoconjugated tetraphenyl porphyrins were selected as photosensitiser. The structural changes in the photochemically treated nucleoprotein complex were followed by optical melting studies. It was found that structural changes caused by the photodynamic action were localised in the protein part but not in the DNA. The inactivation of the virus was in good correlation with the changes of the protein melting temperature.

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1C-3
SINGLE MOLECULE FLUORESCENCE MICROSCOPY OF NUCLEOTIDE EXCISION REPAIR COMPLEXES USING GFP FUSION PROTEINS

Ine Segers-Nolten, Suzanne Rademakers, Wim Vermeulen, Aufried Lenferink, Cees Otto, Jan Hoeijmakers, Jan Greve

Nucleotide Excision Repair (NER) is one of the important DNA-damage eliminating pathways, in which there are involved many different proteins. It is our aim to determine the architecture of NER complexes and to study dynamics of interactions that take place within these structures on a single molecule level.
For this purpose some NER proteins are fused to different mutants of GFP by using recombinant techniques. With Confocal Fluorescence Microscopy and NSOM we will localize different components within a partly reconstituted NER complex. Information about dynamic changes will be gained by monitoring the degree of FRET. The NER proteins that have been selected for this study so far are the XPA protein, which is supposed to function early in NER with a preference for damaged DNA, and the ERCC1/XPF complex, which is responsible for incision at the 5'-side of the damage. There are indications that an association between XPA and ERCC1 may occur during the NER process. The NER-GFP fusion proteins will be combined with fluorescently labeled lesion containing DNA substrates. We will characterize the XPA-DNA binding and the interaction between XPA and the ERCC1/XPF complex. Later other NER proteins, like RPA, TFIIH and XPC, will be included in this study too. First results from single molecule imaging of NER-GFP fusion proteins in association with damaged DNA will be shown.

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1C-4
INFLUENCE OF ADDITIVES ON THE FORMATION OF FREE RADICALS AND THE RELEASE OF BASES AFTER IONIZING IRRADIATION OF DNA

H. Luxenburger, J. Hüttermann

The interaction of ionizing radiation (X-rays and heavy ions) with DNA as critical target for cell inactivation can be examined at the molecular level by studying free radical intermediates. These in turn are precursors of chemically stable products which generate variances like modified bases, the release of bases or strand-breaks. The release of unaltered and modified bases is most probably connected with strand-breaks in DNA.
Here we report on free radicals and product formation from irradiated solid DNA-samples which were used to study the direct radiation action. EPR spectroscopy was applied to explore the radical concentration and to extract patterns from chemically differing radicals. The products were investigated using HPLC, NMR and MALDI-MS. Results from dry DNA were compared with those from 'hydrated' DNA and from DNA in the presence of additives (electron scavangers, radioprotectors), respectively. The protectors can help to analyse the pathway from the radical precursors to the oxidative products whereas the electron scavengers elucidate the reactions leading to the reductive products. Besides X-rays, heavy ions were used for irradiation in order to modify the radiation quality.
The G-values of the released bases are in the order of magnitude 10^{-7} mol J^{-1}. The heavy ion bombardment effected a lower base release than X-irradiation. Both kinds of additives and the increase of the hydration water of DNA provokes the decrease of the G-values particularly in the case of purines.

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1C-5
STRUCTURAL AND DYNAMIC FEATURES OF DNA OLIGOMERS CONTAINING ONE OXIDATIVE DAMAGE

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A 30-mer DNA sequence was synthesized bearing a single oxidative lesion (2'-deoxy-7,8-dihydro-8-oxoadenosine, oxodA) in a definite position, in order to characterize its structural and dynamic properties. The sample was compared with a control, not bearing the damage, and with a previously characterized sample, of identical sequence, containing an oxodG lesion.
γ-Ray footprinting analysis was carried out to resolve the individual strand conformation and to investigate subtle changes of the backbone accessibility to the hydroxyl radicals. UV melting and circular dichroism spectra were performed too. The elastic constant and the structural parameters such as the rise and the hydrodynamic radius were evaluated from fluorescence polarization anisotropy data.
Although no relevant changes in the thermal stability and in the overall structure were observed, large changes of the hydroxyl radical cleavage pattern were present at the oxodA base and in neighbouring bases. An increase of the torsional rigidity was pointed out, probably related to base twist changes at the site of the damage.

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1C-6
THE INFLUENCE OF THE DIFFERENT LESIONS IN THE DNA MOLECULES ON THE AFFINITY OF THE THERAPEUTICALLY ACTIVE NUCLEOSIDES

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Recently we have shown that the DNA molecules extracted from epididymis of the Wistar male rats exposed to low doses of gamma-irradiation and the control DNA molecules interact with some of pyrimidine nucleosides (NUC) in different ways. It had displayed in a shift of melting curves for the DNA-NUC mixtures relative to those for the free DNA samples to the region of higher temperatures. The hyperchromic effects were changing also.

The changes observed in the profiles of the melting curves depend on the total dose and the type of used NUC and point to the formation of the complexes of NUC to damaged DNA. The sites of the radiational damages seem to be led to the formation of locally instable regions in DNA molecule, changing its secondary structure significantly. We have been modeling such nonspecific changes in the structure of the DNA molecule by means of the partial denaturation of the calf thymus DNA in the salt-free aqueous solutions with further its renaturation in NaCl solution.

The analysis of the melting curves of DNA-NUC mixtures allow to conclude that the considered NUC are binding to model and irradiated DNA molecules in similar way and not binding to the control DNA.

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2A-1
DYNAMICS OF THE QUATENARY STATES OF HEMOGLOBIN ENCAPSULATED IN SILICA GELS PROBED BY CARBON MONOXIDE FLASH PHOTOLYSIS AND OXYGEN BINDING

Stefania Abbruzzetti, Maria Bonaccio, Stefano Bruno, Andrea Mozzarelli, Cristiano Viappiani

Encapsulation of hemoglobin in wet, porous silica gels allows to slow down the T to R quaternary transition by several orders of magnitude. CO was photodissociated from R and T state hemoglobin encapsulated in silica gels using a ns Nd:YAG laser. The time course of CO rebinding was monitored by transient absorption. Rebinding to R state hemoglobin was characterized by three exponentials with lifetimes 120 ns, 30 μs, and 150 μs with almost equal relative amplitudes. These values are not affected by the composition of buffer solutions and the presence of allosteric effectors. The kinetics of CO rebinding to T state hemoglobin was measured at different times upon the exposure of hemoglobin gels to CO. The time course is characterized by four lifetimes (120 ns, 140 μs, 2 ms, and 11 ms). The relative amplitudes vary as a function of time reflecting the T to R quaternary transition, and exhibiting a biphasic process with lifetimes significantly affected by the buffer conditions. Oxygen binding by T state hemoglobin silica gels was determined using absorption microspectrophotometry. The oxygen affinity is affected by the buffer conditions, whereas the Hill coefficient (0.75) indicates a significant heterogeneity. Our results suggest the presence in the T state of two non-interconvertible tertiary conformations, characterized by distinct affinities and rate constants of ligand binding.

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2A-2
DYNAMICS OF MYOGLOBIN INVESTIGATED BY PHONON ASSISTED MÖSSBAUER EFFECT

Klaus Achterhold, Fritz Parak

Pulsed synchrotron radiation of the energy 14.4 keV (energy width: 0.9 meV, pulse length: 100 ps, pulse intervals: 100ns–200ns,) can be absorbed by the Mössbauer isotope ^{57}Fe.The reemission occurs with a characteristic delay of 141 ns. If the incoming radiation is energetically detuned by some meV from the Mössbauer level (energy width 4.7 neV), quanta can nevertheless be absorbed if the energy difference is compensated by the creation or the annihilation of phonons during the absorption process. The amount of delayed quanta, detected by fast avalanche photo diodes in the time between the synchrotron pulses, is proportional to the phonon density of states (DOS) at the iron site. In myoglobin the DOS below 4 meV is following a Debye law with a mean sound velocity of 1890 ms^{-1}. A common feature of deoxymyoglobin, wet and dry metmyoglobin is a pronounced peak at 33 meV. An additional density at 30 meV in deoxymyoglobin is absent in metmyoglobin. Dry metmyoglobin has a peak in the phonon density at 42 meV being absent in the wet samples. At the same time the density at 33 meV is dramatically decreased compared to wet metmyoglobin. The DOS is compared with results of complementary methods like Raman scattering. The mean square displacement of the iron due to phonons is discussed in comparison with Mössbauer absorption measurements.

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2A-3
CONFORMATIONAL CHANGES INDUCED ON CYTOCHROME C UPON BINDING OF NITRIC OXIDE. A MOLECULAR DYNAMICS STUDY

Wojciech J. Blicharski, Krzysztof Murzyn

Cytochrome c contains six-coordinational heme group and has been long known to bind nitric oxide as an axial heme ligand. Nitric oxide upon complexing replaces a native ligand (Met80) causing significant structural changes in the vicinity of the heme group.
In this study long, 2 ns molecular dynamics simulations have been performed for wild type yeast cytochrome c and for cytochrome c complexed with nitric oxide, both with inclusion of explicit solvent molecules. Results show that binding of nitric oxide disturbs the native conformation of the heme vicinity inducing changes in secondary structure, mainly on the heme side occupied by nitric oxide. Additionally, the native pattern of hydrogen bonds and buried water molecules is perturbed which lowers the overall α-helical content and causes unfolding of some parts of the protein most detectable in the fragment between Asp60 and Asn70. The radius of gyration for the structure containing nitric oxide increases steadily during the whole simulation time, which suggests that structural rearrangements occur in a longer than the nanosecond time scale covered in MD simulation.
Detected quantitative changes in secondary structure are compared to results of performed circular dichroism measurements indicating two-fold decrease in the total α-helical content for the cytochrome c – NO complex as compared to wild type cytochrome c.

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2A-4
EFFECTS OF PROTEIN STRUCTURE MEMORY IN RATE PROCESSES

Leonid N. Christophorov

Many peculiar ligand-binding or charge-transfer properties of proteins result from the coupling of reaction kinetics to a relaxing protein structure [1]. As a rule, this coupling is described within "sink-Smoluchowski"-type equations [2], thereby implying a single-turnover decay kinetics. However, structural memory that extends beyond the time scale of a single cycle can lead to specific conformational adaptation of the protein under repeated cycling. This essentially nonlinear, nonequilibrium and nonmarkovian mechanism [3] of the emergence of new stable operational states due to the structure-reaction feedback needs a proper theoretical framework which is proposed and developed in detail.
Methods of reliably distinguishing such self-organization effects from apparently similar effects that could be observed in structurally rigid systems (or flexible systems without noticeable conformational memory) under prolonged initiation of the reaction are discussed, putting special emphasis on recent experimental data on primary electron transfer reactions in bacterial photosynthetic reaction centres.
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2A-5
THE HEME POCKET ENERGY LANDSCAPE OF CARBONMONOXY HEMOGLOBIN

Antonio Cupane, Maurizio Leone, Valeria Militello

The heme pocket energy landscape of HbCO is studied by means of FTIR spectroscopy in the temperature interval 300 – 10K. Spectra are analyzed in terms of three taxonomic A substates; the temperature dependence of the relative integrated intensities gives information on free energy difference and interconversion between them. The thermal behavior of peak frequency and half width of the A_1 band is interpreted in terms of a rugged potential well, i.e. in terms of statistical substates of a lower tier (tier 1) within the taxonomic substate A_1. The ruggedness depends on temperature: below 160K the energy barriers separating the statistical substates are much higher than the thermal energy, interconversion is inhibited and constant values of spectral parameters are observed. Above 160K the energy barriers become cooperatively much lower than the thermal energy, interconversion is allowed and a linear temperature dependence of both spectral parameters is observed. Analysis of their thermal behavior in this temperature range enables to characterize quantitatively the overall shape of the potential well and to estimate the linear thermal expansion coefficient of the heme pocket. From this analysis we derive the picture of a very soft hemoglobin pocket at room temperature, characterized by a rather large anharmonic term and linear expansion coefficient. Our data also provide evidence for the existence of at least one lower tier in the hierarchical organization of substates in HbCO.

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2A-6
FLUORESCENCE STUDIES OF LYSOZYME ADSORBED TO SILICA PARTICLES

C. Czeslik, S. Fahsel, H. Herberhold, R. Winter

Adsorption of proteins on solid substrates is a widespread phenomenon, which is also of considerable practical interest. Generally, a detailled understanding of the driving forces of protein adsorption to solid surfaces and the effect of the substrates on the conformation and activity of the adsorbed proteins is needed in order to optimize protein adsorption processes.
In this study, the effect of adsorption to silica particles on the temperature-dependent conformation of lysozyme is investigated by static and lifetime fluorescence spectroscopy. In addition, the temperature-induced changes in secondary structure elements of dissolved lysozyme molecules are characterized by FT-IR spectroscopy. It has been found that the unfolding temperature of lysozyme is lowered by about 14 °C due to adsorption. From lifetime fluorescence measurements, using a multifrequency phase fluorometer, a significant broadening of the fluorescence lifetime distribution of lysozyme due to adsorption has been observed for both the folded and unfolded state. The data indicate that the native structure of lysozyme is modified and destabilized by adsorption to silica particles allowing a different environment to be probed by its tryptophan residues. Finally, first results of pressure-dependent fluorescence and FT-IR studies on the adsorption and unfolding behavior of lysozyme are presented.

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2A-7
SAXS AND SANS DATA REVEAL AN OXYGEN DEPENDENT CONFORMATIONAL CHANGE OF KLH1

Hermann Hartmann, Heinz Decker

For decades keyhole limpet hemocyanin (KLH) from *Megathura crenulata* has widely been used as a potent immunostimmulant, useful hapten carrier and valuable agent in the treatment of bladder carcinoma. As a respiratory protein, it binds oxygen cooperatively, which implies the existence of different conformations. Upon oxygenation two different conformations of KLH1, a deoxy- and an oxy-state, were detected using small-angle X-ray and neutron scattering (SAXS, SANS). The oxy-state is slightly smaller, according to the measured radius of gyration. 3D-reconstruction of KLH1 molecules in the oxy- and deoxy-state, applying a Monte Carlo algorithm to the SAXS and SANS data, reveal a twist of the two non-covalently associated decamers within the native didecameric KLH1 molecule. Massive changes of the surface were observed. As a consequence these conformational transitions of KLH1 molecules may have an influence on surface epitopes and could be important for understanding the molecular basis of the immune response. Comparison of the SAXS/SANS results with a 15 Å 3D-reconstruction of KLH1 from electron microscopy shows a higher similarity with the deoxy-form.

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2A-8
THE FOLD OF HUMAN AQUAPORIN 1

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The fold of human aquaporin 1 is determined from cryo-electron microscopic data at a resolution of 4.5 Å. The monomeric structure consists of two transmembrane triple helices arranged around a pseudo-twofold axis that are connected by a long flexible extracellular loop. Each triplet contains between its second and third helix a functional loop containing the highly conserved so-called fingerprint NPA motif. These functional loops are assumed to fold inwards between the two triplets, thereby forming the heart of the water channel. The helix topology was determined from the sidedness pattern of each of the six transmembrane helices with respect to the membrane, together with constraints defined by the sequence and atomic force microscopy data. The directionality of the helices was determined by collecting the best-fitting orientations resulting from a search through the three-dimensional experimental map for a large number of alpha helical fragments. Tests on cryo-electron crystallographic Bacteriorhodopsin data suggest that our method is generally applicable to determine the topology of helical proteins for which only medium-resolution electron microscopy data are available.

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2A-9
THE PRESEQUENCE OF MURINE 5-AMINOLEVULINATE SYNTHASE; STRUCTURAL ELEMENTS IN SOLUTION

Jorge S. Dias, Brian J. Goodfellow, Glória C. Ferreira, Peter Henklein, Victor Wray, Anjos L. Macedo

5-aminolevulinate synthase (ALAS) catalyses the first step of the heme biosynthetic pathway in nonplant higher eukaryotes and in hepatocytes it is the rate-limiting step, subject to feedback regulation through the end product of the pathway, heme. ALAS is synthesized in cytosolic ribosomes as a pre-enzyme with an amphipatic presequence (PS). During import into the mitochondrial matrix the presequence is cleaved giving the mature enzyme. In yeast it has been shown that this importing process is dependent on the presence of the presequence, indicating that the *target information* is encoded in this region of the pre-enzyme. It was also demonstrated that the presequence contains *heme regulatory motifs* all of which contain a cysteinyl residue.
The murine ALAS-PS of 49 amino acids was chemically synthesized and prepared in H_2O/TFE solutions. NMR and CD studies were carried out which indicate that the PS contains secondary structural elements in solution. Due to solubility problems and aggregation the N- and C-terminal regions of the PS were synthesized separately. The N-terminal region appears to contain more α-helix than the C-terminal region. The interaction of the ALAS-PS with heme was also investigated in order to understand and provide a structural basis for the role of the ALAS-PS in the inhibition of mitochondrial import by heme.

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2A-10
TEMPERATURE DEPENDENT LIGATION EQUILIBRIUM IN H64V-MET-MYOGLOBIN AS A PROBE FOR PROTEIN DYNAMICS

Niklas Engler, Andreas Ostermann, Fritz G. Parak

The sperm whale myoglobin mutant H64V, where the distal histidine is mutated to valine, is known to be five coordinated in the ferric state at room temperature and physiological pH. Conformation changes have been observed by optical absorption spectroscopy as a function of temperature from 20K to 300K. Above the dynamic transition at about 180K the temperature dependence displays the temperature dependent equilibrium between five and six-ligated heme. Below the dynamic transition the equilibrium is frozen in at about 50% of six-coordinated molecules. The temperature dependence of the coordination equilibrium correlates with the dynamics of the iron atom as monitored by Mössbauer spectroscopy. The X-ray structures of H64V-Myoglobin at 300K and 115K are reported with a resolution of 1.5 Å and 1.3 Å respectively. The measurement at high resolutions are possible due to the crystallisation in the space group $P2_1$, whereas all mutant studies up to now have been carried out with crystals in the space group P6. The overall structure at both temperatures is very close to the native myoglobin. The binding of water at the sixth coordination site is possible due to a stabilizing water network extending from the protein surface to the active center. Consequences for the investigation of proteins at low temperatures and the connection to the dynamic transition around 180K are discussed.

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2A-11
AFM OF SURFACE IMMOBILIZED SINGLE HEMOCYANIN MOLECULES

Wolfgang Erker, Heinz Decker

Hemocyanins (Hc) are oxygen carriers in arthropods and molluscs. To understand the cooperative oxygen binding behavior, information about the protein conformations is necessary. We want to investigate the distribution of the conformations and fluctuations of individual Hc molecules with atomic force microscopy (AFM). Our first step was the imaging of single Hc of the tarantula *Eurypelma californicum* in the oxy-state. For AFM measurements the proteins had to be immobilized. Immobilisation by adsorption on maica and measurements in solution were not successful because the tip shifted the Hc. But drying the sample increases the interaction between protein and surface sufficiently. This preparation allowed imaging of single Hc molecules in a quality comparable with that of electron microscopy.
For imaging the proteins in solution a stronger fixation to the surface like covalent attachment were necessary. We tried this both with gold 111 surfaces which react with the cysteines and activated silanes which react with amino groups. In both cases the shape of the Hc altered: they became more flat and broader loosing the finestructure. We suppose that the surface reactivity is to high stretching the protein.
We thank V. Scheumann, W. Knoll, J. Rühe (MPI-P, Mainz) and HJ Butt (Physical Chemistry, Mainz) for technical support and the MWFZ (Mainz) for financial support.

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2A-12
THE MECHANICAL UNRAVELING OF COILED-COIL STRUCTURES. X-RAY AND MODELING STUDIES ON α-KERATIN

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Hard α-keratin is a protein from the intermediate filament family. The main function of these filaments is to ensure the mechanical support of the cell or of biological tissues such as skin and hair.
The mechanical properties of protein are the subject of recent investigation using techniques of nanomanipulation using experimental techniques. The understanding of the mechanical response of the molecule submitted to the external stress is at the atomic level is not straightforward. Using molecular modeling simulation, it is possible to get a better insight in the detailed behavior. Such studies consist in applying different constraints and examining as well the structural properties as the energetic and the force spectra. Preliminary exploration have shown their capability to interpret different protein folding properties on simple molecular models (1). In the present case, a true important structure; heterodimer of keratin molecule; is investigated. The results are discussed on the light of the experimental data. We observed in particular unraveling of coiled-coil structure as well experimentally as with modeling approach (2).
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2A-13
STRUCTURAL EQUILIBRIUM FLUCTUATIONS OF MESOPHILIC AND THERMOPHILIC α-AMYLASE

J. Fitter, R. Hermann, N.A. Dencher, Th. Haußß, J. Heberle

Although the relationship between thermostability and structural flexibility in an enzyme is complex, most studies suggest that increased thermal stability is associated with decreased enzyme flexibility [1,2]. Therefore this relationship was investigated by comparing dynamical properties of a mesophilic α-amylase and its thermophilic homologue at different time scales. FTIR spectroscopy monitoring H/D exchange kinetics of amide protons and quasielastic incoherent neutron scattering measuring picosecond dynamics were used to study dynamical features of the folded state at room temperature. At this temperature fairly similar rates of slowly exchanging amide protons indicate about the same free energy of stabilisation ΔG_{stab} for both enzymes. With respect to motions on shorter time scales, the thermophilic enzyme is characterised by an unexpected higher flexibility as compared to the mesophilic counterpart. In particular the picosecond dynamics revealed a higher degree of conformational freedom for the thermophilic α-amylase. Compared to the mesophilic homologue, the results indicate a higher conformational entropy of the folded state and therefore a smaller ΔS of unfolding for the thermophilic enzyme. The mechanism proposed for increasing thermal stability (larger temperature of unfolding) is characterised by entropic stabilisation and by flattening the curvature of ΔG_{stab} as a function of temperature.

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2A-14
THE ROLE OF THE DISULFIDE BRIDGE IN AZURIN: A MOLECULAR DYNAMICS SIMULATION STUDY

Rita Guzzi, Bruno Rizzuti, Luigi Sportelli

Two molecular dynamics simulations were performed on wild type (wt) and Cys3Ala/Cys26Ala (C3A/C26A) azurin mutant to investigate the role of the disulfide bridge on the structure and dynamics of this protein. Both simulations were carried out under the same conditions for 1.5 ns, using the GROMOS96 package; the starting configuration of the mutant was modelled from the X-ray structure of wt azurin.
The results show that the removal of the disulfide bridge doesn't affect the overall structure of the protein, whereas unexpected alterations of the dynamical properties are observed, even in regions far from the mutation sites. The positional root mean square fluctuations (RMSF) values of the C3A/C26A azurin mutant are considerably reduced with respect to the wt azurin, except for the region including the α-helix, where an increase is observed. The analysis of the dynamical cross-correlation map evidences a dramatic reduction of the anti-correlated motions between the protein residues. It is interesting to note that the only exception to this trend occurs in the region of the mutation sites, where the rising of anti-correlated motions is observed.
These results suggest the hypothesis of a higher stiffness of the mutated protein with respect to the wt form. A possible role played by the disulfide bridge in wt azurin might be to distribute the solvent-induced dynamics on the whole macromolecule, by means of coordinated motions involving different regions of the protein.

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2A-15
PROTON SHUTTLING IN GREEN FLUORESCENT PROTEIN

Volkhard Helms

Wild type and S65T mutant green fluorescent proteins in a cubic solvent box were studied by molecular dynamics simulations of 0.5 to 1 ns length with neutral and anionic forms of its chromophore and of nearby Glu222. The stiff, cylindrical architecture of GFP allows only small structural fluctuations and the overall protein mobility is very similar in all 5 simulations. However, significant differences are observed in the close vicinity of the chromophore. In agreement with experimental observation, wild type GFP with a neutral chromophore and the S65T mutant GFP with an anionic chromophore show the smallest deviations from the X-ray structures that were used as starting coordinates. The simulations results are discussed in the light of the frequently used $A \leftrightarrow I \leftrightarrow B$ model to describe the photophysics of GFP.

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2A-16
PREDICTION OF LONG-TERM PROTEIN MOTION BY LEAP DYNAMICS

Jens Kleinjung, Stephen Martin, Peter Bayley

Prediction of biologically important protein motion by molecular dynamics techniques is still biased by insufficient sampling of conformational space. Leap-dynamics (LD), a combination of CONCOORD essential dynamics and classical molecular dynamics, has been designed for efficient sampling of peptides and proteins in solution [1]. Ca-free calmodulin (apo-cam) consists of two domains (N- and C-domain) linked by a tether sequence, each domain comprising a four-helix bundle that contains two Ca-binding sites.
Apo-cam (PDB entry 1cfd) was submitted to 3 ns of LD simulations at 290 K, 325 K and 360 K. At 290 K the protein shows stable domain folds, although a high degree of conformational variation can be seen at different levels: inter-domain motion, helix packing and secondary structure variation. At 325 K the C-domain unfolds whereas the N-domain remains folded. The simulation at 360 K reveals unfolding of both domains. These results are in good agreement with experimental findings [2]. Collective coordinate analysis of the trajectories gives information about the dominant protein motion. Since both domains interact only weakly, their trajectories can be treated separately, which gives insight into details of domain stability and unfolding transitions. LD is being applied to destabilising point mutations of calmodulin [2].
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2A-17
FACTORS DETERMINING THE ORIENTATION OF AXIALLY COORDINATED IMIDAZOLES IN HEME PROTEINS

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Factors influencing the orientation of the imidazole of histidines axially ligated to heme-iron in heme-proteins were investigated by analyzing crystal structures of the protein data base (PDB) and correlating results with quantum chemical (QC) and molecular force field (MFF) computations.
We found from the PDB that for most of the imidazoles of heme ligated histidines the NH bond points towards the propionic acids (PR) of the heme. This is in particular fulfilled, if the PR of heme point towards the imidazole. An analysis of the imidazole ring conformation using an MFF exhibits that the preferred orientation is due electrostatic interactions of the polar ring atoms of imidazole with the PR groups of heme. In the cytochrome c peroxidase (CcPo) family the hydrogen bond that the NH-group of the imidazole forms with an aspartate is preventing this orientation. In the Hb/Mb family and the subfamily cytochrome b5 MFF computations showed that the backbone of histidine hinders the NH-group of the imidazole to be oriented towards the PR of heme.
With the exception of the CcPo family, specific hydrogen bonds of the NH-group of the imidazole seem to have only a minor effect in determining imidazole ring orientation. QC computations demonstrated that the interaction of the imidazole ring with the porphyrin atom skeleton of heme has no significant influence on its orientation relative to the heme and for bis-histidine ligated hemes. This is also the case for the mutual orientation of axially ligated imidazoles.

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2A-18
INFLUENCE OF TREHALOSE ON THE DYNAMICS OF A PROTEIN: THE C-PHYCOCYANIN

I. Köper, M.C. Bellissent-Funel, W. Petry

Trehalose is a well known bioprotecting molecule. It is found in a number of cells, organisms and biomolecules who can survive conditions of extreme drought or low temperature[1,2]. Despite of a certain number of scientific activities in this field, the protective mechanisms on a molecular lenghtscale still remains unclear.
The C-Phycocyanin (CPC) is a small protein, extracted from the cyanobacteria. Recently dynamics of this protein in hydrated powders as well as in solution has been studied by quasielastic neutron scattering and MD Simulations[4].
The purpose of this work is to investigate the effect of trehalose on the dynamics of the CPC. First we study the sugar in aqueous solution by using different scattering techniques. Neutron spin-echo mesurements are possible, because we were able to deuterate the molecule up to 80 %.
In a second step we investigate dynamics of CPC protein, hydrated with trehalose/water solutions on a time scale from few ps to several ns. Having a completely deuterated form of the protein collective motions of the molecule have been studied by neutron spin-echo spectroscopy. Faster dynamics have been studied by t.o.f. and backscattering spectroscopy.
First results show clear evidence that the internal movements of the protein are slowed down by the sugar when compared to experiments on the protein without trehalose.
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2A-19
HEME SYMMETRY, VIBRONIC COUPLING AND DYNAMICS IN BULKY LIGAND DERIVATIVES OF HEMEPROTEINS

Maurizio Leone, Alberto Boffi, Antonio Cupane, Vincenzo Sanfratello

We report the visible absorption spectra (Q bands) of nicotinate and carbonmonoxy derivatives of soybean (monomeric) leghemoglobin-a and horse myoglobin. The band profiles are analyzed in terms of vibronic coupling of the heme normal modes to the Q electronic transitions, in the framework of the Herzberg-Teller approximation; this theoretical approach allows an estimate of the vibronic sidebands, both for Condon and non-Condon vibrational modes. The local dynamics of the heme pocket, for all the derivatives, is investigated via the temperature dependence, down to cryogenic temperatures, of the spectral line broadening.
The results indicate that leghemoglobin-a better accommodates, with respect to horse myoglobin, the structural distortions imposed within the heme pocket by the bulky nicotinate group. In particular, a x-y splitting of the Q transition has been clearly assessed for myoglobin whereas no spectral splitting upon nicotinate binding has been observed for leghhemoglobin-a.
The analysis of the temperature dependence of the spectral bandwidth shows that leghemoglobin-a behaves as a softer matrix with respect to myoglobin; moreover, the comparison with analogous data obtained for the carbonmonoxy derivatives of the same proteins shows that nicotinate induces, with respect to CO, smaller conformational heterogeneities and smaller anharmonic contributions to the local dynamics.

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2A-20
STRUCTURAL INFORMATIONS PROVIDED BY TRYPTOPHAN PHOTOPHYSICS IN HIV-1 NUCLEOCAPSID PROTEIN

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The HIV-1 nucleocapsid protein, NCp7, is a small protein, characterized by two highly conserved CCHC zinc finger motifs. NCp7 is also characterized by two Trp residues in position 37 and 61, that constitute sensitive intrinsic fluorescence probes of the distal finger motif and the C-terminal domain, respectively. Using steady-state and time-resolved fluorescence techniques, we investigated the structural changes that accompany the binding of zinc and nucleic acids to NCp7. First, we investigated the fluorescence properties of Trp^{37} in the isolated distal finger motif. The weak fluorescence of this residue in the zinc-lacking random structure was related to the efficient quenching of Trp^{37} by the carbonyl peptide bonds, and the protonated His and Cys residues. The binding of zinc induces a large fluorescence increase that was correlated to the high solvent-exposure of Trp^{37} in a strongly folded structure. Moreover, two of the three lifetimes of Trp^{37} in the holopeptide could be attributed to rotamers about the χ^1 angle. The fluorescence properties of (12-53)NCp7, the central two-finger domain of NCp7, were used to evidence the spatial proximity of the two finger motifs. We notably found out that Trp^{37} was located at 7Å from Phe^{16} in the proximal motif. Finally, it was shown that the time-resolved fluorescence parameters of the whole protein correspond to a linear combination of the individual emitting Trp residues in single-Trp-containing derivatives. This property was then used to simultaneously investigate the fate of both Trp^{37} and Trp^{61} in the interaction of NCp7 with nucleic acids.

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2A-21
VIBRATIONAL ENERGY TRANSFER IN A PROTEIN MOLECULE

Kei Moritsugu, Osamu Miyashita, Akinori Kidera

Mode coupling in a protein molecule is studied by a molecular dynamics simulation. The intra-molecular vibrational energy transfer in myoglobin is simulated for the purpose of studying the mode coupling between normal modes in the protein molecule. At first, we define a set of vibrational modes in myoglobin by normal mode analysis. As an initial condition of molecular dynamics simulation, we give small kinetic energy to a specified vibrational mode, i.e., we excite only one mode. As the result of following simulation, we observe that the relaxation of exited vibrational energy is not usual exponential decay and, moreover, the vibrational energy is transferred to only a small number of selective normal modes. An analytical model of weekly coupled harmonic oscillators with the third order coupling terms explains the simulation results almost perfectly, and shows that the selection of modes for energy transfer is based on the relation between the mode frequencies, like Fermi resonance. In addition, the magnitude of a coupling coefficient is determined by how much two modes are overlapped geometrically in the protein molecule.

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2A-22
NEW INSIGHTS INTO NITRIC OXIDE GEMINATE REBINDING TO HEMOPROTEINS

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By means of ultrafast time-resolved absorption spectroscopy, we measured the kinetics and associated spectra of geminate recombination of nitric oxide (NO) to two hemoproteins of endothelial cells involved in blood pressure control. We thus probed dynamic features underlying regulatory mechanisms.
NO-synthase catalyzes the formation of NO from L-arginine and O_2 bound to the heme. The NO produced may also bind to the heme, modulating its own release. NO geminate rebinding to NOS is multiphasic in ferric and ferrous states of the heme, showing a fast bi-exponential picosecond phase followed by a nanosecond one. The rates and relative amplitudes of both phases are strongly modulated by L-Arg: the overall effect is a slow down of NO rebinding. Measurements on the isolated heme domain assign the nanosecond phase to the rebinding of NO still located in the protein core but not in the heme pocket.
Guanylate cyclase (GC) is the receptor of NO and catalyzes the formation of cGMP from GTP. The NO-liganded heme is 5-coordinated. The NO geminate rebinding to GC is ultrafast (τ = 7.5 ps) and mono-exponential with a relative amplitude close to unity (0.97). The His-heme bond does not reform and NO does not escape from the heme pocket. The breaking of the His-Fe^{2+} bond may lead to a closing of the heme pocket, making GC an efficient trap for NO, and allowing an amplification of the signal.

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2A-23
DYNAMICS OF AN UNFOLDED DOMAIN OF ANNEXIN BY NMR: INTERPRETATION OF ^{15}N RELAXATION PARAMETERS

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The isolated D2 domain of annexin I is unable to adopt a tertiary fold but exhibits both native and non-native residual structures and thus constitutes an attractive model for the investigation of dynamics of partially folded states in the context of protein folding and stability. ^{15}N relaxation parameters of the D2 domain were acquired at three different magnetic fields, 500, 600 and 800 MHz. Taking into account the inherent flexibility of partially folded states, we used formalism based on distributions of correlation times were used to reconsider the interpretation of relaxation data beside the usual description in terms of order parameters and discrete correlation times obtained from a model free approach. We showed that all approaches were able to reproduce our experimental data and provided similar dynamical profiles. Our comparative analysis led to consider that the motional parameters obtained from the distributions-based formalisms bring a physical interpretation of the model free parameters $Ss^2$and τ_s in the particular context of unfolded proteins. The study provides meaningful results on the conformational features of the D2 domain structure previously depicted by chemical shift and nOe data. First, the non-native structures can be identified through a high dynamical heterogeneity reflecting the existence of multiple conformers separated by rather high-energy barriers. Second, residual helix segments exhibit distinct dynamical behavior that may be related to their intrinsic helical propensity.

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2A-24
HYDROGEN EXCHANGE IN MYOGLOBIN. A NEUTRON DIFFRACTION ANALYSIS OF MET-MYOGLOBIN AT 1.5Å

Fritz Parak[1], Andreas Ostermann[1], Ichiro Tanaka[2], Nobuo Niimura[2]

Hydrogen bonds are important for the stabilization of the 3-dimensional structure of proteins and thus play an essentiell role for protein dynamics. Neutron diffraction provides the experimental possibility for locating hydrogen atoms directly. So far, there exists only a relative small number of structures determined by neutron crystallography to high resolution. The neutron diffractometer BIX3 at the JAERI equipped with a neutron imaging plate for using a monochromatized neutron beam makes it possible to collect high resolution data sets on protein crystals [1]. We determined the neutron structure of met-myoglobin in the space group $P2_1$ up to 1.5Å. The crystals were soaked in deuterated buffer for years. 88.6% of the possible reflections have been collected with an overall redundancy of 3.1. The overall R_{merge} value of the data set is 10.2%. This high resolution data set provides the possibility to analyse the extent of exchange of hydrogen atoms by deuterium within the protein. The patterns can be interpreted in terms of different degrees of flexibility in the different parts of the protein structure.
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2A-25
CALCULATED PH-DEPENDENT POPULATION OF CO-MYOGLOBIN CONFORMERS

Björn Rabenstein, Ernst-Walter Knapp

X-ray structures of carbonmonoxymyoglobin (MbCO) are available for different pH values. We used conventional electrostatic continuum methods to calculate the titration behavior of MbCO in the pH range from 3 to 7. For our calculations, we considered different x-ray structures determined at pH values of 4, 5, and 6. We developed a Monte-Carlo method to sample protonation states and conformations at the same time, so that we could calculate the population of the considered MbCO structures at different pH values and the titration behavior of MbCO for an ensemble of conformers.
The calculated populations show as expected that the x-ray structures determined at pH 4 are most populated at low pH, whereas the x-ray structure determined at pH 6 is most populated at high pH, and the population of the x-ray structures determined at pH 5 shows a maximum at intermediate pH. The calculated titration behavior is in better agreement with experimental results compared to calculations using only a single conformation. The most striking feature of pH dependent conformational changes in MbCO - the swing-out of His-64 out of the CO binding pocket - is reproduced by our calculations and is correlated with a protonation of His-64 as proposed earlier.

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2A-26
SOLUTION STRUCTURE OF FULL LENGTH CELULASES DETERMINED BY SMALL ANGLE X-RAY SCATTERING

Véronique Receveur, Mirjam Czjzek, Martin Schülein, Bernard Henrissat

Cellulose is a highly stable macromolecule, which paradoxically does not accumulate on Earth albeit photosynthesis produces it at a rate of at least 10^9 t/year. Cellulose is in fact biodegraded by the action of cellulolytic enzymes, called cellulases, which catalyse the hydrolysis of cellulose. Most effective cellulases are made of at least two constitutive domains, a catalytic domain and a non-catalytic cellulose-binding domain (CBD) linked by a glycosylated peptide. Removal of the cellulose-binding domain drastically reduces the catalytic activity of cellulases. This has led to suggest that the spatial arrangement of the two constitutive domains would be essential for optimal activity. The three-dimensional structures of a number of isolated catalytic domains and of CBD have been determined. However it has so far proven impossible to crystallise an entire cellulase containing the two domains. This could be due to the possible flexibility of the linker peptide, or to its heterogeneous glycosylation, or to both. Therefore we performed small angle X-ray scattering experiments in order to elucidate the spatial arrangement of full length cellulases in solution. The values of radii of gyration and of maximum dimensions obtained on various cellulases and on cellulase mutants with modified linkers do show that the linker peptide is quite extended and flexible and that its flexibility depends on its composition. An interpretation of the structural role of the linker is proposed.

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2A-27
REACTIONS OF PROTEINS IN THE GAS-PHASE: DRIFT-TIME MEASUREMENTS OF GAS-PHASE HYDRATION OF PROTEINS

J. Woenckhaus

The relative importance of solvent and intramolecular interactions in determining the 3-dimensional structure of a protein has been the subject of extensive discussions for many years. The development of new ionization methods has made it possible to use mass spectrometry based techniques to study the properties of protein ions in the gas-phase. Hydration reactions of proteins can be performed using an injected drift tube apparatus equipped with an electrospray source. A drift tube, between two QMS, can be used for determining the drift times of the proteins and as an on-axis reaction chamber. We present measurements of the effects of the level of hydration upon protein conformation in the gas phase. Water is known to play a critical role in determining the tertiary structure of proteins, but there is little detailed quantitative information available about the interaction of water with proteins. We report the first measurements of enthalpy and entropy changes for the initial steps in hydration of a gas-phase protein. Our results reveal the presence of a special hydration-site on gas-phase Bovine Pancreatic Trypsin Inhibitor, which may be related to a unique structural water molecule observed for this protein. In hydration measurements of gas-phase Cytochrome C we focused on the question how many water molecules are absorbed depending on the charge state and structure of the gas phase protein. The compact folded conformation of CC^{5+} does not change upon hydration. The partially unfolded conformation of CC^{7+} folds up as water is added.

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2A-28
PHOTOPHYSICS AND PHOTOCHEMISTRY OF THE GFP CHROMOPHORE IN ALCOHOLIC SOLUTIONS INVESTIGATED BY SPECTRAL HOLE BURNING SPECTROSCOPY

Peter Schellenberg[1], Markus Stübner[2]

Hole burning and temperature dependent spectroscopy is performed on the synthetic GFP-analog chromophore in alcoholic solutions. It can be shown that many of the spectral features described for the GFP protein are also observed in the chromophore dissolved in alcohol and alcohol glasses at different pH. It can be demonstrated, that additionally to the neutral form A and the anionic form B, there is a third species present which is in thermal equilibrium with B, and therefore assigned being equivalent to the unrelaxed anionic (I) form of the GFP protein. This I form freezes out at the glass transition temperature.
At low temperature the different forms can be interchanged photochemically: Form A can be transformed to B and I, and B can be transformed to I and A. According to published results on GFP, some of these phototransformations seem to be inhibited in the protein environment. Temperature cycling experiments are also performed to determine the barrier distribution and to distinguish distinct forms of the photoproduct.
We conclude, that the unrelaxed anionic form is distinguished from the relaxed form by a different arrangement of the hydrogen-bonding network between chromophore and matrix. This model can also be applied to the GFP protein.

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2A-29
ANS BINDING TO APOMYOGLOBIN STUDIED BY TIME-GATED FLUORESCENCE CORRELATION SPECTROSCOPY

Andreas Schenk, Carlheinz Röcker, Don C. Lamb, G. Ulrich Nienhaus

We have studied the binding reaction of the organic dye 1,8-anilino-naphtalenesulfonate (ANS) to the heme pocket of apomyoglobin using two-photon excited fluorescence correlation spectroscopy (FCS) to investigate structural fluctuations of the protein in the native and partially denatured states. Fluctuations in the number of fluorescent molecules in a small volume (≈1 fl) due to diffusion, chemical reactions and conformational changes give rise to temporal fluctuations in the fluorescence intensity. Diffusion coefficients as well as rate coefficients can be determined from autocorrelation analysis.
We have employed time-gated FCS to enhance the sensitivity and simplify the data analysis in the case of multiple, interacting fluorescent species. Using pulsed laser excitation and an electronic time gate in the detection channel, fluorescent species can preferentially be suppressed by fluorescence lifetime separation.
The lifetime of ANS increases from about 100 ps to 12 ns upon binding to the hydrophobic interior of apomyoglobin. Time-gated FCS suppresses the background from free ANS in our binding studies of apomyoglobin under native and destabilizing conditions (low pH, guanidine HCl).

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2A-30
FLUORESCENCE AND PROTON NMR STUDIES OF DESTABILIZATION OF SERUM ALBUMIN

Anna Sulkowska

The fluorescence intensity of tryptophanyl groups of serum albumin (BSA) in water solution and in the presence of adenine-fluorochrome which reversible binds to it was measured at various temperatures.
Intensity of the fluorescence is related to molar ratio BSA: adenine and decreases when adenine contain increases. The effect of temperature on quenching of tryptophanyl groups fluorescence by adenine was studied. The reduction of quenching of protein fluorescence in the presence of the same adenine concentration at temperature of 333K was observed.
The proton NMR measurements were recorded for adenine-BSA complexes in D_2O and the broadening of the ligand NMR signal at the presence of protein in relation to the signal in the control system was estimated. The narrowing effect of temperature on ligand NMR signal was calculated from relative coefficient.
Adenine molecule bound to the BSA hydrophobic binding site is released at temperature of 333K. The interaction weaker than those with native protein was observed. The results suggest protein structure changes leading to removal of ligands.

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2A-31
MOLECULAR DYNAMICS SIMULATION SHOWS LARGE VOLUME FLUCTUATION OF PROTEINS

Florence Tama[1,2], Osamu Miyashita[1], Akio Kitao[1], Nobuhiro Go[1]

We present a new approach to study volume fluctuation of protein. From a 1 nsec molecular dynamics simulation, volume fluctuation of human lysozyme has been calculated. We used two different ways for the calculation. In the first one, volume fluctuation is extracted directly from the trajectory. For the second one, a newly developed formalism based on principal component analysis is used. The RMS volume fluctuations obtained from the two analyses agree well to each other. Isothermal intrinsic compressibility is found larger than the one reported by experiment. The difference is discussed and suggested to exist in the uncertainty of the compressibility of hydrated water assumed to deduce the isothermal intrinsic compressibility from the experimental value. Spectrum analysis shows that the low frequency dynamics dominate the total volume fluctuation. The same aspect is found in the study with the principal component analysis. This low frequency region is related to large and slow motions of protein. Therefore a long time dynamics simulation is necessary to describe volume fluctuation of protein.

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2A-32
SOLUTION STRUCTURE AND FUNCTIONAL ASYMMETRY OF A CALCIUM BINDING PROTEIN REVEALED BY NMR EXPERIMENTS

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Calcium Vector Protein (CaVP) is a two domain, calcium binding protein (18.3 kDa) of the EF-hand family. Only the two motifs of the C-terminal domain were found to be functional and the corresponding binding affinities are highly dissimilar (Ka^1 = 4.9×10^6 M^{-1} and Ka^2 = 7.3×10^3 M^{-1}). Due to the aggregation tendency of the integral protein, we expressed separately and studied by NMR the two halves. The isolated domains are stable and have a well-organized tertiary structure which is similar to that of other EF-hand proteins. The Ca^{2+}-saturated form of C-CaVP shows an open structure, with highly-exposed hydrophobic side chains, like in metal-bound regulatory domains. In contrast, N-CaVP structure is not sensitive to Ca^{2+} and exhibits a compact, closed tertiary fold. The ion-induced structural changes in C-CaVP define an original behavior within the EF-hand repertoire. The sequential calcium binding induces a progressive, stepwise transition from a highly disordered protein state into a half-structured conformation and finally into a standard EF-hand pair. Additional NMR studies on the CaVP confirmed that the C-terminal domain shows the same behavior within the integral protein and suggested that the N-terminal domain provides the dimerization interface. Nuclear relaxation measurements (^{15}N) on C-CaVP enabled us to analyze the complex internal dynamics in the partially and fully saturated states and suggested a monomeric behavior for this regulatory domain.

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2A-33
CONFORMATIONS IN SOLUTION OF HOLO AND APO HUMAN CERULOPLASMIN

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Human ceruloplasmin (hCP), a 1046 residue long glycoprotein containing 6 copper atoms, has been studied using Small-Angle X-ray Scattering in its holo and apo forms.
The scattering pattern of the holoprotein differs from the pattern calculated from the crystal structure because of the bound carbohydrate chains (8% (w/w). We modeled them so as to take their contribution to the scattering intensity into account . The resulting curve appears to be very similar to the experimental one.
At s values lower than 0.025 A^{-1} ($s = (2\sin\theta)/\lambda$) the scattering curves of holo and apo –hCP are very different, while they are identical beyond 40 A resolution. The first region is essentially determined by the respective positions of the domains, while the second part is mainly sensitive to the conformation of these domains. Rigid body movements around the flexible interdomain linkers led to the determination of models, the scattering patterns of which displayed an excellent fit to the experimental data. The combination of small-angle X-ray scattering in solution with the use of crystal structures has thus established an essential feature of the conformation of apo-hCP : rather than the molten globule suggested by UV spectroscopy studies, the protein comprises domains in their close to native conformation, linked by very flexible loops, freely moving in solution. The copper atoms of the trinuclear site acts as a staple stabilizing the conformation of the holoprotein.

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2A-34
FTIR SPECTROSCOPIC STUDIES ON DIAMIDE MODELS OF γ-TURN STRUCTURES

E. Vass, J. Samu, M. Hollósi

This work was prompted by the growing interest in the FTIR spectroscopic characterization of folded polypeptide structures, such as β- and γ-turns. The FTIR spectroscopic detection of these structures, often stabilized by ten-membered (C_{10}) or seven-membered (C_7) intramolecular H-bonds, is based on the analysis of the amide I band contour (1700–1600 cm^{-1}).
Herein we report systematic FTIR studies on Ac-Xxx-NHMe (Xxx = Ala, Pro) type γ-turn models performed in various solvents. The assignment of amide I component bands was assisted by ^{13}C isotope labeling, as well as by parallel studies on Ac-Xxx-OMe analogues. In DMSO and D_2O practically no H-bonded γ-turn structures were detected. In TFE the spectra showed significant absorptions, arising from the acetyl CO group, between 1625-1605 cm^{-1} (the lowest value was found for Xxx = Pro) suggesting the presence of H-bonded γ-turn structures. The considerably higher frequencies of the corresponding bands in CH_2Cl_2, however, point to a strong interference of solvational and conformational effects in TFE, especially in the case of small linear peptides. The intense and surprisingly low-frequency acetyl amide I band of Ac-Pro-NHMe results from the higher propensity of Pro to form a strongly H-bonded γ-turn, the tertiary amide character of the Ac-Pro linkage, and finally, the presence of bifurcated H-bonding schemes in protic solvents (TFE, D_2O).
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2A-35
^{57}FE- AND ^{183}W-RSMR ON MYOGLOBIN – TEMPERATURE DEPENDENT INVESTIGATIONS OF PROTEIN DYNAMICS

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The γ-quanta, emitted by a Mössbauer source, are Rayleigh scattered by the electrons of the sample (RSMR). The energy of the radiation scattered in a certain angle is analysed using a Mössbauer absorber. This allows to separate the elastic and inelastic part of the scattered radiation with the energy resolution of the Mössbauer effect. Depending on the lifetime of the Mössbauer level the dynamics of the sample can be observed on different timescales. Using ^{57}Fe (wavelength λ = 0.86 Å) and ^{183}W (λ = 0.267 Å) motions can be detected, which are faster than 100 ns and 0.2 ns respectively. In contrast to the incoherent neutron scattering, which measures the dynamics of the hydrogens, RSMR enables to investigate the dynamics of the heavier atoms like C, N, and O. No enrichment of the sample with Mössbauer isotopes is necessary. The RSMR measurements have been performed on polycrystalline horse heart myoglobin between 0.03 $Å^{-1} \leq \sin(\vartheta)/\lambda \leq 0.45$ $Å^{-1}$ at 5 different temperatures from 85 K up to room temperature. The experimental data, taken with both Mössbauer isotopes fit together although their characteristic times are different. Therefore, the relevant modes of motions are faster than 0.2 ns. Most of the angular dependence of the elastic and inelastic scattered quanta can be explained by normal modes. The quasi diffusive protein specific motions, which occur at temperatures above 180 K, are taken into account by the Brownian Oscillator model.

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2A-36
CHANGES IN PROTEIN DYNAMICS FROM 77 K UP TO PHYSIOLOGICAL RANGE OBSERVED BY TRP PHOSPHORESCENCE

F. Tölgyesi, B. Ullrich, J. Fidy

Tryptophan phosphorescence, especially its lifetime, studied as a function of temperature gives valuable insight into the changes in the protein conformation, and dynamics.
We investigated these parameters for several proteins (alkaline phosphatase, a-crystallin, HIV1 protease, apoazurin, alcohol dehydrogenase) from cryogenic temperatures up to the physiological range. We observed the characteristic change in protein dynamics at a low temperature, around 200 K, that is already known, but we observed another change at a physiological temperature, around 30 °C, that can be interpreted as the activation of large amplitude motions in the protein. Examples will be given, how the observed parameters signal changes in protein dynamics and stability with complex formation (e.g. HIV1 protease with its inhibitor) or with aggregation (e.g. bovine lens α-crystallin).

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2B-1
NEURAL NETWORK FOR GENOME ANNOTATION

Nora Benhabiles, Peter Lackner, Francisco Domingues, Manfred Sippl

We implemented a neural network (NN) which correlates ProFIT scores to a single confidence value for a specific sequence/structure alignment. A representative data set of the fold space was derived with the CATH V1.6 classification to train, validate and test the neural network. We have selected 524 homologous and analogous positive pairs and 30724 negative pairs. This set is divided into 3 equal and disjoint parts to get the training, testing, and validating sets. The NN topology is of 5 inputs nodes, 4 hidden nodes and 2 output nodes with the Stuttgart Neural Network Simulator (SNNS). The inputs are the pair and solvation energies, two alignment scores and the alignment length. The minimum false positive rate reached with the current implementation is of 0.28% for 37.14% correct positive pair predicted. The rate of false positive encourage us to use the method for large scale genome annotation.

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2B-2
RATIONAL DESIGN OF BIOACTIVE LIPOPEPTIDES

Jean-Marc Bonmatin, Isabelle Moineau, Françoise Peypoux

Studies of cyclic bacterial lipopeptides remained of academic interest since knowledge on both biosynthesis and structure-activities relationships (SAR) were lacking. Interest enlarged after we collected data on their structure, biosynthesis and after bio-assays. Surfactins and Lichenysins, from *Bacillus* species, are not only very powerful biosurfactants (metal removal, oil recovery...) but also valuable antibacterial, antitumoral and anti-mycoplasma agents.
The common arrangement is a cyclic heptapeptide with a *LLDLLDL* chiral sequence, linked *via* a lactone bond to a β-hydroxy fatty acid (C_{13}–C_{15}). The biosynthesis is catalyzed non-ribosomally by a multienzyme complex involving a multicarrier thiothemplate mechanism. Controlling the culture, we favoured production of selected peptidic variants. Used with the screening of *Bacillus* strains, this strategy led to the Surfactin and Lichenysin G families. Variants were separated and mass and NMR spectroscopies provided structure elucidation. Here, fine SAR have evidenced the respective role of the polar and hydrophobic domains in interfacial and membrane properties. Specific residues required for an improved activity were tested leading to the selection of the most efficient lipopeptide in terms of surfactant, chelatant and haemolytic properties.
This comprehensive approach permits advances in the rational design of lipopeptide for medicinal, pharmaceutical and environmental applications. Such applications are in expansion since chemical synthesis and the tool boxes for genetic engineering are well developed.

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2B-3
DERIVING AN ENERGY FUNCTION THAT RECOGNIZES NATIVE PROTEIN STRUCTURES

Jochen Farwer, Ugo Bastolla, Ernst-Walter Knapp

To simulate protein folding one needs an energy function, which is able to recognize native protein structures. Such an energy function can be derived by using threading. In a threading procedure decoy structures for a given sequence of length N are generated by considering native structures from proteins of length ≥N. An energy function is appropriate, if the native structure is recognized among a large number of decoy structures generated by threading.
In the simplified protein model, which we use, residues are represented by C_α-atoms only. Residues which are closer than a certain distance, say d = 11Å, are considered to be in contact. A simple energy function contains 210 contact parameters U_{ij} (one for each amino acid pair). The energy for a specific sequence in a given conformation is the sum of the corresponding contact energies U_{ij}. The energy parameters are optimized by maximizing the similarity of the native structure with structures of low energy.
Using 186 target proteins of length $N \leq 200$ and a set of 420 protein structures to generate decoys including the target proteins, the optimized energy function recognizes 92.5% of the target proteins. For the future it is planed to use such an energy function for Monte Carlo methods to simulate protein folding and to make protein structure prediction.

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2C-1
UNFOLDING AND INTERMOLECULAR ASSOCIATION IN GLOBULAR PROTEINS ADSORBED AT INTERFACES

Sarah Adams*, Rebecca J. Green†, Ian Hopkinson†, Richard A.L. Jones*

The conformational transitions that occur on heating solutions of globular proteins, unfolding and intermolecular association are compared with the analogous transitions undergone by proteins adsorbed at interfaces. Adsorption of protein to a variety of surface coatings and the subsequent changes in protein structure observed upon heating were monitored using attenuated total reflection Fourier transform infrared (FTIR-ATR) spectroscopy. The effects of surface hydrophobicity, by utilising a variety of polymer coatings, and surface charge, using a range of different lipid layers, have been considered.

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2C-2
REFOLDING OF RECOMBINANT METALLOPROTEASES AND NATIVE LECTINS

Leila Maria Beltramini, Heloisa S. Selistre-de-Araujo

The mechanism by which proteins fold and refold to their native conformation is a fundamental problem in biophysics and molecular biology. Spectroscopic and chromatographic methods and biological assays have been used as powerful tools to study the refolding process. We have studied the refolding of members from two protein classes: a recombinant snake venom metalloprotease (rsvMP) and its pro-enzyme rpro-svMP, and tetrameric lectins from the *Artocarpus* genus. Both rsvMP and rpro-svMP are monomeric proteins having three disulfide bonds. The lectins frutalin and jacalin are tetrameric, assembled by non covalent bonds. RsvMP and rprosvMP were recovered from inclusion bodies by solubilization in 6M buffered urea, purified under denaturing conditions, and refolded by dialysis with gradual urea removal in the presence of Ca^{++} and Zn^{++}, which also led to the activation of the pro-enzyme. Native jacalin and frutalin were denatured by guanidine hydrochloride overnight at 25 °C. Refolding of both lectins was achieved by dilution and dialysis with gradual guanidine removal in PBS D-galactose. Refolded metalloproteases and lectins were similar to the native forms as judged by circular dichroism and fluorescence emission spectra, chromatography and biological assay. For the lectins, the sugar-binding site promoted the refolding process, a fact compatible with the nucleation-condensation model proposed for protein folding. Refolding of the metalloproteases was promoted by the divalent ions.

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2C-3
A CD INVESTIGATION OF THE NATIVE AND NON-NATIVE CONFORMATIONAL STATES OF THE GRB2 N-TERMINAL SH3 DOMAIN. EFFECT OF BINDING TO A PROLINE-RICH PEPTIDE FROM SOS

J.A. Bousquet, C. Garbay, B.P. Roques, Y. Mély

SH3 domains are small protein modules which interact with proline-rich peptides. The structure of the N-terminal SH3 domain from Grb2, an adaptor protein in the intracellular signaling pathway to Ras, was investigated by CD spectroscopy. About the native conformation, CD data well agree with the compact β-barrel structure revealed by NMR spectroscopy. From the CD spectral changes induced by varying pH, ionic strength, temperature, or hydrophobicity of the environment, evidence for the existence of distinct non-native conformations was obtained. Along the free energy scale, these appear to distribute to two conformational ensembles. Both of them consist of loose partially unfolded conformations, differing by the native-like or non-native properties of their secondary structure. Most of these conformations exist in mild or non-denaturing conditions. Instead of these, only the native all-β conformation possesses a condensed protein exterior, appropriate for the binding to the VPPPVPPRRR decapeptide from Sos. Upon binding, the native conformation undergoes a local tertiary structure change in an hydrophobic pocket at the binding site. This is accompanied by the PP-II helix folding of the proline-rich peptide. Interestingly, in the near-UV domain a significant change in the CD spectral contribution of an aromatic exciton was observed, thus allowing quantitative tracking of the binding process.

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2C-4
REFOLDING OF THE FRUTALIN: A POTENTIALLY USEFUL BIOTECHNOLOGICAL TOOL

Patricia Targon Campana, Ana Cristina de O. Monteiro, Andressa P.A. Pinto, Derminda I. de Moraes, Leila Maria Beltramini

The circular dichroism (CD) has been used as a powerful tool to study the mechanism by which proteins fold to their native conformation. This fundamental problem have been studied in our laboratory by the refolding of Frutalin, a lectin from *Moraceae*. It is a tetrameric lectin, D-galactose specific and its native CD spectrum was identified as being dominated by β-sheet. It showed be a potent stimulant of human linfocitos, actived natural killer cells "in vitro", and leukocytes migration in mice pleural cavity "in vivo". "In vitro", the initial state in this puzzle is the unfolded protein by thermal denaturation and induced by GndHCl. The thermal unfolding conditions used were 60 °C for 30 to 60 min. Our results indicate that the refolding is promoted by the freezing process in the presence of 0.1 M D-Galactose-PBS followed by three-fold concentration in a Centriprep 3. Positive hemagglutination occurred for both the native and refolded forms. Frutalin was also denatured using GndHCl 4 M and the refolding ocurred. Refolded Frutalin samples from GdnHCl, were filtered by Size Exclusion Chromatography on the Superdex 75 column and eluted with PBS containing 0.1 M of D-galactose. Two fractions were separated: one was correspondent at position of the native form and other at the position of denatured form. These results was confirmed by CD, fluorescence spectra and hemaglutination activity of the fractions.

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2C-5
FOLDING OF CYTOCHROMES MONITORED BY FTIR SPECTROSCOPY

Kate A. Dalton, Ekkehard Kauffmann, Klaus Gerwert

Cytochrome c is frequently used as a model for protein folding. The protein has a single heme covalently attached to the polypeptide backbone. Cytochrome b_{562} is comprised of a four helix bundle. However, this protein, which consists of 106 residues, noncovalently binds a single heme which is ligated at Met 7 and His 102 to the heme iron. Cytochrome b_{562} is also different to the other cytochromes which share the same four helical structural fold as their hemes are covalently bound.

The refolding of cytochrome b_{562} and cytochrome c is followed here by time-resolved FTIR spectroscopy which can provide information about the formation of tertiary and secondary structure.

Photoinduced electron transfer has been used as a trigger for folding of cytochromes. In this approach folding is triggered by electron transfer from a caged electron taking advantage of the fact that it is possible to find denaturing conditions at which the ferricytochrome is unfolded whilst the ferro-cytochrome is folded. Submillisecond time-resolved FTIR data will be presented.

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2C-6
ON THE MECHANISM OF MOLECULAR CHAPERONE FUNCTION. THE "FETTRED FOLDING" MODEL

Alexander P. Demchenko

Monomeric molecular chaperones of Hsp70 subfamily possess only one site of interaction with hydrophobic segments of unfolded protein substrates. The exact mechanism of their participation in folding process and the role of ATP hydrolysis in this mechanism are not clear. We suggest that the chaperone on binding at the earliest steps of folding prevents an early non-specific collapse of hydrophobic segments, this limits the conformational search and allows spontaneous formation of sites determining the native fold in other parts of the globule. Dissociation of the complex occurs at the later step, when the hydrophobic sites are ready to combine correctly. This requires the primary weak binding to be switched to strong binding after successful search, and the reverse switch from strong binding to weak binding after well-controlled time period to allow a rapid substrate release. Essential for this mechanism is the coupling between two cycles of events, capture-holding-release of substrate protein and ATPase cycle, which allows to switch between weak and strong binding and make the whole process controlled and uni-directional.

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2C-7
"IN VITRO" REFOLDING OF RECOMBINANT HUMAN BMP2 PROTEIN

Jesús M. García-Cantalejo, Diana González-Muñoz, Jesús Zurdo, José Sanz-Casado, José L. López-Lacomba

Bone Morphogenetic Protein 2 (BMP2) is a member of the superfamily of TGFβ-like proteins. The aminoacid sequence contains 7 cysteins that gets an homodimer final form composed by two monomers linked by a disulphide bond. Moreover, each monomer contains three internal disulphide bonds. BMP2 has been produced by both eukaryotic and prokaryotic expression systems. Production in prokaryotic presents majors problems to get a correct and biologically active form that has been solved in the presence of zwitterionic detergents.
Here we shown the *E. coli* production and subsequent "in vitro" refolding of mature human BMP2 in the presence of chaotrope reagents, urea and guanidinium hydrochloride, high ionic strength and a redox system. The protein can be correctly folded "in vitro" by using moderate quantities of chaotrope reagents in the folding buffer. Production and quantification of biologically active protein is determined by the induction of alkaline phosphatase activity after BMP2 treatment of a mouse myoblast cell line.

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2C-8
UREA DENATURATION OF HUMAN SERUM ALBUMIN LABELLED WITH ACRYLODAN

Fermin Moreno, José González-Jiménez

We have studied the urea denaturation of human serum albumin (HSA) labelled at the residue Cys-34 with Acrylodan (AC). This aminoacid residue is located in the subdomain I, very far from the single tryptophan 214 of the protein. Denaturation data have been obtained by fluorescence and CD and they have been analysed according to the method of Pace et al., modified by Santoro and Bolen.
The effect of the urea concentration on the protein was followed by the intrinsic protein fluorescence and by the fluorescence of the AC bound to the protein (360–480 nm). There is a large fluorescence quenching of the AC bound to HSA, by addition of the denaturing agent, without appreciable shift in the emission maximum, even at 8M urea. The denaturation profiles obtained are different, however, by both types of fluorescence, although the protein fluorescence profiles are equal whether the protein is labelled or not.
The CD spectra exhibit two minima at 220 and 209 nm, whose intensities decrease with the urea concentration. There is not any appreciable effect of AC on the HSA behaviour, which agree with the results obtained by protein fluorescence. The denaturation profiles obtained by CD, however, are significantly different than those obtained by fluorescence.
We conclude that: a) HSA is not totally unfolded at 8M urea; b) the denaturation of the several domains of the protein follows different denaturation profiles; and c) the protein is denatured by a non-two-state model.

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2C-9
RELATIONSHIPS BETWEEN TOPOLOGY, SEQUENCE AND FOLDING PATHWAYS

R. Guerois, L. Serrano

Recent studies showed that folding mechanisms are conserved in spectrin and Src SH3 domains, two proteins with similar topologies and low sequence homology (30%). These results suggest that protein topology rather than sequence details is determinant in the folding mechanism. To test further this hypothesis; we analysed the folding transition state of sso7d, a protein sharing similar topology to that of SH3 domains, with no sequence homology. Unexpectedly, drastic shift in the position of the folding nucleus is observed for sso7d with respect to SH3 domains. A folding algorithm, FOLD-X, was developed to investigate the relation ships between topology, sequence and folding mechanism. Predictions obtained using FOLD-X are in very good agreement with the experimental data.

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2C-10
SANS STUDIES ON THE *E. COLI* CHAPERON SYSTEM

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Chaperones are proteins that assist misfolded proteins to find their nativestate. The *E. coli* chaperonin GroEL binds and releases denatured substrate protein in an ATP dependent process, accompanied by its co-chaperonin GroES.
Small Angle Scattering (SAS) allow one to obtain structural information about proteins in solution: an indirect Fourier transformation of the scattering curves results in the distance distribution function of the molecules. Small Angle Neutron Scattering (SANS) allows one to study parts of complexes selectively: deuteration of subunits and variation of the solvent D_2O content enhances the scattering from certain parts of a protein complex and attenuates or eliminates the signals of other parts. We are able to prepare partially deuterated GroEL and GroES that can be made invisible in 99% D_2O. This reduces considerably the signal to noise ratio in neutron scattering experiments. Simultaneously, it allows us to use protonated substrate proteins in experiments where previsously each substrate protein had to be deuterated.
In our experiments, we have studied the interaction of GroEL with its co-chaperonin GroES, co-chaperonin GP31 and various substrate proteins.

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2C-11
MICROSECOND TIME-RESOLVED FTIR-SPECTROSCOPY WITH A CONTINUOUS-FLOW MIXER ON A MICROCHIP

Ekkehard Kauffmann*, Nicolas C. Darnton#, Robert H. Austin#, Klaus Gerwert*

We present a new continuous flow mixing cell for FTIR spectroscopy. It combines a micro fabricated analytical device with silicon infrared optics. The design is based on diffusive mixing in the regime of laminar flow[1] and reaches a time resolution of up to 250 µs. Because of its 8 µm optical pathlength it is suitable for FTIR spectroscopy of aqueous solutions.
β-lactoglobulin, a predominantly β-sheet protein, can refold to 80 % helical content upon addition of trifluoroethanol (TFE)[2]. The refolding is initiated in the micro-mixer and monitored by FTIR spectroscopy. The main phase of the refolding has a rate constant of 125 s^{-1}. This is greater by three orders of magnitude than the unfolding rate in guanidine hydrochloride (GdmCl) or the refolding from the GdmCl denatured state. We conclude that TFE, besides stabilizing α-helices, reshapes the energy landscape of the protein drastically and flattens activation barriers.
With this mixing chip it will be possible to investigate the dynamics of a large variety of protein reactions by time-resolved FTIR spectroscopy.

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2C-12
HETEROGENEITY IN THE FOLDING INTERMEDIATES OF BARSTAR DETECTED BY TIME-RESOLVED FLUORESCENCE RESONANCE ENERGY TRANSFER

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Heterogeneity of folding intermediates has been proposed as a hallmark of protein folding process. A triple mutant of barstar having a single tryptophan (W53) in its core and a single cysteine sidechain was prepared. The sulfhydral sidechain of the cysteine was coupled to 5,5'-dithio-bis-[2-nitrobenzoic acid] (DTNB). The TNB group quenched the fluorescence of W53 to the extent of ~95% by fluorescence resonance energy transfer (FRET) in the native state. The quenching gets relieved in the denatured state.
Fluorescence decay kinetics of W53 was analyzed as a distribution of lifetime by the unbiased Maximum Entropy Method (MEM). The distribution of lifetimes was translated into a distribution of distance between the W53 (the donor) and the TNB group (acceptor). Such distance distributions could reveal the structural heterogeneity of the intermediate species involved in the folding process. This FRET-based method has also been used in detecting small changes in the structure under various native-like and denaturing conditions.

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2C-13
STABILITY OF A SMALL PROTEIN INVESTIGATED BY COMPUTER SIMULATION

Giulia Morra, Ernst-Walter Knapp

The cold shock protein CspB from Bacillus Subtilis is a small β-barrel protein (67 residues). This fast folding protein has been largely investigated, because of its simple two state mechanism. A large body of experimental data concerning the thermodynamic stability of various mutants of CspB is also available.
In this work we want to investigate by computer simulation how the stability of the folded state of this protein and of its mutants changes with pH and ionic strength. For that purpose we consider electrostatic interactions and conformational changes. The protonation patterns are determined by solving the Poisson-Boltzmann equation, without considering explicit water molecules, for the native structure and one unfolded reference structure. Since the number of titratable groups is small enough, the difference in free energy can be evaluated by direct summation method. In this way we can calculate energy and entropy of the folded and unfolded state. Thus, we estimate those contributions to the stability, which are provided by different protonation states of titratable groups.

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2C-14
INTERMEDIATE STATE DETECTED BY SAXS DURING HEAT DENATURATION OF A SMALL β-PROTEIN

Javier Pérez, Daniela Russo, Patrice Vachette, Michel Desmadril, Dominique Durand

The heat denaturation process of a small β-protein, neocarzinostatin, was followed by Small Angle X-ray Scattering. A close analysis of the data clearly shows that the unfolding transition involves more than two states, what previous fluorescence and microcalorimetry measurements had only suggested.
From ambient temperature to 59 °C, the experimental patterns compare well with the curve calculated from the atomic coordinates, with a radius of gyration of 14.0 ± 0.5 Å. Above 60 °C, the protein progressively unfolds and reaches a highly unfolded state at 77 °C. Heat denatured NCS is then well described by the worm-like chain model of Porod and Kratky. The radius of gyration has a value of 26.2 ± 0.5 Å. The transition from native to unfolded NCS appears to be highly cooperative, with a sigmoidal shape of the square of the radius of gyration *vs* temperature plot. However, the absence of isoscattering point common to all scattering curves suggests the existence of at least one non-native state before reaching the complete unfolded state. This has been confirmed by a Singular Value Decomposition of the whole experimental data set. Using a three state thermodynamic model, a least-square calculation yields a representation of the intermediate structure, or ensemble of structures. This appears to be a non globular state, with a radius of gyration of 23.4 Å, close to but clearly distinguishable from the unfolded state.

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2C-15
HEAT-SHOCK PROTEIN 90 (HSP90): MODIFICATION OF TERTIARY AND QUATERNARY STRUCTURES BY DIVALENT CATIONS

Cyrille Garnier, Pascale Barbier, Claudette Briand, Vincent Peyrot

HSP90 is one of the major stress protein which overall structure remains unknown. The aim of this study was to investigate tertiary and quaternary structures of the HSP90 and the influence of divalent cations. By analytical ultracentrifugation (UCA) and native PAGE, we showed that HSP90 was in equilibrium between monomeric and dimeric forms. The elution of the HSP90 dimeric form on size exclusion chromatography and UCA analysis indicated that this protein was an elongated protein. Mg^{2+} and Ca^{2+} induced a protein concentration dependent increase of the sedimentation coefficient reflecting an oligomerization process. In presence of cations, we evidenced, by electrophoresis and size exclusion chromatography, an oligomeric form when HSP90 was cross-linked by EDC. In the opposite, when HSP90 was cross-linked before cations addition only dimeric forms were observed. The sedimentation coefficient of cross-linked dimers was higher in presence of cations than in absence. In the same way, the cations induced a shift of cross-linked dimer elution profile toward lower molecular weight on size exclusion chromatography. These two results were explained by a tertiary structural conformational change, which was confirmed by far-UV circular dichroism experiments. To sum up, divalent cations induced a HSP90 tertiary conformational change leading to an oligomerization process *in vitro*. These structural changes could be involved in the *in vivo* regulation of HSP90 functions.

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2C-16
SINGLE MOLECULE PROTEIN FOLDING WITH CYTOCHROME C

Christian Rischel[1,2], Lars Elkjær Jørgensen[1], Zeno Földes-Papp[3], Rudolf Rigler[3]

Investigation of protein folding on single molecules can give new information about pathways and timescales. In our experiments, we detect single protein molecules by their fluorescence, and in order to relate protein conformation to fluorescence intensity we incorporate a donor-acceptor pair, such that the donor fluorescence depends on the donor-acceptor distance by virtue of the FRET mechanism.
Cytochrome C from yeast is a protein of 109 amino acid residues, with a heme group attached covalently to cysteines at position 20 and 23, and with a single free cysteine at position 108. We have attached a fluorescent dye molecule to the free cysteine, either BODIPY 507/545 or Alexa 488 (both from Molecular Probes). The absorption band around 535 nm of the heme group makes it a good acceptor for these fluorophores. For both dyes, the fluorescence is strongly reduced in the folded state of the protein but only reduced by 10-25% in the GuHCl-denatured state.
In the first single-molecule experiments we have measured the timescale of rapid structural fluctuations in the denatured state by FCS. This information is important in relating the folding time to the absolute height of the free energy barrier.

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2C-17
PICOSECOND DYNAMIC OF HEAT DENATURED PROTEIN IN SOLUTION REVEALED BY QUASI-ELASTIC NEUTRON SCATTERING

D. Russo, J. Pérez, M. Desmadril, D. Durand

To define the folding pathway of a protein it is very important to characterise the structure and dynamics of the native and unfolded states under various conditions. The characterisation of the internal dynamics of non native proteins remains a major limiting factor to understanding the protein unfolding process and define its landscape energy. Dynamical events in folded and unfolded proteins occur on a wide range of time scales, and picosecond motions make a particularly important contribution to the internal fluctuation of the atoms from their mean position. Quasi elastic incoherent neutron scattering is a very powerful technique to investigate the self correlation in motions of protein atoms. Quasi elastic neutron scattering experiments performed on neocarzinostatin (NCS), a small β-protein, reveal an important change in the picosecond internal dynamics when the protein unfolds by heating. All internal dynamics parameters (characteristic average relaxation time, amplitude of the diffusive motions, fraction of "immobile" scatters, mean-square vibrational amplitude) undergo abrupt modifications when the temperature is raised above 65 °C. In agreement with NMR experiment, the dynamic image at 21 °C is characteristic of a rigid structure where the only revealed diffusive movements are that of the loops side chain. Arising temperature the constraint backbone and beta sheet side chains hydrogen atoms contribute to the picosecond dynamics, with a low amplitude.

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2C-18
BOUND STATE CONFORMATION AND DISAGGREGATION OF MODEL PEPTIDE SUBSTRATES OF THE CHAPERONE SECB: ESR AND FLUORESCENCE STUDIES

Vikram G. Panse, Pia D. Vogel, Wolfgang E. Trommer, Raghavan Varadarajan

SecB is a homotetrameric chaperone that forms part of the protein translocation machinery in *E. coli*. We have investigated the bound state conformation of the model substrate bovine pancreatic trypsin inhibitor (BPTI) as well as the conformation of SecB itself by using proximity relationships based on site-directed spin labeling and pyrene fluorecence methods. The data suggests that SecB binds a collapsed coil of reduced unfolded BPTI, which then undergoes a structural rearrangement to a more extended state upon binding to SecB. In addition ESR shows that also SecB undergoes a conformational change during this process. Similar studies using the insulin B chain revealed that SecB does not act as a catalyst in the dissociation of aggregated B chains but rather binds to a small population of free B chains with high affinity, thereby shifting the equilibrium from the aggregate towards individual B chains.

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2C-19
APPLICATIONS OF LASER INDUCED PH-JUMP IN THE CHARACTERIZATION OF FAST EVENTS IN PROTEIN FOLDING

Cristiano Viappiani, Stefania Abbruzzetti, Mauro Carcelli, Paolo Pelagatti, Dominga Rogolino

Laser-induced pH-jump allows to achieve a step increase or decrease of the pH of the solution within a few nanoseconds. The methodology requires the use of suitable photoactivatable caged compounds releasing irreversibly either a proton or hydroxide. The sudden change in pH allows the investigation of the proton transfer processes and the following structural rearrangements occurring on the proteins upon the change in the ionization of some of the aminoacidic residues. We report the characterization of some of these caged compounds, in particular a novel photoactivatable caged hydroxide. An application of a caged proton to study fast events in protein folding is also reported. The kinetics of the protonation and dissociation of the non-native axial histidine ligands (His26 and His33) in unfolded Fe(III) cytochrome *c* (cyt *c*) in 3.1 M GuHCl has been monitored by transient absorption detection. We show that deligation of the nonnative histidine ligands from the heme in GuHCl unfolded Fe(III) cyt *c* is a process characterized by a complex kinetics, extending from the microseconds to the milliseconds. The transient absorption data nicely reproduce the deligation kinetics previously measured with stopped-flow pH-jump in the milliseconds. In addition, we evidence a submillisecond phase, which is related with the proton transfer processes occurring on the protein and leading to the protonation of the histidine residues.

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2C-20
INITIAL CONFORMATIONAL DYNAMICS IN CYCLIC AZOBENZENE PEPTIDES

Sebastian Spörlein, Christian Renner, Raymond Behrendt, Luis Moroder, Wolfgang Zinth, Josef Wachtveitl

Femtosecond time resolved spectroscopy of model-peptide-compounds is the most direct approach to study the mechanisms underlying the fast reactions in protein folding. The ultrafast photoisomerization of azobenzene can be utilized to switch between structural motifs in cyclic peptides. In a monocyclic molecule containing the photochromic (4-amino)phenylazobenzoic acid (APB) and the active site fragment of thioredoxin reductase, the light induced reversible *trans/cis*-isomerization leads to a conformational transition from a very rigid, highly constrained *trans*-APB-peptide to a more flexible *cis*-APB-peptide.
Real time observation of the ultrafast photoreactions of the isolated chromophore azobenzene, the linear and the cyclic APB-peptides is performed using fs-time resolved absorption spectroscopy in the visible spectral region.
Azobenzene shows a fast photoisomerization within a few 100 fs, but for the linear APB-peptides the ps-components dominate. The clearly increased lifetime of the excited state may reflect a slower isomerization due to the larger molecular mass of the molecule. Interestingly, in the cyclic peptide the *trans/cis* isomerization occurs faster than in the linear peptide. In contrast, the restriction of conformational space (*cis/trans*) leads to a drastically slower reaction dynamics. Only in the cyclic system very slow components were found and may be indicative for the response of the peptide backbone upon azobenzene isomerization.

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2C-21
MOLECULAR DYNAMICS-SIMULATIONS OF AFM UNFOLDING EXPERIMENTS

Holger Wagner, Helmut Grubmüller

The tension of muscle sarcomeres upon stretch is mainly due to the spring-like behaviour of the protein titin [Lab95]. These mechanical properties of titin upon enforced unfolding have been studied through AFM-experiments [Rie98], optical tweezers [Kel97,Tsk97], and molecular dynamics (MD)-simulations [Lu98]. The AFM-experiments result in force profiles showing a sawtooth pattern corresponding to the successive unfolding of single immunoglobuline (Ig)-domains.
We performed MD-simulations of the enforced unfolding of single Ig-domains of titin. These simulations provide a model for the unfolding pathway and for the microscopic interactions during unfolding. The pathway of the enforced unfolding is compared with the pathway derived from MD-simulations of thermic unfolding.
Considering activated processes in a two state-model, computed unfolding forces have been rescaled to the millisecond time scale of AFM-experiments. Thereby, good agreement between measured and computed unfolding forces was obtained.
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2C-22
USING PRESSURE-JUMP TECHNIQUES FOR STUDYING PROTEIN FOLDING

J. Woenckhaus[1], R. Köhling[1], C. Royer[2], R. Winter[1]

Most studies dealing with protein folding have been carried out at atmospheric pressure using temperature or the chemical composition of the solvent as experimental variables. We present data on the pressure-induced un/refolding of SNase WT using pressure-jump techniques in combination with small-angle synchrotron X-ray scattering and FT-IR spectroscopy, which monitor changes in the tertiary and secondary structural properties of the protein. Application of pressure above 2 kbar at 25 °C leads to a two-fold increase of the radius of gyration and a large broadening of the pair-distance-distribution function, indicating a transition from a globular to an ellipsoidal structure. Analysis of the IR amide I' spectral components reveals that the pressure-induced unfolding process is accompanied by an increase in disordered structures while the content of ß-sheets and α-helices decreases. A (T, p)-phase diagram was obtained. Due to a large positive activation volume, the pressure-induced unfolding of SNase follows a relatively slow kinetics on the timescale of minutes. The refolding reaction is an order of magnitude faster. The first-order changes in β-sheet, α-helical and random structure occur on a similar timescale as the changes in protein compactness, thus indicating that they dependent upon the same rate-limiting step. The data are compared with results obtained using other trigger mechanisms.

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2C-23
A CONTRIBUTION TO THE *AB INITIO* GLOBULAR PROTEIN FOLDING

Denis Znamenski, Jacques Chomilier, Jean-Paul Mornon

We present an enhanced version of a procedure for *an ab-initio* prediction of protein structures called RUSSIA (Rigid Unconnected Secondary Structure Iterative Assembling). It assembles secondary structures, predicted from their sequences, considered as rigid blocks. Alpha helices are constructed with a radius of 2.3 Å and 3.6 residues per turn. Beta sheets are modelled by helicoid surfaces with a variable degree of twist. The interactions between side chains are determined by a 20 x 20 symmetric matrix of pairwise distances between each pair of amino acids.
Maximal loop lengths were introduced in the algorithm to constrain the reciprocal positions of secondary structures.
Using either hydrophobic or, if available, topologically conserved residues as attractants, a set of tightly assembled tertiary structures was generated by successive adjustments made by translations/rotations of the secondary structures. The end criterium for the algorithm is the minimum of the greatest distance between centres of hydrophobic areas of secondary structures and the centre of gravity of the protein. The most compactly assembled structures among all generated ones were selected. An application of this algorithm to 4 α-helix bundles (ferritin, myohemerythrin, granulocyte-macrophage stimulating factor) resulted in r.m.s. differences between model and native structures of 3Å, 1.8Å and 2.4Å, respectively. The results for α-helix and β-sheet as well as two β-sheets assembling will be presented.

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2C-24
UNFOLDING PROPERTIES OF ANNEXIN I DOMAIN 2 IN MOLECULAR DYNAMICS SIMULATIONS. COMPARISON WITH NMR DATA

Tru Huynh, Jean-Michel Neumann, Jeremy C. Smith[1], Alain Sanson

MD simulation, in conjunction with experiments, on small proteins can provide much information on the folding processes. The interest of annexins for folding studies stems from their hierarchical structure: the domain, five helices in a right handed super helix topology and a domain circular association. This hierarchical simplicity renders the examination of their folding pathways of particular interest. The study of isolated domains of annexin I has also revealed an important hierarchy in stability: domain 1 remains stable in aqueous solution and constitutes an autonomous folding unit, domain 2 and domain 3 do not. Results will be presented concerning MD simulations of the isolated annexin I domain 2 with explicit water molecules at several temperatures ranging from 300 K to 450 K, using the CHARMM program. A dynamic of 10.1 ns was ran at 450 K as well as secondary dynamics. The simulated structures of the domain 2 unfolded state fit remarkably well the NMR data: the residual local - native and non-native - structures and helix propensities. This allows a reliable description of the ensemble of structures that characterize the molten globule state and its hydration state. These simulations also highlight what could be the important role of local nucleation structures and secondary structure frameworks in the building of a restricted ensemble of related folding pathways.

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2C-25
UNFOLDING AND FOLDING OF THE LAMBDA CRO REPRESSOR PROTEIN

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In the Cro protein, two monomeric units form a dimer by aligning the C termini of each monomer, allowing the formation of a β-ribbon across the dimer. The N-terminal parts form small subdomains that consist of three α-helices and a short N-terminal β-strand. The thermal unfolding of the wild-type protein and a disulfide bridged variant (Cro-V55C) has been scrutinized by IR spectroscopy and dynamic light scattering. A well defined intermediate was observed for the Cro-V55C dimer, in which the protein has a lost its secondary structure in the N-terminal region and is a tetramer. The remaining structure formed by the C-terminal parts of each polypeptide chain unfolds during a second transition, which also involves the dissociation of the tetramers. 2D-IR spectroscopy and T-jump experiments in combination with time-resolved IR spectroscopy reveal that the first thermal transition of Cro-V55C is a three-step process, which starts with the unfolding of the short N-terminal β-strand. Kinetic IR studies of refolding of Cro-V55C from its intermediate state show that no secondary structure is formed within the first seconds, suggesting that oligomerization of the protein strongly decelerates its folding. A quite different picture was obtained for refolding of Cro-V55C from the thermally unfolded state, where a significant amount of secondary structure is formed within the dead time of the experiment. Moreover, the N- and C-terminal parts of the polypeptide chain seems to refold as independent domains.

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2C-26
FORCED UNFOLDING OF A SINGLE PROTEIN DOMAIN

Pierre-François Lenne, Stephan M. Altmann, Arnt J. Raae, Jari Ylanne, Matti Saraste, J. K. Heinrich Hörber

Before the advent of the AFM, it was necessary to heat or expose a protein to chemical denaturants to unfold it. Now, a protein can be mechanically unfolded by manipulating it with the tip of an AFM. Unfolding force curves present a saw-tooth like patterns in which each peak force is interpreted as an unfolding event. We studied spectrin domain unfolding by manipulating engineered polymeric protein consisting of identical spectrin domains with AFM. Spectrin is a vital and abundant protein of the cytoskeleton. We demonstrate that the unfolding of spectrin domains can occur in a stepwise fashion during stretching. The force-extension patterns exhibit features that are compatible with the existence of at least one intermediate between the folded and the completely unfolded conformation. We explain some difficulties inherent to the use of "conventional" AFM for protein unfolding where molecules are extended at constant speed. In particular, the study of the unfolding kinetics suffers from the fact that the force is highly non-linear during this loading. We introduced a novel AFM with a Multiple Sensor System. We demonstrate that with this instrument we can drive force curves in a stepwise ramped fashion with precision and stability typical for contact mode AFM. After each extension step, force is recorded and may reveal transitions between a folded and an unfolded states. This opens the possibility to explore details in the mechanical stability of proteins.

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2D-1
THE MOLECULAR BASIS OF THE VERSATILITY OF REGULATORY INTERACTIONS OF CA^{2+} CALMODULIN

Stephen Martin, Peter Bayley

Stimulation of mammalian cells raises the intracellular concentration of Ca^{2+} from < 100nM to > 1μM, and the translation of this signal into activation of a multiplicity of enzymic and other processes depends mainly on the action of calmodulin, (Cam). The question is how the necessary selectivity of activation of diverse targets can be regulated by this single protein molecule. We have simulated equilibrium and kinetic processes associated with the binding of 4 Ca^{2+} ions, two each to the two domains of calmodulin, (which have significantly different Ca affinities and co-operativites). We have also considered additional regulatory effects of physiological concentrations of $[Mg^{2+}]$ = 1-5mM both in Ca^{2+} binding, and in the subsequent interactions with different targets, based upon knowledge of X-ray and nmr structures of complexes with typical target sequences. The versatility of calmodulin action is illustrated by the different binding and activation profiles (vs $[Ca^{2+}]$) deriving from : a) the differential affinity of the domains for Ca^{2+} (C-dom > N-dom); b) the relative affinity of domains for Mg^{2+} vs. Ca^{2+} (N-dom > C-dom); c) the different conformational effects of Ca^{2+} and Mg^{2+}; d) the different modes and domain specificities of binding and the affinities of different target sequences for Ca_4.Cam; e) the potential role of partially saturated calcium-bound species (e.g. Ca_2.Cam; $Ca_2.Mg_2$.Cam) as equilibrium and kinetic intermediates in regulatory systems.

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2D-2
A DISSOCIATIVE TRANSITION STATE LIKE CHARGE DISTRIBUTION IN GTP IS INDUCED UPON RAS-BINDING

Marco Blessenohl, Christoph Allin, Alfred Wittinghofer, Klaus Gerwert

FTIR difference spectroscopy is used to determine the molecular GTPase mechanism of the small GTP binding protein H-*ras* p21. The reaction is initiated by the photolysis of caged GTP bound to Ras. The phosphate vibrations are assigned using ^{18}O labeled caged-GTP. The binding causes an unusual frequency downshift of the GTP β-(PO_2^-)-phosphate vibration, whereas the α-(PO_2^-)- and γ-(PO_3^{2-})-phosphate vibrations shift up. The frequency shifts indicate a lowering of the GTP non bridging β-(P-O) bond order and an increase of the α- and γ -(P-O) bond order. These changes can be explained by an increase of negative charges at the non bridging β-oxygens and a decrease at the α- and γ- non bridging oxygens as compared to unbound GTP. Recently we were able to assign the bridging P_β-O-P_γ vibration, which is significantly downshifted compared to unbound GTP, indicating a weakening of the bridging β,γ-bond upon binding to *Ras*.
Therefore, we propose that H-*ras* p21 provides a specific binding niche that preprograms a dissociative transition state for the intrinsic GTPase mechanism.
In complex with the GTPase activating protein NF-1, the reaction is accelerated by 5 magnitudes and an intermediate can be accumulated only for wildtype NF-1.

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2D-3
LIPOSOMES WITH INCORPORATED HAPTENS AS MODEL FOR STUDYING THE INTERACTION OF ANTIBODIES WITH POLYVALENT ANTIGENS

Olga D. Hendrickson, Boris B. Dzantiev, Anatoliy V. Zherdev, Alexander P. Kaplun

Unilamellar liposomes loaded with antigen-lipid conjugates were proposed as a tool to study the interaction between antibodies and polyvalent antigens with migrating determinants. Herbicide atrazine was chosen as a model antigenic hapten. Its carboxylated derivative was coupled to dimyristoylposphatidylethanolamine (DMPE). Anti-atrazine antibodies were conjugated with peroxidase label using periodate technique. Seria of liposomal antigens were obtained from mixtures of dimyristoylposphatidylcholine (DMPC) and the atrazine-DMPE conjugate by their multiple extrusion through LiposoFast-1 injector (generously provided by *Avestin*). Range of DMPC:conjugate molar ratio was 1:9 - 1:999. Kinetic and equilibrium constants of the reaction between liposomal antigens and antibodies were measured by ELISA. Concentration and kinetic dependencies were compared for antibodies and their monovalent derivatives; the contribution of bivalent immune complexes into binding constants was characterised. A mathematical model was proposed to describe the formation of the immune complexes with different composition and affinity; properties of the model were analysed using Mathbrain 1.51E program.
The work was made possible by Russian Foundation for Basic Research (award #00-04-49405).

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2D-4
PROTEIN-PROTEIN DOCKING AND POISSON-BOLTZMANN CALCULATIONS FOR COMPLEXES OF CYTOCHROME C WITH BC1-COMPLEX AND WITH CYTOCHROME C OXIDASE

Dagmar Flöck, Volkhard Helms

Cytochrome c participates in the mitochondrial and bacterial respiratory chains by shuttling electrons between two membrane proteins, bc1-complex and cytochrome c oxidase (COX). During recent years, crystallographic information of the structures of the individual proteins and measurements of binding affinities and kinetics have become available. However, understanding fine details of the transport mecha-nism requires models of the productive electron transfer complexes.
In this work, complexes of cyt c and bc1-complex and of cyt c and COX were obtained by protein docking with the FTDOCK program that searches over rotational and translational space for best surface complementarity and electrostatic correlation. The complexes were further refined by rigid body energy minimization employing an empirical atomistic force field. Accurate binding energies were then calculated by solving the Poisson-Boltzmann equation for wild type and mutant proteins using the UHBD program.

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2D-5
DISSOCIATION KINETICS OF *RAPANA THOMASIANA* HEMOCYANIN

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The dissociation kinetics of the hemocyanin from the gasteropod *Rapana Thomasiana* was investigated by the light scattering stopped flow technique, at 363 nm. The native protein is stabilised in its associated form (102S di-decamer of 8.5-9 MDa) at neutral and slightly alkaline pH by the presence of divalent cations, such as Mg^{2+} or Ca^{2+}. For instance at 1 mM Ca^{2+}, the protein remained fully associated at pH 8 and partially associated even at pH 10, without denaturation. In contrast, the protein was completely dissociated into monomers (250-300 kDa) by an excess EDTA at any pH above 8.5. The kinetics of dissociation was therefore induced by rapid pH jumps (from 7 to 9), as follows: the protein solution (pH 7, 0.5 mM Ca^{2+}) was rapidly mixed with alkaline EDTA (pH 9.2, 0-100 mM EDTA). The kinetics reached a maximum value near 10 mM EDTA, but above an unexpected progressive slowdown was observed. Both effects (increase and decrease of the dissociation rate constants with EDTA) were accounted by the following kinetic model: at concentrations below the maximum rate, EDTA chelates Ca^{2+} ions, favouring the protein dissociation rate, whereas above the maximum rate, free Ca^{2+} ions compete with sites on the protein, which prevent dissociation by binding EDTA.

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2D-6
BARRIER FLUCTUATIONS AND THE HOFMEISTER EFFECT

A. Dér, A. Neagu, Monica Neagu

The Hofmeister effect consists in changes of protein solubility triggered by neutral electrolyte cosolutes. Based on the assumption that salts cause stochastic fluctuations of the energy barriers, a kinetic theory of this phenomenon is proposed. An exponentially correlated noise, of amplitude proportional to the salt concentration, is added to each energy barrier, and the time-dependence of the mean protein concentration is calculated. It is found that the theory yields the well-known Setschenow equation if the noise correlation time is low in comparison to the aggregation time constant. Experimental data on salting-in agents are well fitted, while in the case of salting-out cosolutes, two independent dichotomic fluctuations are needed to fit the data. This may result from the fact that, in both cases, the low-concentration regime is dominated by salting-in electrostatic contributions, while at high concentrations electron donor/acceptor interactions become important; these have opposite effect in the second case.

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2D-7
BINDING OF CAPTOPRIL TO APO-ANGIOTENSIN CONVERTING ENZYME

Ortiz-Salmerón E., Barón C., García-Fuentes L.

Angiotensin converting enzyme (ACE; EC 3.4.15.1) is a zinc metallopeptidase that play a central role in bood pressure regulation. It is a dipeptidyl carboxypeptidase which converts angiotensin I into potent vasopressor peptide angiotensin II [1]. ACE is a target for antihypertensive drugs, such as captopril (D-[3-mercapto-2-methylpropanoyl]-L-proline). A calorimetric study was performed to characterize the binding of captopril to apo-ACE in buffer cacodylate at pH 7 over a temperature range of 15 to 30°C. These results have been compared with those obtained to the binding of this inhibitor to holo-ACE [2]. Calorimetric measurements indicate that captopril binds to two sites in the monomer of apo-ACE, this binding being enthalpically unfavorable at low temperatures and being dominated by a large positive entropy change. The temperature dependence of the free energy of binding $\Delta G°$ is weak because of the enthalpy-entropy compensation caused by a large heat capacity change, $\Delta C_p = -4.92 \pm 0.17$ kJ/K mol of monomeric apo-ACE. This negative value of ΔC_p is consistent with burial of a large non-polar surface area upon binding, and slight conformational changes in the vicinity of the active sites.
Supported by Grant PM97-0003 from DGICYT, Ministerio de Educación y Cultura, Spain
[1] Ehlers, M.R., and Riordan, J. F. (1989) Biochemistry 28, 5311
[2] Ortiz-Salmerón, E., Barón, C., and García-Fuentes, L. (1998) FEBS Letters 435, 219

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2D-8
POLYMERIZATION OF AMYLOID β-PEPTIDE AT DIFFERENT PH INVESTIGATED WITH FLUORESCENCE CORRELATION SPECTROSCOPY

Aladdin Pramanik, Niklas Bark, Lars O. Tjernberg, Lars Terenius, Rudolf Rigler

The deposition of Alzheimer amyloid β-peptide (Aβ) fibrils in brain is a key step in Alzheimer's disease. Studying of the Aβ polymerization and its inhibition is a potential strategy for drug design relevant for Alzheimer's disease. Fluorescence correlation spectroscopy (FCS) is a powerful biophysical tool for studying molecular interactions of biological importance (Rigler, J. Biotech. 41 (1995) 177-186). Using FCS we have for the first time followed the Aβ polymerization in real time, detecting monomers, oligomers and large aggregates simultaneously (Tjernberg et al., Chem. Biol. 6 (1999) 53-62). The Aβ aggregation shows high cooperativity and the critical concentrations for cooperative transitions differ at different pH. The Aβ polymerization at pH 7.4 occurs above a critical concentration of 50 μM and proceeds from a mixture of Aβ monomers and dimers into large aggregates, without any detectable amounts of oligomers, and reaches a maximum after 40 min. At pH 8.0 when Aβ exists only in monomers, the aggregation initiates after 6 h and occurs above a critical concentration of 300 μM, and oligomers are detectable. The monomeric state of Aβ leads the aggregation process to initiate after several hours over higher critical concentration of Aβ, suggesting that several hours are necessary for the formation of dimers from monomers. In conclusion, dimer/tetramer formation of Aβ is an important factor in the Aβ aggregation.

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2D-9
THE INTERACTION BETWEEN CATALASE AND NAFCILIN BY EQUILIBRIUM DIALYSIS MEASUREMENTS

Gerardo Prieto, Félix Sarmiento, Pablo Martínez-Landeira, Juan Manuel Ruso

Binding isotherms of nafcilin to bovine catalase were determined by equilibrium dialysis in which aliquots of catalase solutions were placed in dialysis cells and equilibrated with nafcilin solutions at pH 3.2, 7.4 and 10.0 for over 120 hours at 25°C. The main characteristic of the isotherm is the large number of drug molecules boud (ν) per protein molecule.
Standard Gibbs energies per drug bound ΔG_ν were obtained from the isotherms using the Wyman potential. The curves of ΔG_ν as a function of ν pass through minima corresponding to the most tightly bound ligands at low values of ν and tend to limiting values of ΔG_ν (*ca* -18 kJmol^{-1}) at high values of ν.

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2D-10
THE INTERACTION BETWEEN CATALASE AND NAFCILIN BY ζ-POTENTIAL MEASUREMENTS

Félix Sarmiento, Gerardo Prieto, Pablo Martínez-Landeira, Juan Manuel Ruso

An experimental investigation on the adsorption of nafcilin on bovine catalase at different media (pH 3.2, pH 7.4 and pH 10.0) at 25 °C by measurements of the zeta potencial (ζ- potencial) has been performed. At pH 7.4 and 10.0 the protein surface has a negative electrokinetic charge while at pH 3.2 the nafcilin ions affect the ζ-potencial causing a change in the neighbourhood of the point zero charge (p.z.c.) from positive to negative values. From the p.z.c. we have calculated the Gibbs energies of adsorption and the number of sites (N_1) of adsorption. The results are compared with previous studies on the interaccions between catalase and anionic surfactants and penicillins with other proteins.

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2D-11
A NOVEL METHOD OF MONITORING GEL FORMING INTERACTIONS IN GASTRIC MUCUS

Catherine Taylor, Adrian Allen, Peter W. Dettmar, Jeffrey P. Pearson

Mucus is a unique biological secretion in that it is able to form gels. Mucus gels have dominantly solid like behaviour but are also able to flow and re-anneal over time. Mucus gels have previously been studied using non-destructive rheological methods to determine the solid like (G', elastic modulus) and liquid like (G", viscous modulus) behaviour. We have developed a novel rheological technique where mucus gel interactions are broken down and reformed, which allows monitoring of gel formation.
Mucus gel is subjected to an up/down stress sweep with continual monitoring of G' and G" using a Bohlin CVO 50 rheometer. With increasing shear stress gel breakdown is induced (G" > G'). Decreasing shear stress then allows reformation of the gel (G' > G").
Fresh pig gastric mucus could be repeatedly broken down and allowed to recover within the test system without showing signs of loss of gel structure. For any gel sample recovery occurs at a slightly lower shear stress than breakdown (e.g. recovery 58Pa, breakdown 74Pa) but there was no change in either value with successive breakdowns.
There was a characteristic pattern in the recovery of G' and G" with marked agreement in values between different gel samples. A peak in G" occurs close to the point G' regains dominance.
Gastric mucus is a resilient gel showing stability to repeated breakdown.
A method now exists that can monitor gel forming interactions in mucus and will allow investigation of their nature.

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2D-12
DIMERIC FORMYLTRANSFERASE FROM METHANOPYRUS KANDLERI IS ENZYMATICALLY ACTIVE. AN ULTRACENTRIFUGE STUDY

Christos Tziatzios, Seigo Shima, Rudolf K. Thauer, Ulrich Ermler, Helmut Durchschlag, Dieter Schubert

Formylmethanofuran: tetrahydromethanopterin formyltransferase (Ftr) from the hyperthermophilic archaeon *Methanopyrus kandleri* is an enzyme involved in the pathway of carbon dioxide reduction to methane. It is composed of only one type of subunits of molecular mass 32kD; its activity depends on the presence of lyotropic salts. When crystallized, the enzyme is a tetramer. Analytical ultracentrifugation was applied to analyze the oligomeric structure and the structure-function relationships of the enzyme in solution. We have reported that formyltransferase is in a monomer/dimer/tetramer association equilibrium, the association constants depending on the concentration and type of salt present. According to the effects of strong and weak lyotropic salts on the oligomerization and activity of the enzyme, monomeric Ftr is enzymatically inactive; it remained open whether the dimer or the tetramer, or both of them, represent the functional unit. We have now studied, by analytical ultracentrifugation, a subunit interface mutant with altered association behaviour. At 500 mM potassium phosphate (pH 7.2), the mutant was found to possess one third of its maximal activity. Under these conditions, the sedimentation equilibrium profiles showed that the mutant protein was present as monomer and dimer only. The dimer content amounted to approx. 83%. Thus, dimeric Ftr is enzymatically active.

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2D-13
KINETIC COMPETITION AMONG PROCESSES AND REVERSIBILITY IN PROTEIN UNFOLDING/AGGREGATION: THE CASE OF BOVINE SERUM ALBUMIN

S.M. Vaiana, M.U. Palma

A great interest is currently focussed on understanding protein folding, unfolding, misfolding and aggregation in terms of basic laws of physics. This is due to the challenging complexity of the problem, as well as to its direct relation to so far unharnessed pathologies such as Alzheimer's, Amyloidoses, and Transmissible Spongiform Encephalopathies.
The present study builds on previous work from our group showing the occurrence of multiple feedback between processes on different length scales (protein conformational change, solution demixing, protein cross-linking and aggregation). BSA is a very useful model system, exhibiting all typical features of pathological protein coagulation. We measure dynamic light scattering and circular dicroism in temperature jump kinetic experiments. Our data widen the scenario of three interacting processes, to include a fourth one. We show that the "intermediate" form initiating aggregation is the result of an increase of β-structure content of single proteins, and of a concurrent process of oligomerization. These two processes produce non-native oligomers, which remarkably behave as larger units exhibiting their own collective behavior with spinodal instability, critical divergences, demixing and final cross-linking and aggregation. In accord with previous findings of our group, progress from oligomers to final aggregation is therefore remarkably more complex than a plain stepwise accretion into larger and larger polymers.

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2D-14
DISSOCIATION-EQUILIBRIUM CONSTANT AND BOUND CONFORMATION FOR WEAK ANTIBIOTIC BINDING INTERACTION WITH DIFFERENT BACTERIAL RIBOSOMES

Laurent Verdier, Josyane Gharbi-Benarous, Gildas Bertho, Nathalie Evrard-Todeschi, Pascale Mauvais, Jean-Pierre Girault

This study characterises the low-affinity antibiotic binding interactions by the T_2 (CPMG). Three different compounds, a ketolide 'telithromycin' (HMR 3647), a macrolide 'roxithromycin' and a lincosamide 'clindamycin' belonging to the macrolide-lincosamide-streptogramin B (MLS_B) class of antimicrobial agents were examined against *Esherichia. coli*, *Streptococcus pneumoniae* and *Staphylococcus aureus* strains. T_2 (CPMG) measurements represent a sensitive method and they can be used to characterise equilibrium binding of low-affinity antibiotics to bacterial ribosomes. This analysis revealed binding ketolide, macrolide and lincosamide to the different strains of ribosomes with $K_D = 10^{-4} - 10^{-3}$. Additionally, this paper demonstrates that the three MLS_B antibiotics exist in these ribosome binding sites in certain conformations by a study using 2D transferred nuclear Overhauser effect spectroscopy (TRNOESY). TRNOE experiments resulted in constraints used on molecular modelling to obtain the conformation of the antibiotic in its bound state.This study allowed us to compare the bound structure at the bacterial ribosomes for the active antibiotics and to bring to the fore an interesting relationship between the conformation of the antibiotic when interacting and its activity.

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2D-15
ANALYSIS OF AMYLOID FIBRILS FORMED BY A SH3 DOMAIN UNDER DEFINED SOLUTION CONDITIONS

Jesús Zurdo[1], Iñaki J. Guijarro[2], Christopher M. Dobson[1]

Amyloid fibrils are highly ordered protein aggregates associated with a range of debilitating human conditions including Alzheimer's disease, type II diabetes, systemic amyloidosis and prion-related disorders. Recently, proteins unrelated to any known human disease have been found to convert in vitro into higher order structures with all the characteristics of the disease-related amyloid fibrils. The analysis of different model polypeptides provides a new way to dissect amyloid formation and aggregation processes.
We have carried out studies on the PI3-SH3 domain in order to analyse the modifications experienced by the precursor prior amyloid fibril formation and their consequences on its aggregation pattern, the kinetics of the process and the final fibril structure. Combination of CD, NMR diffusion, FTIR and EM techniques show that compact precursors show a higher propensity to aggregate, whereas more expanded ones experience a much more slower kinetics of self-association related to well defined amyloid fibrils with morphological characteristics also regulated by solution conditions. We have been able to distinguish between two different kind of interactions between protofilaments and differentiate between the FTIR beta-like pattern associate to either aggregates or regular amyloid fibrils. All these studies provide new strategies to explore in order to better understand misfolding, protein aggregation and amyloid formation processes.

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2D-16
SELF-ASSOCIATION OF A PEPTIDE IN WATER: MOLECULAR AND SUPRAMOLECULAR ORGANIZATION OF LANREOTIDE

C. Valery, F. Artzner, B. Robert, M.L. Torres, R. Cherif-Cheik, M. Ollivon, M. Paternostre

Lanreotide is an octapeptide used in acromegaly therapy. Ipsen Pharma have realized a new formulation, Autogel®, allowing a monthly drug administration instead of a three times/day injection. Autogel® is a liquid crystalline gel (birefringence) obtained from water and lanreotide acetate. The molecular (IR and Raman spectroscopies) and the supramolecular organization (X-Ray scattering, optical and electron microscopies, calorimetry (DSC), turbidity) of the peptide within the gels are studied. Spectroscopic techniques indicate the presence of β-sheet conformation and turns in samples containing at least 5% w/w of peptide. A disulfide bond existence is proved by Raman spectroscopy from 5% w/w to Autogel® (30% w/w). Small/wide angle X-ray scattering (ESRF, LURE) and microscopies show three scales of organization from 5 to 10% w/w: 36nm diameter hollow columns organized in a hexagonal lattice and composed of twisted fibers containing twisted columns of the elemental entity (2x2x2nm). Scattering objects exist at lower concentrations (about 2% w/w) and columnar mesophase at higher ones (20 to 30% w/w). DSC exhibits changes in the states of water correlated to lanreotide concentration. Turbidity assays point out an aggregation limit concentration about 3% w/w.

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2D-17
BINDING STRENGTH BETWEEN LOW-DENSITY-LIPOPROTEIN AND SULFATED POLYSACCHARIDES MEASURED BY SINGLE MOLECULE FORCE SPECTROSCOPY

Gilberto Weissmüller, Gilza M.P. Prazeres, Paulo M. Bisch, Ana M.F. Tovar, Paulo A.S. Mourão

Glycosaminoglycan (GAG) chains that project from proteoglycans of the arterial wall are responsible for the formation of complexes with plasma low-density-lypoproteins (LDL). This is a step in the normal exchange of components between the circulating plasma and the arterial wall. However, during atherosclerosis, this process contributes to continuous focal deposition of cholesterol-rich lipoproteins, mainly LDL, in lesions. Also, glycosaminoglycans induce structural alterations in LDL molecules that may potentiate their atherogenic effects.
The interaction LDL-GAG has been studied with a variety of biochemical, calorimetric and spectroscopic techniques. These methods do not provide direct measurements of the binding forces and, since the nature of the involved binding sites is not well known, this information is very difficult to obtain from their measurements. However, using an atomic force microscope (AFM), we can directly access the binding strength between these molecules by force spectroscopy.
In this study we probed the interaction of LDL adsorbed to a mica surface with different sulfated polysaccharides attached to the AFM sensor tip, under various buffer conditions. We show that dextran sulfate, chondroitin sulfate and dermatan sulfate have different binding strengths with LDL and that both Ca^{++} concentration and the ionic strength modulates the binding forces.

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2E-1
$[Fe_4S_4]^{2+/3+}$-CLUSTERS IN NATIVE, MUTATED AND UNFOLDED HIPIP *C. V.* INVESTIGATED BY FIVE SPECTROSCOPIES

A.W.E. Dilg, E. Babini, I. Bertini, F. Capozzi, K. Grantner, O. Iakovleva, C. Luchinat, M. Mentler, F.G. Parak

HiPIPs are electron transfer proteins in photosynthesis bacteria like *Chromatium vinosum* containing a cubane-like $[Fe_4S_4]$-cluster. The cluster which is investigated here by Mössbauer spectroscopy, Cw and pulsed EPR, ^{57}Fe-ENDOR and EXAFS exists in a reduced diamagnetic $[Fe_4S_4]^{2+}$- and in an oxidized paramagnetic $[Fe_4S_4]^{3+}$-state. The net spin of the oxidized cluster is S = 1/2 as observed by EPR. Mössbauer and NMR investigations on oxidized HiPIPs show the necessity to introduce two antiparallel coupled spectral components representing a pure ferric iron pair and a pair of two indistinguishable iron ions in an oxidation state between two and three ("ferric-ferrous pair"). Electronic relaxation has to be taken into account even at T = 5 K to avoid contradictions of Mössbauer, pulsed EPR and ^{57}Fe-ENDOR [1]. A Mössbauer analysis of a Cys77Ser-mutant from the HiPIP *C. vinosum* indicates a shift of the electron cloud of the cluster towards the serine. The presence of the cluster in two electronic isomers and their appearance in NMR, EPR and Mössbauer experiments is discussed. Measurements of protein dynamics on *C. vinosum* are presented where the molecule has been in the oxidized, the reduced, the native, the C77S mutated, the folded and the partially unfolded form.
[1] A.W.E. Dilg, G. Mincione, K. Achterhold, O. Iakovleva, M. Mentler, C. Luchinat, I. Bertini, F.G. Parak (1999) JBIC 4:727-741

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2E-2
MÖSSBAUER STUDIES ON IRON CENTERS IN D1/D2/CYTOCHROME B_{559}

O. Iakovleva, A.Garbers, J.Kurreck, G. Renger, F.Parak

Mössbauer studies show that D1/D2/Cytochrome b_{559} isolated from spinach is deprived of all detectable non heme irons. This cytochrome attains the low-potential form with the iron center in the pure low-spin ferric state. Its g_z –value is equal to 2.93. Recent studies on the (bis)imidazole complexes propose that the differences in the redox potentials and in the g -values come from the different orientations of the imidazole planes of the axial ligands. Parallel orientation implies the low-potential form while in the case of the high-potential form the imidazole planes are tilted versus each other. The present study shows that the imidazole planes in D1/D2/Cyt b_{559} are parallel. The simulation of the Mössbauer spectra is done in accordance with the crystal field theory of Griffith and Oosterhuis, and the obtained agreement is very good. A rhombic symmetry is assumed. It is shown that the g -tensor and the A -tensor share the common principal (molecular) axes system. The coordinate system of the electric field gradient is rotated about molecular z-axis by $\alpha = 90°$. The influence of the electron spin relaxation is taken into account.

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2E-3
INTERMEDIATES IN THE REACTION OF CYTOCHROME P450CAM WITH PEROXY ACETIC ACID

V. Schünemann, A.X. Trautwein, C. Jung, D. Mandon, R. Weiss

Cytochromes P450 are found in numerous organisms where they catalyze a whole variety of reactions, such as aliphatic and aromatic hydroxylations, epoxidations, heteroatom oxidation, and N- and O-dealkylation, by transfer of an active oxygen from its heme unit to the substrates.
Here we present a first complementary Mössbauer and EPR study on reaction intermediates of substrate-free and substrate-bound cytochrome P450cam from *Pseudomonas putida* prepared by the freeze-quench method from ^{57}Fe- and ^{56}Fe-labeled P450cam using peroxy acetic acid as the oxidizing agent.
When reacting the substrate-free P450cam for 8 ms reaction time the reaction mixture consists of ~90% of ferric low-spin iron with g-factors and hyperfine parameters of the starting material; the remaining ~10% are identified as a tyrosyl radical (S = 1/2) by its EPR and as ferryl iron (S = 1) by its Mössbauer signature.
Thus the reaction of substrate-free P450cam with peroxy acetic acid leads to the oxidation of both a tyrosine residue to a tyrosine radical and of the iron(III) center to iron(IV). In the case of substrate-bound P450cam neither a tyrosine radical nor a iron(IV) is formed. These findings together with EPR relaxation studies indicate that Tyr-96, which is hydrogen-bonded to the substrate camphor, is the candidate for the observed tyrosine radical.
This work was supported by the Deutsche Forschungsgemeinschaft.

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2E-4
ELECTRONIC AND STRUCTURAL VERSATILITY OF IRON-SULFUR CLUSTERS

A.X. Trautwein, R. Benda, V. Coropceanu, Ch. Meier, V. Schünemann, H. Winkler

Fe-S proteins presumably were among the first catalysts nature had to work with. H. Beinert described these proteins as ancient structures which are still full of surprises (JBIC 5, 2, 2000). Our own research in this field indeed revealed a number of exciting and surprising properties of Fe-S clusters:
(i) tunability of electron (hole) localization/delocalization by super exchange, double exchange and symmetry distortion with the consequence to influence redox properties. Collaboration with K. Wieghardt et al., Beardwood et al., A.Kostikas et al.
(ii) prismane cluster (6Fe-6S) with peculiar electronic properties and "prismane" protein with peculiar structural properties – the latter does not contain prismane clusters but rather two 4Fe clusters, one with unexpected iron coordination ("hybrid" cluster). Collaboration with W. R. Hagen et al., P. Lindley et al.
(iii) evidence for reversible interconversion between 2Fe-2S and 4Fe-4S clusters in anaerobic ribonucleotide reductase and biotin synthase from *E. coli*. Collaboration with H. Fontecave et al., A. Marquet et al.
(iv) evidence for a 8Fe cluster in the FeFe cofactor of the Fe-only nitrogenase from *Rhodobacter capsulatus*. Collaboration with A. Müller et al.
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2E-5
PH DEPENDENT HG(II) COORDINATION IN METALLO-THIONEINS: A ^{199M}HG TDPAC-STUDY

W. Tröger[1], B. Ctortecka[1], T. Butz, P. Faller[2], M. Vašák[2] and the ISOLDE Collaboration

Mammalian metallothioneins (MT) are ubiquitous, cysteine-rich proteins of low molecular weight which bind d^{10} metals ions such as Zn(II), Cd(II), Cu(I) and Hg(II) in metal thiolate clusters. They play an important role in the metabolism and in the modulation of the essential trace element zinc and copper and in the binding of toxic heavy metals. The latter suggests also the involvement in cellular detoxification mechanisms. Several 3D structures have been solved for mammalian MT. Divalent metals are tetrahedrally coordinated by both bridging and terminal thiolates in cluster structures.
Here, we report on Hg (II) coordination studies in MTs by time differential perturbed angular correlation (TDPAC) spectroscopy. With TDPAC the local environment of the a TDPAC probe, here ^{199m}Hg, can be studied by the correlation of two subsequent γ quanta emitted by the probe. In MT mainly two- and fourfold Hg(II) coordinations were found depending on Hg(II) concentration and pH. At acidic pH twofold coordinations dominate whereas at basic pH 8.6 also higher coordination numbers occur.

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2E-6
INTERPRETATION OF MÖSSBAUER SPECTRA IN THE ENERGY AND TIME DOMAIN WITH NEURAL NETWORKS

H. Paulsen, R. Linder, F. Wagner, H. Winkler, S.J. Pöppl, A.X. Trautwein

Neural networks (NN) have been used for the analysis of Mössbauer and nuclear forward scattering (NFS) spectra. The task of the NN is to give a reasonable and fast estimate of hyperfine parameters, e.g. the isomer shift, the electric field gradient tensor, and the magnetic hyperfine interaction tensor. The fast estimate of these parameters is obligatory when a fast decision is required whether and under which conditions (temperature, applied field) further masurements have to be performed, especially if time is a limiting factor like for recording NFS spectra at 3rd generation machines. Additionally, such an estimate is useful in providing a set of start parameters for, e.g. a conventional spin-Hamiltonian analysis.
Different network architectures have been tested for a single parameter analysis. Best results have been achieved so far with feed-forward networks with (i) two hidden layers (20 neurons each) and the improved backpropagation algorithm APROP as learning rule and (ii) with one hidden layer with 5 neurons and the variable metric minimization algorithm MINIM included in the program NETFIT. For the performance of the NN, preprocessing of the data turned out to be as important as the choice of the network architecture. The NN were able to perform a single parameter analysis with the raw spectra, but suitable preprocessing of the data allowed to reduce the size of the network, which is important with regard to a simultaneous multi-parameter analysis.

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2F-1
BINDING MECHANISM OF ZN^{2+} TO THE C-TERMINAL ZINC FINGER OF THE HIV-1 NUCLEOCAPSID PROTEIN: CONTRIBUTION OF THE COORDINATING RESIDUES

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The two Cys-X_2-Cys-X_4-His-X_4-Cys retroviral type zinc fingers (CCHC motifs) of HIV-1 nucleocapsid protein, NCp7, strongly bind Zn^{2+} both *in vitro* and in mature virions. The integrity of the Zn^{2+} saturated CCHC motifs proves to be critical in the main functions of NCp7. All mutations affecting the Zn^{2+} coordination result in non-infectious viruses. To further understand the central role of Zn^{2+} binding in NCp7 structure and functions, the coordination of Zn^{2+} to the C-terminal zinc finger, (35–50)NCp7, is investigated by equilibrium and kinetic (stopped-flow) techniques, by monitoring the fluorescence of Trp^{37}. The role of each coordinating residue in the binding process is elucidated by comparing the binding parameters of the wild type to those of four mutants lacking one of the Zn^{2+} ligands. Each mutation in (35-50)NCp7 induces a sharp decrease in the metal affinity, but does not abolish Zn^{2+} binding. The stabilizing effect of the three Cys appears similar and two-fold higher than that of the His. The equilibrium binding constants of all the peptides show a steep pH dependence in agreement with the pK_a values of the coordinating residues. Furthermore, the dynamics of Zn^{2+} binding involves the formation of an intermediate complex that slowly rearranges to form the optimized Zn^{2+}-peptide complex geometry. Taken together, our data allow us to propose a molecular mechanism of the Zn^{2+} binding.

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2F-2
THERMODYNAMIC ANALYSIS OF THE BINDING OF GLUTATHIONE TO GLUTATHIONE S-TRANSFERASE

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Glutathione transferases (EC 2.5.1.18) (GST) are a multigene family of dimeric proteins which play a key role in the detoxication of electrophilic xenobiotics (1). The homodimeric glutathione S-transferase from *Schistosoma japonicum* is expresed in *E. coli* by the Pharmacia pGEX plasmid. The binding of substrate glutathione reduced (GSH) was studied by isothermal titration calorimetry (ITC) at pH 6.5 over a temperature range of 15 to 30 °C. Calorimetric measurements in various buffer systems with different ionization heats suggest that protons are released during the binding of GSH. Moreover, equilibrium experiments for GSH binding to GST were performed at pH 6.5 by quenching of the enzyme intrinsic fluorescence. Fluorimetric and calorimetric measurements indicate that GSH binds to two sites in the dimer of GST. This binding is enthalpic and entropically favorable at all temperatures checked, resulting in an enthalpy-entropy compensation. The temperature dependence of the enthalpy change yields the heat capacity change (ΔC_p) of -0.132 ± 0.024 kcal mol^{-1} K^{-1}.

[1] Salinas,A.E., and Wong M.G. (1999) Curr.Med.Chem. 6, 279–309.

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2F-3
SATURATED AND UNSATURATED PHOSPHOLIPDS AND THEIR PHASE BEHAVIOUR DURING PLA_2 HYDROLYSIS

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Phospholipase A_2 (PLA_2) hydrolyzes the ester bond of phospholipids to release fatty acids and lysolipids. The products exhibit a great diversity of function. The enzymatic hydrolysis of a substrate can be described as proceeding in two steps. The first consists of enzyme adsorption and the second step is catalytic hydrolysis. The influence of lipid phases on the course of a reaction was investigated. Gel phase domains are known to exist in biological membranes and their role in enzyme regulation needs to be evaluated. Time -resolved small and wide angle synchrotron diffraction was utilized to investigate the phase changes during the reaction. Grazing incidence synchrotron diffraction was utilized to study structures of lipid monolayers before and after PLA_2 adsorption and reaction, in addition microscopy and FT-IR experiments were performed.
The adsorption takes place best when the phospholipid is in a gel phase inducing a change in the conformation of the headgroup. This pre-orientation of the substrate facilitates the diffusion into the catalytic active site of the enzyme. The velocity of the reaction is nevertheless very slow in high ordered phases. It is high in gel phases with a large number of defects or in a coexistence regions of gel and fluid phases. During the reaction a phase separation occurs and fatty acid enriched domains are formed. The phase separation may play a key role in the biological function of the PLA_2-products, particularly the second messenger function of arachidonic acid.

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2F-4
REACTION MECHANISM OF *DROSOPHILA* ALCOHOL DEHYDROGENASE

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The mammalian alcohol dehydrogenase (ADH) has been in the center of interest as a model system for enzymatic reactions during the last decades. Numerous experimental and several theoretical investigations have been carried out.
The ADH of insects catalyzes in essence the same reaction but employs a totally different mechanism without a metal center, while the mammalian enzyme has a zinc complexed in its active site. The most important residues involved in the reaction in the *Drosophila* enzyme have been shown to be Ser138, Tyr151 and Lys155 by site directed mutagenesis.
Based on crystal structures and experimental results it was concluded that the reaction mechanism involves a deprotonated Tyr and/or Ser. Lys seems to be of special importance for the binding of NAD^+, but seems not to be directly involved in the reaction mechanism.
By kinetic measurements, several pK values could be determined. Nevertheless, it was not possible to determine, if Ser or Tyr deprotonates, because the measured pK values could not be assigned unambiguously
In the work presented here we carried out electrostatic calculations and Monte-Carlo titrations to determine the protonation pattern of the whole protein. From our computations we found that both Ser138 and Tyr151 were protonated in the resting state of the enzyme. Also all other residues close to the active site possessed normal pK values. Based on these results, EVB calculations for the deprotonation and the oxidation reaction are reported.

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2F-5
FREE ENRGY ANALYSIS OF ENZYME – INHIBITOR BINDING

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The determination of free energies of binding of inhibitors to enzymes are of immense practical interest in structure based drug design efforts, proceeding from the available crystal structure data. We present here a methodology to estimate and analyze the molecular thermodynamics of enzyme-inhibitor binding thus bridging the gap between structure and thermodynamics. We further analyze the energetics of complexation in terms of free energy components, thus facilitating an identification and hence an optimization of forces favoring complexation to obtain desired binding constants. The complexes of protease family of enzymes are adopted for this study. The model incorporates explicit all atom accounts of the energetics of electrostatic interactions, solvent screening effects, van der Waals components and cavitation effects of solvation combined with a Debye-Huckel treatment of salt effects as well as entropic considerations. In general we note that van der Waals and hydrophobic effects favor complexation while electrostatics tend to be case specific. A detailed analysis of binding in the systems mentioned above with an overview of the thermodynamics of protein-ligand binding will be presented and discussed.

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2F-6
ASPARTATE TRANSCARBAMYLASE SHORT TIME DYNAMICS STUDIED BY INELASTIC NEUTRON SCATTERING

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E. coli aspartate transcarbamylase (ATCase) is a 310 kDa allosteric enzyme which catalyses the first committed step in pyrimidine biosynthesis. The crystallographic structure of ATCase of E. coli has been determined at 2.5 Å resolution. The binding of the substrates, carbamyl phosphate and aspartate, induces significant conformational changes [1]. The protein switches from a T state (quaternary structure in the absence of ligand) towards a R state (quaternary structure of ATCase saturated with ligand). The same quaternary structure change is observed upon binding of the bi-substrate analogue PALA (N-(phosphonacetyl)-L-aspartate) [2]. The passage of the T state to the R state results in an increase of 5% of the radius of gyration of the protein, as measured by small angle X-ray scattering [3].
Owing to the large neutron incoherent scattering cross-section of the hydrogen atom and the abundance of this element in proteins, inelastic neutron scattering experiments give a global view of protein dynamics as sensed via the individual motions of its hydrogen atoms. We present incoherent inelastic and quasi-elastic neutron scattering results of the local dynamics (few angstroms), at short time (few tens of picoseconds), of ATCase in T and R conformations.
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2F-7
SYSTEMATIC SEARCH FOR A "BACK DOOR" IN ACETYLCHOLINESTERASE

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Acetylcholinesterase (AchE) [4] is an enzyme that terminates the signal transmission at the cholinergic synapses of neurons by rapid hydrolysis of the neurotransmitter acetylcholin.
To allow substrate release from the active site of AChE a "back door" mechanism has been proposed. Such a back door is not evident from the structure of the protein; accordingly different models have been suggested and are discussed controversally [1,3]. Using, Conformational Flooding' molecular dynamics simulation [2] we have performed a systematic search and have identified and characterized flexible regions that should allow substrate exit with a low activation energy.
Our results provide an unbiased back door model.
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2F-8
ENZYMATIC ACTIVITY OF RIBONUCLEASE H: SINGLE-MOLECULE FLUORESCENCE STUDIES

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Single-molecule fluorescence techniques open up the possibility to observe individual enzyme molecules at work and yield insight into static and dynamic heterogeneities in enzyme activity. Here we present a single-molecule study on ribonuclease H (RNase H), an endonuclease that specifically hydrolyzes RNA hybridized to a complementary DNA strand.
To observe the enzymatic activity of RNase H, we developed a model substrate consisting of a hybrid duplex in which a DNA strand is labeled with either a fluorescent dye (Cy3, Cy5) or quencher (dabcyl, QSY) at the 3'-end, and a complementary DNA-RNA-DNA (mixed) strand is labeled with either Cy3 or dabcyl at the 5'-end.
Enzyme molecules were labeled with Cy3, biotinylated and bound to a streptavidin-coated glass surface. Using a home-built confocal scanning microscope, single enzyme molecules were localized on the surface, and time histograms of the fluorescence intensity were recorded in two separate channels. The enzymatic process was studied by observing the fluorescence at a single enzyme spot: as the enzyme cuts the substrate, the quencher-derivatized fragments fall apart and the Cy3 fluorescence of the complementary strand is restored by tracking the energy transfer (FRET) from the Cy3 bound to the enzyme to the Cy5-labeled duplex in the presence or absence of Mg^{2+}.

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2F-9
RUBISCO: INVESTIGATION OF A LARGE CONFORMATIONAL TRANSITION ARISING AT SUBSTRATE BINDING

Jürgen Schlitter, Michael Spitzer, Günter Wildner

Rubisco is a key enzyme of the Calvin cycle, fixing carbon dioxide for photosynthesis or oxygen for photorespiration to the same substrate ribulose bisphosphate. Its relative specificity for carboxylation has increased from cyanobacteria over higher plants to red algae by a factor 5 in a so far not yet understood way. Only recently a hypothesis was put forward that connects specificity with the dynamics of the binding niche closing mechanism[1]. The hypothesis is supported by a kinetical model, X-ray structures and mutagenesis experiments[2]. When the substrate has entered its binding niche, it is successively covered by a loop, a lysine and the C-terminal tail. The large conformational change involves displacements of up to 30 Angstroms. The tail contains both conserved residues and other, partially charged residues which may influence the dynamics of the conformational change and the crucial lifetime of the closed conformation. The dynamics can be investigated by means of biophysical experiments and simulations. They are undertaken to characterize the processes quantitatively and possibly to find a correlation between the known structures, parameters of the enzyme kinetics, and the conformational dynamics on the other hand.

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2F-10
STRUCTURAL TRANSITION OF A LIPASE (HLL) ADSORBED ON MODEL SURFACES: A FTIR-ATR STUDY AT LIQUID/SOLID THE INTERFACE

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All lipases, whose tridimensional structures are known up to now, exist under two forms: an open form and a closed form. The closed form shows a lid, which hinders the catalytic triad. When the lipases are adsorbed on a lipid substrate, the lid is open and the protein active.
Our goal is to understand at a molecular level, which are the interactions responsible for the opening mechanism. We investigate the structural changes of the *Humicola Lanuginosa Lipase* (HLL) by FTIR-ATR spectroscopy with model surfaces at the liquid/solid interface. To discern the nature of the involved interactions (hydrophobic or electrostatic), we studied the influence of synthetic surfaces with controlled hydrophobicity and polarity. The hydrophobic and polar surfaces were obtained respectively by grafting alkylsilane or aminosilane onto the silicon ATR element.
No adsorption of HLL on the studied polar surfaces occurs. On hydrophobic surfaces, the formation of HLL monolayers was obtained in the concentration range of 10^{-5} to 10^{-7} M. The structural and solvation changes between the solution and adsorbed states of HLL suggest a rearrangement of the lipase tertiary structure in contact with the hydrophobic surface. The 15% of Asp and Glu side-chains still protonated in the adsorbed protein could be explained by an increase in hydrophobicity of the adsorbed state, consistent with the open state of the lipase.

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2F-11
HINGE-BENDING DOMAIN MOVEMENT AND CATALYSIS OF E. COLI 5'-NUCLEOTIDASE (5'-NT)

Norbert Sträter, Thomas Knöfel, Robert Schultz-Heienbrok

Single crystal X-ray structure analysis of 5'-NT in four crystal forms yielded nine independent observations of protein conformers. Three conformers can be classified as open conformations and six molecules are in a closed state. The open and closed states differ in the relative orientation of the two protein domains by a maximum of 95.7° whereas the maximum rotational difference of the protein domains within the open and closed forms is 10.4° and 8.0°, respectively. The phosphatase active site is located between the two domains. The N-terminal domain harbours the catalytic dimetal center, whereas residues responsible for substrate binding are located in the C-terminal domain. Substrates and inhibitors bind to both enzyme forms showing that the substrate is translated by ~20 Å into the catalytic center via the hinge-bending domain movement. The domain rotation is facilitated by changes in the main-chain conformation of residues of an inter-domain helix.
The temperature factor distribution over the amino acid residues shows that a limited domain movement of the smaller C-terminal domain is also present in one of the crystal forms. Thus, the crystallographic data not only provide static snapshots of the protein conformational landscape but also a direct observation of the global conformational flexibility. Normal mode calculations and kinetic data on mutants which are trapped in the open or closed forms via engineered disulfide bridges further characterize the domain movement.

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2F-12
SPECTROSCOPIC CHARACTERIZATION OF RECOMBI-NANT ADENINE PHOSPHORIBOSYL-TRANSFERASE (RAPRT) AND RAPRT-HIS FROM *LEISHMANIA TARENTOLAE*

C.S. Caruso, O.H. Thiemann, L.M. Beltramini

Human cells can synthesize purine nucleotides by two different mechanisms: the *de novo* and salvage pathways. The parasitic protozoa preferentially use the salvage pathway. For this reason, its component enzymes have been suggested as potential drug design targets. One such enzyme, APRT, converts the purine base adenine and α-D-5-phosphoribosyl 1-pyrophosphate (PRPP) to inosine monophosphate (IMP) as part of the salvage pathway. The *aprt* gene of *L. tarentolae* was subcloned into pQ30 vector (QIAGEN) and Pet29a+ vector (NOVAGEN) and transformed into *E.coli* SG13009-pREP4 and into *E.coli* BL21(DE3), respectively. Despite reasonable over expression of both enzymes, rAPRT-His precipitated at concentrations above 0,5 mg/ml. At concentrations below 0.3 mg/ml both enzymes are dimeric (26 kDa) and gave CD spectra which presented two minima at 209 and 222 nm, a maximum at 195 nm and a crossover at 200 nm. The CD spectrum of rAPRT, between 205 and 235 nm, was slightly more intense than that of rAPRT-His. The fluorescence spectra of both enzymes were measured in the absence of bound ligand and in the presence of adenine, PRPP and Mg^{2+}. The fluorescence intensity was the same for both enzymes with a maximum in fluorescence emission at 335 nm. The fluorescence intensity of rAPRT decreased only in the presence of PRPP. In conclusion, when rAPRT binds PRPP, there is probably a structural change that interferes with the tryptophan environment.
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2F-13
THE DEACYLATION STEP OF ACETYLCHOLINESTERASE: COMPUTER SIMULATION STUDIES

Peter Vagedes, Björn Rabenstein, John Marelius, Johan Åqvist, Ernst-Walter Knapp

The deacylation step of Acetylcholinesterase (AChE) was simulated using the empirical valence bond method (EVB) in combination with free energy perturbation (FEP) calculations. Before the enzyme structure was used to simulate the reaction, the protonation pattern of the acylated enzyme and the free enzyme was determined by a Monte Carlo titration. As a result it was found that Glu199, which is located close to the catalytic triad, is protonated in the free and acylated enzyme. Also the EVB simulation of the reaction showed, that the uncharged Glu199 is favorable to stabilize the transition state of the deacylation step. This is in agreement with experiments, demonstrating that the Glu199Gln mutation does not have a significant influence on the kinetics of deacylation. The EVB calculations yielded an energy barrier of the deacylation step that is 11–12 kcal/mol lower in AChE as compared to a reference reaction in water. The largest calculated rate of the deacylation reaction is $k_{cat} = 5.5 \cdot 10^2\ s^{-1}$ and thus only by a factor of 30 smaller than the experimental value. Furthermore the deacylation is not hindered by the presence of choline, the product of the acylation step, indicating that choline may be released only after deacylation of AChE.

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2F-14
KINETIC ANALYSIS OF ENZYME SYSTEMS WITH SUICIDE SUBSTRATE IN THE PRESENCE OF A REVERSIBLE, COMPETITIVE INHIBITOR

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The inactivation of enzymes induced by a substrate takes place in enzymes acting on a substrate following a branched path mechanism consisting of a catalytic route and an enzyme inactivation route. Such substrates are called suicide substrates. Suicide substrates are useful in the study of kinetic and chemical mechanisms of enzymes and provide powerful instruments for the study of the structure and function of enzyme molecules.
In this contribution we present a general kinetic analysis of enzyme catalyzed reactions evolving according to a Michaelis-Menten Mechanism, in which a competitive, reversible inhibitor acts. Simultaneously, enzyme inactivation is induced by an unstable suicide substrate, i.e., it is a Michaelis-Menten mechanism with double inhibition: one originating from the substrate and another originating from the reversible inhibitor. Rapid equilibrium of the reversible reaction steps involved is assumed and the time course equations for the reaction product has been derived under the assumption of limiting enzyme. The goodness of the analytical solutions has been tested by comparison with the simulated curves obtained by numerical integration. A kinetic data analysis to determine the corresponding kinetic parameters from the time progress curve of the product is suggested.

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2F-15
KINETIC AND MOLECULAR-MODELLING STUDY OF THE INTERACTION OF A CLASS-A β-LACTAMASE WITH β-LACTAM COMPOUNDS

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β-Lactamases are widespread in the bacterial world, where they are responsible for resistance to penicillins, and related compounds. Detailed structural and mechanistic understanding of these enzyme can be expected to guide the design of new antibacterial compounds resistant to their action. The most common β-lactamases are the class A enzymes, such as the clinically significant *Staphylococcus aureus* PC1 β-lactamase. In this work the interactions between *S. aureus* PC1 enzyme and a set of characteristics β-lactam compounds were studied. This set involves substrates such as penicillin G, cephalotin, cephamycins (cefmetazole and cefoxitin), moxalactam and imipenem. The values of k_2 (acylation rate) and K_S (dissociation constante of the Henri-Michaelis complex) were determined for most of this substrates. The structures of the Henri-Michaelis complexes that these compounds form with the *S. aureus* enzyme were optimised using the AMBER force field implemented in the MacroModel program 6.0. Results obtained allow to determine the most important electrostatic interactions between the substrate (carboxylic group, β-lactam carbonyl group and the β-lactam side) and the enzyme (Ser-70, Ser-130, Asn-132, Lys-234, Ser-235, Gln-237 and Arg-244). An average of these interactions has been used to derive a relationship to the kinetic data K_S . An increase of 0.2–0.5 Å in this average value was found to result in an increase in K_S by about one order of magnitude.

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2F-16
COMPUTER SIMULATIONS OF ENZYMATIC REACTIONS IN DNA PHOTOLYASE

Daniel Winkelmann, Peter Vagedes, Ernst-Walter Knapp

DNA photolyase catalyzes the repair of cyclobutane dimers in adjacent pyrimidine bases of DNA caused by UV-induced lesion. The light-harvesting cofactor of DNA photolyase absorbs a photon and provides the energy via an exciton transfer to the second cofactor $FADH^-$. The catalytic mechanism involves an electron transfer from the excited $FADH^-$ to the pyrimidine dimer, which decomposes via a radical anion and a final back transfer of the electron to the FADH and dissociation of the reactive complex.
Before we study the repair reaction of the DNA photolyase in detail the protonation pattern of the crystal structure of DNA photolyase will be determined by a Monte Carlo titration.The electron transfer and following rearrangment reactions will be studied by computer simulation using the empirical valence bond method (EVB) in combination with free energy pertubation (FEP) calculations.

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2F-17
KINETIC PROPERTIES OF HORSERADISH PEROXIDASE IN THE PRESENCE OF Cd^{++}

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Horseradish peroxidase activity was studied at pH 4.0, with o-dianisidine and 8×10^{-4} M H_2O_2 as substrates, in the presence of various concentrations of Cd^{++}. When the enzyme was incubated for 5 min at room temperature with 4–6 mM Cd^{++}, K_m (0.06 mM) was unaffected but V_{max}, expressed as μmoles/min/pmole enzyme, was decreased from 0.25 to 0.20 for 4 mM Cd^{++} and to 0.17 for 6 mM Cd^{++}. When the enzyme was incubated for 20 min at room temperature with 4–6 mM Cd^{++}, K_m was still unaffected and V_{max} was decreased to 0.19 for 4 mM Cd^{++} and to 0.16 for 6 mM Cd^{++}. However, when the incubation time was increased to 60 min, both K_m and V_{max} were affected: K_m increased to 0.08 mM for 4 mM Cd^{++} and to 0.1 mM for 6 mM Cd^{++} while V_{max} decreased to 0.16 for 4 mM Cd^{++} and to 0.13 for 6 mM Cd^{++}. When the Cd^{++} concentration was raised to 25–100 mM, both K_m and V_{max} were affected, regardless of the incubation time. Double-reciprocal plots showed that, depending on Cd^{++} concentration and incubation time, either non-competitive or mixed inhibition was observed when horseradish peroxidase was incubated with Cd^{++}. Moreover, our studies showed that under appropriate conditions, the non-competitive inhibition could be changed to mixed type competition and vice versa. In addition, the inhibition observed after incubation for up to 20 min in Cd^{++} 4–100 mM was reversible with excess o-dianisidine, but it was not after 60 min incubation in Cd^{++} 50–100 mM.

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3A-1
EFFECT OF OLEIC ACID ON MEMBRANE LIPID STRUCTURE

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During the last years several studies have shown that the membrane structure plays a crucial role in various cell functions. For this reason, agents that modulate the membrane structure can be used to study the roles of lamellar and nonlamellar phases in cellular functions. In this work we show that oleic acid (OA) alters the membrane structure, mainly inducing the formation of nonlamellar phases. Differential scanning calorimetry experiments, on 1-palmitoyl-2-oleoyl phosphatidylethanolamine (POPE) vesicles, demonstrate that OA favors the formation of nonlamellar structures at concentrations (10–200 μM) lower than those altering membrane fluidity (over 200 μM). Fluorescence quenching of fluorescent phosphatidylethanolamine (PE) derivatives by KI and acrylamide, in PE and PC liposomes, suggest that fluorophores are differentially accessible to the quencher in lamellar and nonlamellar structures. The presence of OA in PE liposomes favors the coexistence of lamellar and nonlamellar structures, in agreement with DSC results. Moreover, the presence of OA in PE liposomes induced significant and marked increases in vesicle diameter but not PC vesicles, as measured by electron microscopy. In summary, OA induces nonlamellar phase propensity/formation, making this molecule an useful tool for studying membrane lipid structure-function relations.

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3A-2
COCHLEATE STRUCTURES FORMED IN CRYSTALLINE DMPG

Alfred Blume, Walter Richter, Gert Rapp, Patrick Garidel

Aqueous dispersions of 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol (DMPG) in 100 mM NaCl at *pH* 7 form a stable crystalline gel phase when stored for 7 days at 4 °C (initial state). We studied the structure of this initial state as well as the thermotropic phase behavior by differential scanning calorimetry, freeze-fracture electron microscopy, FT-IR spectroscopy, and synchrotron x-ray diffraction.

The low temperature phase is characterized by two different morphological aggregates: flat, crystalline multilamellar sheets and cochleate cylinders. This phase melts into a liquid-crystalline phase at 40 °C with a high phase transition enthalpy of 79 $kJ \cdot mol^{-1}$ in contrast to the usually observed phase transition at 24 °C from the gel phase.

The set of reflections observed in the wide angle x-ray region resembles that of the crystalline L_c phase observed in phosphatidylcholines. The FTIR spectra show that the chains are tightly packed in an orthorhombic subcell lattice. The frequency of the C=O band (1732 cm^{-1}) and the reduced half width indicate a highly ordered hydration shell in the backbone region. The phosphate bands also show that the residual water of hydration in the headgroup region is well ordered. The freeze-fracture electron microscopic and X-ray data reveal very tightly packed lamellar aggregates. The lamellar repeat distance found for this phase is very small (4.75 nm) and indicates that only a very small water layer is present in between the lipid bilayers.

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3A-3
MEMBRANE CURVATURE INDUCED BY AMPHIPHILIC POLYMERS

Rumiana Dimova, Hans-Günther Döbereiner, Reinhard Lipowsky

We investigate the curvature response of phospholipid membranes to partitioning of the class of amphiphilic molecules C_iE_j. The shapes and thermal fluctuations of giant vesicles are monitored as a function of bulk concentration of C_iE_j via phase contrast microscopy. We exchange the media solution and observe the morphological response of a selected vesicle. We employ a wide concentration range both below and above the respective CMCs, which are measured with dynamic light scattering. Vesicles morphology is quantified via real time imaging of the equatorial vesicle contour [1]. Time sequences of 2d contours are Fourier transformed and analyzed in terms of static and dynamic correlation functions. The thermal fluctuation spectrum encodes the bending modulus and the spontaneous curvature of the membrane. In this study, we are interested in the curvature induced by grafting polymers from solution onto the bilayer. Recently, the functional dependence of membrane curvature on the grafting density has been theoretically studied in detail [2]. Experimentally, we quantify the surface concentration of C_iE_j by measuring the partition coefficient from bulk solution into the membrane employing isothermal titration calorimetry. Using the partition coefficient, the measured spontaneous curvature for various bulk concentrations can be compared to theoretical predictions.

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3A-4
MEASUREMENT OF SPONTANEOUS CURVATURE VIA FLUCTUATION SPECTROSCOPY

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The bending elastic properties of a fluid membrane are characterized by its bending modulus and spontaneous curvature. Whereas there are various methods used by a number of groups to measure the elastic modulus, experiments in order to obtain the second parameter were performed only very recently [1]. In this study, the mean shape of giant vesicles is used to extract the spontaneous curvature as a function of solute asymmetry across the membrane [2]. Here, we present a novel technique which allows to deduce both elastic parameters from the thermal shape fluctuations. Experimental fluctuation spectra are obtained via real time image analysis of equatorial vesicle contours in the focal plane of a phase contrast microscope. They are compared to theoretical spectra obtained via MC simulations [3]. We demonstrate the validity of our technique exploring temperature-induced shape instabilities of prolate vesicles, where the thermal fluctuations show characterisitic features connected to the soft mode of the respective instability. We employ the new method to measure spontaneous curvature induced by sugar [1] and pH [4] asymmetry.

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3A-5
PHASE BEHAVIOR OF ACETYLENIC PHOSPHOLIPIDS WITH DIFFERENT HEADGROUPS

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A series of acetylenic di-stearoyl phospholipids was synthesized which differ in the position of the triple bond (C4, C14) as well as in the polar group (PC, PE, PG, PA, PS).
In general, the main transition temperatures of aqueous dispersions were depressed in comparison to those of the appropriate saturated lipids as detected by DSC.
X-ray diffraction revealed that with the introduction of the triple bonds an ordering force in the chain region appears which also influences the bilayer stacking and hydration. Thus, usually subgel phases were detected which directly transformed into the liquid crystalline phase on heating. The headgroup variations showed that as a function of the shape, charges, and conformational freedom of the polar groups differences exist in the phase behavior. PS and PG headgroups may compensate the ordering effect of the acetylenic chains and form normal gel phases under certain conditions.
Best indexing results of the additional short spacings in the subgel phase of PA14 were reached under the assumption of a molecular lattice similar to that of the saturated lipid. From this analysis arguments were derived for the explanation of the ordering force of the triple bond.

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3A-6
FLUID FREE BILAYERS: A NEW MODEL FOR BIOLOGICAL MEMBRANES

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Highly hydrated lipid bilayers freely floating at 15 to 20 Å above adsorbed ones on smooth silicon substrates have been prepared. Samples were precisely characterised in-situ by means of neutron and synchrotron radiation specular and off-specular reflectivity at several temperatures between the lipid gel and liquid crystalline phases. Around the chain melting temperature a spectacular simultaneous increase in both inter-bilayer distance and bilayer fluctuation was observed, but no unbinding. It might be a direct observation of the balance between energy minimisation and entropy repulsion, leading to an estimation the dimensionless parameter $A\kappa/(k_BT)^2$, where A is the Hamacker constant and k the bending modulus. Well above the chain melting temperature the bilayer, although in the fluid phase, is structured and stable; the inter-bilayer distance decreases back to about 20 Å. Such free bilayer is a flexible model system for physical and biophysical studies of membranes.

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3A-7
SPECTROSCOPIC AND X-RAY DIFFRACTION STUDIES OF THE HYDRATION OF DIPHYTANOYLLECITHIN AND -CEPHALIN

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The lyotropic phase behaviour of Diphytanoylphophatidylcholine (DPhPC) and its ethanolamine analogue (DPhPE) has been probed by FT-IR spectroscopy according to the protocol described previously [1] and small-angle X-ray scattering (SAXS) varying hydration via the relative humidity (RH). Because of its high bilayer stability, DPhPC was frequently used as a model lipid in biochemical and biophysical studies [2]. Recent NMR data had shown that its superstructure is strongly influenced by the water content [3].
As IR-spectroscopic data reveal, DPhPC imbibes much more water than DPhPE. Methylene marker bands indicate extremely high disorder of the acyl chains of either lipid in the whole RH range studied. Phase transitions are indicated to occur at about 80 % RH in DPhPC and towards 0 % RH in DPhPE.
First SAXS results show that DPhPC and DPhPE exhibit lamellar and hexagonal phases in the ranges of high and medium hydration, respectively, and that both lipids undergo phase transitions upon dehydration. The phases adopted in the dry lipids are not assignable at present.
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3A-8
FROM LIVING POLYMERS TO MEMBRANES: A MICELLE-TO-VESICLE TRANSITION IN LIPID-DETERGENT SYSTEMS

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A theoretical analysis of transient structures in mixtures of dimyristoylphosphatidylcholine (DMPC) and sodium cholate (NaC) capable of the temperature-induced micelle-to-vesicle transition was carried out. The theoretical model is based on the Helfrich theory of curvature elasticity and Ginzburg-Landau approximation of free energy. Connection between Helfrich curvature elasticity energy and enthalpy of mixing was established. An analysis of the free energy shows that nonideal rod-like aggregates with the elliptical cross section are formed due to NaC-DMPC nonideal mixing in a certain range of detergent/lipid ratios. On the basis of dynamical equation solution it was shown that rod-like micelles are unstable and subject to temperature-induced curvature deformations leading to their fusion. Fusion along the main axis of the rods leads to linear growth of micelles with formation entangled micelles. Experimentally the linear growth of micelles is manifested in temporary increase of turbidity in light scattering experiments. Further fusion of the worm-like micelles occurs down side surfaces of micelles and results in formation of bilayer structures. Transformation of bilayers to vesicles is followed by partial decrease in turbidity. Processes of fusion and decay of rod-like micelles (living polymers) during the temperature-induced micelle-to-vesicle transition are analysed in the report.
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3A-9
THE INTERACTION OF VISCOELASTIC NETWORKS OF ANIONIC LIPIDS WITH WATER

Peter Grabitz, Thomas Heimburg

Close to the lipid chain melting transition the membrane curvature elasticity assumes a maximum. The likelihood of transitions in vesicular shape is therefore especially high close to the melting temperature. We have found various lipid systems that display structural transitions from a closed vesicular structure to a viscoelastic membrane network close to the chain melting reaction of lipids. These structural transitions occur mainly in anionic lipid systems and are observable with many methods, including calorimetry, densitometry, or viscometry as measured in frequency dependent low shear viscometers. This includes various phosphatidylglycerols as well as zwitterionic membranes loaded with the angiotensin receptor antagonist Losartan® or with charged peptides. The driving force for the structural change consists in an interaction with water, which differs in the different vesicular states. The structural transitions lead to unique heat capacity traces which display several C_p-maxima. The importance of the interaction with the aqueous medium is demonstrated in pressure calorimetry. There is a striking similarity of these transitions with other solvent driven structural transitions as the pre- and main transition which are rather a melting transition coupled to a structural change. We suggest that this kind of transition may play an important role in membrane fusion events like exocytosis.

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3A-10
OPTICAL DEFORMATION OF SOFT BIOLOGICAL DIELECTRICS

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Two counterpropagating laser beams are used to stretch soft dielectrics such as red blood cells or vesicles with forces up to 400 pN. The deformation forces act on the surface between object and surrounding medium and are significantly higher than the trapping forces on the object. Radiation damage is avoided since a double-beam trap does not require focusing for stable trapping. Ray-optics was used to calculate the stress profile on the surface of the trapped object. Measuring the net forces and deformations of well defined elastic objects validated this approach. Optical stretching bridges the gap between optical tweezers and AFM for elasticity measurements. (Physical Review Letters, in press)

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3A-11
NEUTRON DIFFRACTION STUDIES OF FULLY HYDRATED PHOSPHOLIPID BILAYER STRUCTURE

T. Gutberlet, T. Hauß, J. Katsaras

Neutron diffraction is a very useful method in structure determination of lamellar phospholipid membranes, which are of basic relevance to understand physical properties and function of biological membranes. Neutron diffraction offers the possibility of contrast variation by deuteration of molecular subgroups, thus labeling specific regions to be studied in the bilayer membrane, or of additives (e.g. drugs, peptides, proteins) and solvents. Distribution and penetration of water can be studied easily by exchange of H_2O and D_2O at all stages of hydration and in excess water conditions. In this contribution applications of neutron diffraction measurements on DMPC using contrast variation by deuteration and H_2O/D_2O exchange will be presented. Low resolution scattering length density profiles will be shown, where the distribution of water and molecular subgroups within the bilayer is derived of directly. The results demonstrate the current possibilities of neutron diffraction in structure determination of biological model membranes.

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3A-12
THE IMPACT OF AMPHIPHILES ON MEMBRANE STABILITY

H.H. Heerklotz, J. Seelig

The membrane perturbation must be quantified, e.g., for the characterization of non-specific effects of antibiotic peptides or for the classification of detergents used in membrane protein solubilization. The basic idea of the study is to correlate the stress-inducing incorporation of a molecule into a lipid bilayer with its self-association to essentially stress-free micelles. As a model study, we have measured the POPC membrane/water partition coefficients, K, of 14 non-ionic surfactants and compared them with the CMC. Generally, K·1/CMC within one order of magnitude. The small but significant deviations from this rule serve to classify "strong" (K·CMC < 1) and weak detergents (K·CMC > 1). Strong detergents (e.g., decyl and dodecyl maltoside, C_mEO_n with m = 10, 12 and n = 7, 8, tritons, di-C_7-PC) destabilize the membrane already at low concentrations and lyse the membrane already at less than one detergent per lipid. Weak detergents (e.g., octyl glucoside, $C_{12}EO_5$, $C_{12}EO_6$, CHAPS) do not drive micelle formation until the membrane consists predominantly of detergent molecules. These results were used for comparison with the antibiotic lipopeptide surfactin. In this case, the electrostatic interactions in a micelle had to be taken into account. The result shows that the peptide destabilizes the membrane far more efficiently than the "strongest" synthetic detergents. This stimulates further studies on the nature of the detergent-like action of antibiotic peptides.

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3A-13
RELAXATION PROCESSES AND KINETICS OF TRANSITIONS IN LIPID MEMBRANES

Vesselka Ivanova, Peter Grabitz, Thomas Heimburg

Relaxation phenomena in the lipid bilayer have a putative importance for dynamic processes in biological membranes. In model membranes, close to the lipid chain melting transition, the relaxation following an external distortion is very slow. Experimentally, relaxation times up to seconds have been found at the melting temperature. Outside of transition regimes typical relaxation times are much faster, on the micro- to millisecond timescale. We have studied relaxation processes in membranes using FTIR, temperature perturbation and pressure perturbation calorimetry. We found that relaxation times are largely increased in the melting regime. To analyze relaxation phenomena we model the melting process with Monte-Carlo simulations. These calculations are designed to describe macroscopic fluctuations of state on a length scale larger than single lipids. This are mainly fluctuations of lipid domains which dominate the thermodynamic response of the system close to transitions. We collected the information of the simulation into histograms describing the probability distribution of membrane states. From the shape of the histograms we derive the thermodynamic forces acting on the lipid system after an external distortion, based on a linear non-equilibrium thermodynamics approach. The result of the simple model is that the relaxation times are proportional to the heat capacity, independent of the nature of the distortion. These findings were compared to our experimental data.

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3A-14
INFLUENCE OF TRIFLUOPERAZINE ON THE PHASE BEHAVIOUR OF ZWITTERIONIC OR CHARGED PHOSPHOLIPID BILAYERS

Andrzej B. Hendrich, Olga Wesolowska, Krystyna Michlak

Trifluoerazine (TFP) is well known as an antipsychotropic drug and (more recently) as a compound reversing multidrug resistance in cancer cells. Contrary to other widely used phenothiazine derivative -chlorpromazine interaction of TFP with lipid bilayers was not extensively studied. We performed calorimetric experiments on TFP mixtures with dipalmitoylphosphatidylcholine (DPPC), dimyristoyl-phosphatidylcholine (DMPC) and dimyristoylphosphatidylglycerol (DMPG). For the TFP:lipid mole ratios higher than 0.04 and 0.06 (for DPPC and DMPC respectively) the deconvolution of transition profiles into two gauss components was possible. The concentration-dependent decrease of transition temperatures was accompanied by increase of the distance between gauss components. Deconvolution of TFP:DMPG transition profiles was not possible for all studied mole ratios (0.01–0.1). Molar transition enthalpies of DMPC and DMPG were decreased up to 60% by 0.1 mole ratio of TFP in comparison to initial values for pure lipids. Transition enthalpy of DPPC was not changed. These data suggest that TFP induces phase separation in zwitterionic but not in charged phospholipid systems and strength of interaction depends on lipid chain length.

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3A-15
RAMAN MICROSPECTROSCOPY OF PHOSPHOLIPID-PEPTIDE BLACK FILMS

F. Lhert, F. Capelle, D. Blaudez, C. Heywang, J.-M. Turlet

Black films are made of two monolayers of amphiphilic molecules separated by an interstitial water core of thickness less than 100Å. Phospholipids are able to form such films which constitute an inverted membrane model.We studied black films of DMPC (dimyristoyl-phosphatidylcholine) by Raman microspectroscopy in the range 600–4000 cm^{-1}. The DMPC vibrational bands are very well defined and provide information about structural and conformational order of the alkyl chains, whereas the liquid water band centered at 3400 cm^{-1} gives an estimation of the aqueous core thickness. At 29 °C, DMPC is in the liquid crystal phase: the water thickness is only 22 ± 4Å, and the value of the lateral order parameter is representative of a fluid phase.We also studied the effect of melittin on the organization of the DMPC film. Melittin is a positively charged peptide known to interact strongly with membranes. It was added to the phospholipid solution before the film formation.

– for a peptide-DMPC molar ratio as low as $2 * 10^{-3}$ (which is significantly less than those commonly used in previous reports), we observed an ordering of the DMPC chains.

– for molar ratios higher than $2 * 10^{-3}$, the water core thickness increases gradually to 260 Å at a ratio of $1 * 25 \cdot 10^{-2}$, likely because of electrostatic repulsion between the monolayers.

– at ratio higher than $2.5 * 10^{-2}$ melittin induces a huge swelling of the film that reaches a thickness of several microns.

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3A-16
TERNARY MIXTURES OF PHOSPHOLIPID, FATTY ACID AND LYSOLIPID: AN ESR STUDY

Pernille Høyrup, Thomas H. Callisen

PLA_2 hydrolyses phospholipids producing lysolipid and fatty acid. The reaction is charaterised by a lag-phase of slow hydrolysis followed by a so-called burst at which the reaction is very rapid. The burst occurs when 5-10 % of the phospholipid has been hydrolyzed.

Ternary mixtures of phospholipid and the two hydrolysis products has been studied using ESR spectroscopy of three different spin probes: 5-doxyl-stearic acid, 5-doxyl-PS, and CAT16. From the ESR spectra we extract the lipid chain order and the ratio between spin probe free-in-solution and in lipid environment. In the case of the 5-doxyl-fatty acid and the CAT16 mixtures containing up to 10 % hydrolysis products have a lipid chain order that is either constant or slightly lower than that of pure phospholipid. Mixtures containing 10 % hydrolysis products exhibit maximal lipid chain order. The fraction of spin probe free-in-solution has a local maximum at 15 % product content. The offset of this peak is at 10 %. The spectra of 5-doxyl-PS reveal increasing chain order up to 10 % product content. The chain order is almost constant in the range of 10 and 50 % product content. The 5-doxyl-PS partitions only in the phospholipid matrix.

These results suggest that the hydrolysis products are included in the substrate matrix until saturation is reached at 10 % hydrolysis. Further hydrolysis is connected to lower lipid chain order in exclusion of products from the phospholipid matrix.

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3A-17
ELECTROSTATIC BINDING OF PENTALYSINE TO NEGATIVELY CHARGED LIPID VESICLES

Wigand Hübner, Andreas Erbe, Patrick Garidel, Maria A. Requero, Alfred Blume

The thermodynamic parameters for the binding of pentalysine to anionic lipid vesicles containing DMPG were obtained from high sensitive isothermal titration calorimetry (ITC).
The binding of pentalysine to negatively charged DMPG and DMPC:DMPG vesicles in the temperature range of 30–60 °C (liquid-crystalline phase) is exothermic. With increasing temperature the binding enthalpy ΔH becomes more exothermic, whereas ΔG is nearly temperature independent. At temperatures above 50 °C, the electrostatic binding of pentalysine to charged membranes is only driven by enthalpy.
The temperature dependence of ΔH can be explained by a release of water molecules from apolar binding sites. Infrared spectroscopic investigations support this explanation. The observed frequency shift of the methylene stretching bands upon binding of pentalysine to DMPG vesicles in the liquid crystalline phase is the result of a tighter packing of the acyl chains with increased conformational order.
As expected, the apparent binding constant for pentalysine binding to DMPC:DMPG vesicles is lower than for binding to pure DMPG vesicles.

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3A-18
LONG-CHAIN FATTY ACIDS ENTER AND LEAVE CELLS BY FREE DIFFUSION

Frits Kamp, Barbara E. Corkey, James A. Hamilton

Whether long-chain (> 12 carbons) fatty acids (FA) can enter cells by free diffusion in the same way as hydrophobic weak acids such as short-chain FA, has been a matter of controversy. Using vesicles of egg phosphatidylcholine we have shown that: (1) FA dissociate from albumin in less than 1 second; (2) FA bind to phospholipid bilayers with high affinity; (3) the pKa of FA bound to bilayers is 7.6; (4) FA can flip-flop across bilayers extremely fast in their un-ionized form, thereby causing changes in internal pH; and (5) FA dissociate very fast from bilayers. These characteristics challenge the idea that specific membrane proteins (FAT, FATP) are necessary to transport FA across the plasma membrane. We present new data in favor of the diffusion mechanism of FA transport. These include changes in cytosolic pH and extracellular [FA] accompanying movement of FA into and out of various cell types. The rates of the pH changes vary with FA chain length and are different in various cell types. When FA are delivered to adipocytes, the cytosolic pH drops in within seconds. Other ionizable amphiphiles such as sulfonylureas and fatty amines can also enter cells by free diffusion. These observations are important in studies of the regulation of FA uptake in various tissues. In the studies of drug delivery and multi-drug resistance proteins, the rate at which certain drugs cross the plasma membrane in their unionized form has to be considered.

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3A-19
IRRAS STUDY OF PHOSPHOLIPID MONOLAYERS CONTAINING BMP OR GANGLIOSIDE GM1

Andreas Kerth, Arne Gericke, Alfred Blume

Infrared reflection absorption spectroscopy (IRRAS) is a valuable tool to investigate the conformational order in monolayer films at the air/water interface. In this study, phospholipids containing perdeuterated acyl chains, namely DPPC-d_{62} and DMPC-d_{54}, were used together with the ganglioside GM1 and bis(monoacylglycero)-phosphate BMP.
The frequency of the respective methylene stretching vibrations decreases with increasing surface pressure, indicating an ordering of the acyl chains upon compression. In mixtures of phosphatidylcholines with GM1 or BMP (4:1), the hydrocarbon chains of both film compounds could be analysed separately. For example, in the DPPC-d_{62}/GM1 4:1 film, the frequency change of DPPC-d_{62} upon compression is nearly unaffected compared to the pure phospholipid film whereas the acyl chains of the ganglioside remain more unordered in the mixed film.
From angular dependent measurements of monolayers in the condensed film state and consecutive analysis of the methylene stretching vibration intensities the respective tilt angles of the hydrocarbon chains were derived.

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3A-20
EFFECT OF FATTY ACIDS ON NONLAMELLAR-PHASE PROPENSITY

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During the last years several studies have shown that the membrane structure plays a crucial role in various cell functions, such as signal transduction and membrane protein activity. For this reason, agents that modulate the membrane structure can be used to study the roles of lamellar and nonlamellar structures in cellular functions. Differential scanning calorimetry was used to investigate the effect of various fatty acids (14:0, 16:0, 18:0, 18:1 cis and trans, 18:2 and 18:3) on the thermotropic behavior of 1,2-dielaidoyl phosphatidylethanolamine (DEPE). The presence of fatty acids (phospholipid:fatty acid, 20:1, mol:mol) induced significant decreases in DEPE lamellar-to-hexagonal phase transition temperature (T_H). These changes in T_H were modest for saturated fatty acids (1.4–2.6 °C for myristic, palmitic and stearic acids) and marked for unsaturated fatty acids (3.5–13.5 °C for oleic, elaidic, linoleic and linolenic acid). Elaidic and oleic acid appeared to have the greatest effect on T_H values, indicating that more than one double bond did not induce further changes in membrane structure than those induced by 18:1 fatty acids. Conversely, elaidic and oleic acid did not induce marked changes in the DEPE solid-to-liquid phase transition (0.3–1.2 °C), indicating that these fatty acids have little effect on membrane fluidity at the above molar ratio. The present results show that monounsaturated fatty acids have important effects on membrane structural properties, facilitating the *in vitro* formation of nonlamellar structures. For this reason, elaidic and oleic acid are useful tools for the investigation of the roles associated with nonlamellar phases in biological membranes.

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3A-21
TEMPERATURE-DEPENDENT UV SPECTRA AS A TOOL FOR STUDIES OF PHASE STATES IN BIOCOLLOIDAL PHOSPHOLIPID SYSTEMS

Olga V. Korzovska, Longin N. Lisetski

Electronic spectra are known to be sensitive to supramolecular organisation of organic ordered structures. So, it was of certain interest to study the electronic spectra changes taking place under phase transitions in model biological membranes. Our objects were lyotropic colloidal systems based on hydrated phosphatidylcholines doped with biomembrane components (peptides, steroids, etc.) or some biologically active substances (e.g. drugs and anaesthetics). Temperature-dependent spectra D(T) in near UV range were obtained for these systems and, in parallel, for thermotropic liquid crystals in which the type of molecular arrangement was similar to that in lyotropic colloids investigated. Comparative analysis of the results allowed us to clearly distinguish between the effects on the molecular level typical for conventional electronic absorption spectra, and specific effects related to changes of the phase state and supramolecular structure on the level of short- and long-range ordering. Thus, outside the region of lecithin absorption bands D was shown to decrease with temperature in all cases, whereas within the absorption bands D(T) nature proved to be dependent on features of intermolecular interactions. Possible explanation can be done in terms of disordering of dichroic chromophore groups. In general, the influence of peptides and steroids on the D(T) dependences confirm the picture of their action upon the bilayer, assumed earlier from our DSC measurements.

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3A-22
EFFECTS OF STRUCTURE AND FLUORINE SUBSTITUTION ON THE INTERACTION OF LIPOPHILIC IONS WITH CELL MEMBRANES

Markus Kürschner, Vladimir L. Sukhorukov, Wolfdieter A. Schenk, Ulrich Zimmermann

The tungsten carbonyl anion $[W(CO)_5SC_6H_5]^-$ and its fluorinated analogue $[W(CO)_5SC_6F_5]^-$ markedly changed the electrical properties of the plasma membrane of mammalian cells as shown by the single-cell-electrorotation technique. Both compounds gave rise to an additional antifield peak in the rotational spectra of cells indicating that the plasma membrane of treated cells displayed a strong dielectric dispersion in the kHz-MHz frequency range. This means that these tungsten complexes act as lipophilic ions which are able to introduce mobile charges into the plasma membrane. From the rotational spectra the ion transport parameters, such as the surface concentration, partition coefficient and translocation rate constant of the lipophilic anions across the membrane could be evaluated. These transport kinetics reflect the structure, environment and mobility of the ions which can be used as field sensitive molecular probes in studies of living cells. Comparison of the membrane transport parameters for the two anions showed that the fluorine substituted analogue was more lipophilic, but its translocation across the plasma membrane was by at least one order of magnitude slower than that of the parent hydrogenated anion. Whereas a hydrocarbon spacer inserted between the aromatic ring and the thiolate group, $[W(CO)_5SCH_2C_6H_5]^-$, had only little effect on the ion transport.

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3A-23
A MULITNUCLEAR SOLID-STATE NMR STUDY OF PHOSPHOLIPID-CHOLESTEROL INTERACTIONS: SPHINGOMYELIN-CHOLESTEROL BINARY SYSTEMS

Wen Guo, Volker Kurze, James A. Hamilton

Interactions of sphingomyelins (SpM) with cholesterol (Chol) have been the subject of numerous studies, yet specific interactions between Chol and SpM and the influence of the sphingosine backbone are not fully understood. ^1H, ^{31}P, and ^{13}C MASNMR techniques were used to obtain high resolution spectra from multilamellar dispersions of unlabeled brain SpM and Chol at various mixing ratios. In addition, we obtained ^2H NMR spectra of oriented lipid membranes with specific ^2H labels. Our findings show the disruption of the gel-phase of pure SpM by Chol below the main phase transition temperature and an increase in acyl chain ordering and membrane rigidity with increasing Chol incorporation in the liquid crystalline phase. We observed a solubility limit of Chol in SpM at ~50 mol%, the same value as previously reported for DPPC membranes. Our investigations show closer interactions of Chol with the paraffinic compared to the acyl chain of SpM at low Chol concentrations and an onset of stronger interactions with the acyl chain at > 25 mol% Chol. Polar interactions between Chol and interfacial moieties of SpM showed sensitivity to the sphingosine structure only at low and intermediate Chol concentrations. At the solubility limit, apolar van der Waals interactions between Chol and the hydrocarbon chains of SpM dominated and resembled those of Chol with the acyl chains of DPPC. These studies show subtle but not large differences in the way Chol interacts with DPPC and SpM.

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3A-24
SPECIFIC INTERACTIONS OF PHOSPHOLIPID MEMBRANE COMPONENTS BY EVIDENCE OF DSC PHASE TRANSITIONS DATA

Longin N. Lisetski, Olga Korzovska, Valentina D. Panikarskaya

Among the most important physical properties of biological membranes is their liquid crystalline state. Thus, it is known that only L_α-phase of membrane phospholipids is biologically relevant, i.e. ensuring proper functioning of cell membrane. Besides, all membrane components are able to affect the ordering of lipid molecules within a bilayer and, therefore, to change their phase state. So, clarifying mechanisms governing changes in the lipid ordering due to interactions of the membrane components is a point of high biomedical importance.
For this purpose, studies of model membranes were carried out by differential scanning calorimetry (DSC) which clearly reflect specific ordering of different parts of the molecules and cooperativity of phase transitions. The model membranes used were colloidal systems based on hydrated dipalmitoylphosphatidylcholine (DPPC) doped with amino acids, peptides, steroids, drugs etc.The results obtained allow determination of specific features of dopants affecting lipid bilayer parameters as well as separation of effects caused by their interaction with hydrophilic and hydrophobic parts of the bilayer. E.g., for antimicrobial agents decamethoxinum and aethonium, it is argued that their therapeutic action is probably based on effective fluidizing of membrane.

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3A-25
STABILITY OF LIPID BILAYER IN MODEL SYSTEMS AND METHODS OF ITS MODIFICATION

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The stability of liposomal structure is provided during an establishment of a dynamic equilibrium between processes of oxidation – POL and reduction – AOA in medium and liposomal vesicles. The equilibria shift to the oxidized or antioxidized system of liposome by changing the intensity and direction of POL processes may be used as an instrument for regulation of liposome properties.
Suspensions were prepared from lyophilized phosphatidilkholinic liposomes using ectericidum and ectericidum with chlorophill. Irradiation of suspensions by the light of He-Ne laser was carried out during 15 minutes. Coherent irradiation is shown to induce the shift of dynamic equilibrium in all objects studied. The irradiation of ectericidum leads to further prevalence of POL processes. In the case of "ectericidum + liposome" the effects of induced oxidation are attenuated because of activation of AO system of liposoluble vitamins.The POL processes as well as AO responses proceed more intensively in the case of "ectericidum + liposomes + chlorophill", the total AOA being unchanged. It testifies to affinity of a given heterogeneous liposomal system towards a dynamic equilibrium. The observations allow to assert, that AO homeostasis is provided in heterogeneous synthetic systems on the basis of the natural precursors. The dynamic equilibrium of heterogeneous bilayer lipid systems is sensitive to the composition of media of suspending and irradiation with low-intensive laser radiation.

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3A-26
DIPYRIDAMOL INTERACTION WITH MODEL MEMBRANES ASSESSED WITH SURFACE-SENSITIVE SCATTERING AND SPECTROSCOPY

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Submolecular level structural investigations of lipid monolayers on aqueous subphases using x-ray and neutron reflectivity measurements have been undertaken to study the organization of particularly the lipid headgroups [1,2]. In a next step, we study the association of the water soluble vasodilator dipyridamole (DIP) to obtain a molecular level comprehension of its interaction with membranes. Despite relatively small binding constants – as obtained from fluorescence quenching measurements on vesicle systems [3] – membranes are affected in their lateral organization already at very small DIP concentrations: While dipalmitoylphosphatidylcholine (DPPC) monolayers are condensed upon binding of the drug in a concentration range well below 1 mol%, such monolayers are expanded at larger concentrations (1 mol% and up) indicative of partial interpenetration of the pharmaceutical into the membrane. For various DIP derivatives it has been reported that their biological activity correlates with their partitioning into membranes. We have studied details of membrane reorganization inferred by DIP with reflectivity and FTIR reflection-absorption spectroscopy, addressing specifically the impact of the drug on the DPPC headgroup.
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3A-27
FLUID-FLUID MEMBRANE MICRO-HETEROGENEITY: A FRET STUDY

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Large unilamellar vesicles of dimyristoylphosphatidylcholine / cholesterol mixtures were studied using fluorescence techniques (steady-state fluorescence intensity and anisotropy, fluorescence lifetime and fluorescence resonance energy transfer (FRET)). Three compositions (cholesterol mole fraction 0.15, 0.20 and 0.25) and two temperatures (30 °C and 40 °C) inside the coexistence range of liquid-ordered (lo) and liquid-disordered (ld) phases were investigated. Two common membrane probes, N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-dimyristoylphosphatidylethanolamine (NBD-DMPE) and N-(lissamine™-rhodamine B)- dimyristoylphosphatidylethanolamine (Rh-DMPE), which form a FRET pair, were used. The lo/ld partition coefficients of the probes were determined by individual photophysical measurements and global analysis of time-resolved FRET decays. While the acceptor, Rh-DMPE, prefers the ld phase, the opposite is observed for the donor, NBD-DMPE. Accordingly, FRET efficiency decreases as a consequence of phase separation. It was possible to detect very small domains (< 20 nm) of lo in the cholesterol-poor end of the phase coexistence range. On the other hand, domains of ld in the cholesterol-rich end of the coexistence range have comparatively large size. These observations are probably related to different processes of phase separation, nucleation being preferred in formation of lo phase from initially pure ld, and domain growth being faster in formation of ld phase from initially pure lo.

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3A-28
PRESSURE INDUCED CUBIC TO CUBIC PHASE TRANSITION IN MONOOLEIN HYDRATED SYSTEM

Michela Pisani, Sigrid Bernstorff*, Claudio Ferrero#, Paolo Mariani

Synchrotron X-ray diffraction has been used to investigate structure, stability and transformation of the Pn3m bicontinuous cubic phase in the monoolein-water system under hydrostatic pressure. The experimental results show the occurrence of a Pn3m to Ia3d cubic phase transition when the mechanical pressure increases up to 1–1.2 kbar, depending on water concentration. The underlying mechanism for the phase transition has been explored in searching for relationships between the structural parameters derived from the two cubic phases. Since the lipid concentration is rather low and the external pressure increases the cell sizes, thus reducing the principal curvatures, a tentative analysis of the pressure effects on the energetics of these structures has been exploited. A simple theoretical model based on curvature elastic contributions has been used: calculations show that pressure reduces the spontaneous curvature Ho of the monoolein layer and determines a sign inversion of the Gaussian curvature modulus Kg. As a negative value of Kg favors the Pn3m cubic phase, the induced transition to the Ia3d cubic appears dictated by changes in the spontaneous curvature. The calculated curvature elastic energy show that the phase sequence induced by pressure then results from a delicate interplay between the different free energy contributions.

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3A-29
DIFFERENTIATION OF HYDROCARBON CHAIN PACKING MODES OF DHPE IN MONOLAYER AND BULK PHASES

Annette Meister, Günter Förster, Gerald Brezesinski, Alfred Blume

The model lipid 1.2-Dihexadecyl-sn-glycero-3-phosphorylethanolamine (DHPE) was used for the estimation of hydrocarbon chain packing modes in monolayers and bulk phases. Temperature dependent X-ray powder diffraction- and FTIR-measurements were applied to characterize the gel phases in the bulk. GIXD- and IRRAS-measurements were used to describe the monolayer phases. Until now the indexing of the X-ray data was based on trial and error which prevents an unambiguous calculation of the lattice parameters. Additional lattice energy calculations with the Cerius^2 software package are a powerful tool to discriminate packing modes of long-chain lipids by applying a continuous energy criterion. They allow a consideration of packing frustrations or freedom of hydrocarbon chains in monolayer and bilayer structures. The difference between the temperature dependent X-ray data of DHPE in the monolayer and the bulk phase is caused by different paths in the van der Waals energy hyper-surface corresponding to different chain packing modes.
FTIR- and IRRAS-measurements were carried out to confirm the packing modes and to get additional information about the setting angles between the backbone plane of a zigzag chain fragment ($-CH_2-CH_2-$) and the b-axis of the lattice. The measured setting angles were compared with those obtained after the energy minimization process.

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3A-30
STEROL EVOLUTION AND THE PHYSICS OF MEMBRANES

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Sterols are important molecular components of the plasma membranes of eucaryotic cells. Using deuterium NMR spectroscopy in conjunction with statistical mechanical modelling we present a unifying picture of how the evolution-engineered differences in molecular chemistry between cholesterol and its precursor lanosterol are manifested in the physical properties of model membranes in terms of molecular order and phase equilibria. Cholesterol optimizes the stability of a particular membrane phase, the liquid-ordered phase, that is a liquid and at the same time exhibits high molecular conformational order.

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3A-31
CONVENTIONAL SPIN LABEL ESR IN POLYMER-LIPID MICELLAR DISPERSIONS

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Conventional Electron Spin Resonance spectroscopy has been used to study aqueous micellar dispersions formed by the polymer-lipids poly-(ethylene glycol) linked to dipalmitoylphosphatidylethanolamine (PEG-DPPE). We consider PEG with different chain length, i.e., PEG:350, PEG:2000 and PEG:5000. Using spin labelled phospholipid at different positions along the acyl chain (n-PCSL, n = 5, 7, 10, 12 and 16), we compare the thermotropic phase behaviour and the segmental chain dynamics of micelles of PEGs-DPPE to those of the common micelles of Cetyltrimethylammonium bromide (CTAB) and to those of the bilayers of dipalmitoylphosphatidylcholine (DPPC).
In contrast to lamellar dispersions of DPPC, that show the pre and the main phase transitions, neither PEGs-DPPE nor CTAB micellar dispersions exhibit endothermic transitions. However, the spectral anisotropy is less affected by temperature in micelles of PEGs-DPPE than in those of CTAB. Moreover, a gradient of increased mobility when moving from the polar-apolar interface toward the end of the acyl chain is observed in bilayers of DPPC and in micelles of CTAB. In PEGs-DPPE micelles, instead, the segmental chain motion is comparable almost along the entire acyl chain length.

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3A-32
ADSORPTION OF TRITON X-100 TO PHOSPHATIDYLCHOLINE AND SPHINGOMYELIN MONO- AND BILAYERS

T. Nyholm, J.P. Slotte

The ability of Triton X-100 to solubilise a membrane is known to depend on the membrane lipid composition. Certain membrane domains, rich in cholesterol and sphingolipids, have been shown to be resistant to Triton X-100 solubilisation at low temperatures. In this study we have measured how Triton X-100 adsorbs to model membranes containing phosphatidylcholines and sphingomyelins. The adsorption of Triton X-100 to monolayers at 22 °C is influenced both by Triton X-100 concentration (more adsorption at higher subphase concentration) and the initial surface pressure (less adsorption at higher surface pressure). Monolayers of pure phospholipids resisted Triton X-100 adsorption in the following order: DPPC > D-*erythro*-*N*-24:1$^{\Delta 15}$-SM = D-*erythro*-*N*-16:0-SM > EPC. Inclusion of 30 mol% cholesterol to the phospholipid monolayers decreased the adsorption of Triton X-100. The detergent-to-lipid molar ratio did not exceed 1:9 in the monolayer experiments. We also studied the effect of Triton X-100 on diphenyhexatriene steady-state dynamics in small unilamellar vesicles containing either DPPC or D-*erythro*-*N*-16:0-SM. Adsorption of Triton X-100 to SUVs of both DPPC and D-*erythro*-*N*-16:0-SM decreased linearly the T_m of the gel/liquid phase transition. The change was similar with both SUV types. The detergent-to-lipid molar ratio did not exceed 1:10 in the SUV experiments. Our results show that SM is not more resistant to Triton X-100 adsorption than its acyl-matched PC at temperatures above 20 °C.

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3A-33
SPECIFIC INTERACTIONS IN THE MIXED LANGMUIR MONOLAYERS OF AMPHIPHILIC MEMBRANE COMPONENTS

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The lipid-water interface can substantially modify interactions of biomembrane components, affecting organisation and functioning of the membrane. π-A diagrams were obtained, using a standard Langmuir trough, both for phosphatidylcholine monolayers containing amphiphilic dopants and for the pure compounds involved. Cholesterol, stearoyl-L-alanine and decamethoxinum (a bisquaternary ammonium antimicrobial agent) were used as dopants which represent typical classes of membranotropic substances.
As expected, the presence of cholesterol led to a certain increase in the collapse pressure and a decrease in average surface area reflecting condensation effect of cholesterol on the monolayer in L_α-phase. Stearoyl-L-alanine (a "two-dimensional analog" of the proper amino acid) caused no deviation from linearity on vs. concentration plots with lecithin. On the contrary, decamethoxinum strongly disorders phospholipid layers due to strong interaction with hydrophilic parts. This suggestion is in a good agreement with our data previously obtained by SIMS, which indicate that decamethoxinum is able to make supramolecular aggregates with 1 to 3 phospholipid molecules.

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3A-34
STATIC AND DYNAMIC LIGHT SCATTERING MEASUREMENTS OF OSMOTICALLY INDUCED SHAPE CHANGES OF LARGE UNILAMELLAR VESICLES

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Static and dynamic light scattering measurements have been used to characterize osmotically induced changes to the radius of gyration (Rg) and effective hydrodynamic radius (Rh) of large unilamellar vesicles. These results are compared to theoretically predicted changes to Rg and Rh for several thermodynamically stable vesicle shapes such as prolate ellipsoids, oblate ellipsoids and their limit shapes. Measurements were performed in media with a variety of ionic and nonionic solutes (such as NaCl and sucrose) on vesicles of pure dioleoylphosphatidylglycerol (DOPG), dioleoylphosphatidylcholine (DOPC), and several lipid mixtures. We interpret results as trajectories through the phase diagram of the area difference elasticity (ADE) model and comment on the effects of lipid composition, solute composition and vesicle size on stable vesicle shapes.

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3A-35
THE FREQUENCY DEPENDENCE OF THE EFFECT OF THE APPLIED ELECTRIC FIELD ON PHOSPHOLIPID VESICLE SHAPE

Primoz Peterlin, Sasa Svetina, Bostjan Zeks

External AC electric field deforms flaccid phospholipid vesicles in aqueous solution into approximate rotational ellipsoids, with the rotational axis aligned with the direction of the applied field. At low frequencies, the deformation is prolate. As the frequency is increased, a transition into oblate deformation is observed. A theoretical explanation for this phenomenon was sought. The shape of vesicle is determined by minimising its total free energy, consisting of membrane bending energy and the work done by the force of electric field during vesicle deformation. The latter contribution is calculated by first solving the Laplace equation for electric potential in case of a spherical shell, immersed in a medium with different electric properties. From the potential, Maxwell stress tensor is calculated. Evaluated on the membrane-water boundaries in the direction perpendicular to it, scalarly multiplied by the local membrane displacement and integrated over the vesicle surface, it yields the sought term. Described theoretical model provides both qualitative and quantitative agreement with the experiment, and can in turn be used for determining electrical parameters of the observed system.

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3A-36
THE DIPOLE POTENTIAL OF LIPIDS CONTAINING ALKYL THIO FATTY ACIDS

Uwe Peterson, David A. Mannock, Ruthven N.A.H. Lewis, Peter Pohl, Ron N. McElhaney, Elena E. Pohl

The large intrinsic membrane dipole potential, Φ_d, is important for protein insertion and functioning as well as for ion transport across natural and model membranes. The origin of Φ_d is controversial. From experiments carried out with lipid monolayers a significant dependence on the fatty acid chain length is suggested, whereas in experiments with lipid bilayers the contribution of additional -CH_2-groups is negligible small compared to the one of phospholipid carbonyl groups and lipid bound water molecules. To compare the impact of -CH_2-groups near and far from the glycerol backbone, we have varied the lipid composition by incorporation of sulfur atoms in different positions of the fatty acid chain. Φ_d of symmetrical lipid bilayers containing one heteroatom was obtained from the charge relaxation of oppositely charged hydrophobic ions. We have found that the incorporation of an S-atom into lipid membranes decreases Φ_d. The effect ($\Delta\Phi_d$ = −21mV) is most pronounced for S-atoms near the lipid head group. From the moderate changes in Φ_d induced by the heteroatom the permeability of respective lipid vehicles designed for drug delivery is predicted to vary little.
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3A-37
EFFECT OF MONOVALENT IONS ON THE PHASE BEHAVIOUR OF MULTILAMELLAR PHOSPHOLIPID VESICLES

Stine S. Korreman, Niels Boye Olsen, Dorthe Posselt

The effect of adding 0-200 mM alkali halide salts to multilamellar phospholipid (diacylPC) vesicles has been studied close to the main transition by use of two different techniques - Small-Angle X-ray Scattering (SAXS), and Piezoelectric Bulk Modulus Gauge (PBG). By SAXS, it is observed that addition of salts has pronounced effects on the swelling behaviour. In the fluid phase well above T_m, the repeat distance level is shifted, and for temperature $T \rightarrow T_m^+$ the anomalous swelling in repeat distance changes dramatically. The observed effects are dependent on salt concentration and furthermore exhibit an ion specificity, which may be related to the Hofmeister series of the ions. Using the PBG, the velocity of sound in a sample is measured with great precision by producing a standing wave in a spherical geometry. The velocity of sound shows an anomaly for $T \rightarrow T_m^+$ as well, and also here the anomaly changes with addition of salt. We suggest that both the observed swelling effects are driven by changes in the Hamaker constant and in the decay length of the hydration force. The latter change has its origin in hydration of ions, and is especially prone to be ion specific according to the Hofmeister series. We interpret the anomalous swelling as the proceeding of a critical unbinding of the lamellae upon approaching the main transition temperature, and propose that addition of salt alters the proximity of the critical temperature of unbinding to the main transition temperature.

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3A-38
LOCATION OF SOME DRUGS IN DMPC LIPOSOMES STUDIED BY FLUORESCENCE ANISOTROPY USING DIPHENYLHEXATRIENE AND TRIMETHYLAMMONIUM-DIPHENYLHEXATRIENE

Catarina Rodrigues, Paula Gameiro, Salette Reis, José L. Lima, Baltazar de Castro

The presence of chemical drugs may alter the mobility of neighboring lipids and modify membrane freedom of movement. Knowledge of their molecular location within membranes is important to understand stucture/function relationships. Fluorescent probes, through fluorescent anisotropy studies, provide information not only about membrane fluidity but also about drug location in membranes. Diphenyl-hexatriene (DPH) is deeply buried as expected from its hydrophobic nature and pack well with fatty acyl chains. Trimethylammonium-diphenylhexatriene (TMA-DPH), with a cationic trimethylamino group attached to DPH phenyl ring, is located more shallowly than free DPH.
We report the effect of the drugs *rifampicine, isoniazide, chlordiazepoxide and griseofulvine* in the fluorescence anisotropy of the fluorescent probes DPH and TMA-DPH, in unilamellar vesicles of dimyristoylphosphatidylcholine (DMPC). The study shows that rifampicine and chlordiazepoxide interacts with the fluorescent probes and increases anisotropy values before and above phase transition and so, stabilize the lipid matrix. In contrast, for isoniazide and griseofulvine no changes were detected, leading us to think of a more superficial interaction with vesicles i.e., more at the polar head level than a deeper one. These results are in agreement with what was expected from the values of the drugs partition coefficient determined for DMPC/Hepes buffer (pH 7.4) liposomes.

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3A-39
CHOLESTEROL EFFECTS ON THE PHOSPHATIDYLCHOLINE BILAYER NNON-POLAR REGION: A MOLECULAR SIMULATION STUDY

Tomasz Róg, Marta Pasenkiewicz-Gierula

A 15-ns, constant temperature and pressure molecular dynamics (MD) simulation of the fully hydrated, liquid-crystalline dimyristoylphosphatidylcholine (DMPC) bilayer membrane containing ~22 mol% of cholesterol (Chol) was performed. The bilayer reached thermal equilibrium after 7 ns. The primary aim of the analyses of a generated 8-ns trajectory was to investigate the atomic-level mechanisms of the Chol ordering effect on the hydrocarbon chains of PCs. A strong increase in the chain order, measured as Smol parameter, in the DMPC-Chol membrane as compared to the pure DMPC membrane, was observed along the whole chain. Alkyl chains of PCs, which were the nearest neighbours of Chol, were the most ordered. For these molecules, a decrease in the number of *gauche* conformation/myristoyl chain as well as in the tilt of the chain, were observed. However, differences between PCs neighbouring the α and β face of Chol were apparent. In general, DMPCs near the Chol β face were less ordered, especially for a few first segments, which were in the proximity of the β face methyl groups.

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3A-40
COUNTERION EFFECT ON THE INTERACTION BETWEEN AMPHIPHILIC QUATERNARY AMMONIUM SALTS AND LIPID BILAYERS WITH AND WITHOUT CHOLESTEROL

Bozenna Rózycka-Roszak, Hanna Pruchnik, Adriana Mucha

The interaction of dodecyltrimethylammonium halides (DTAX) (1) and dodecylbenzyldimethylammonium halides (DBeAX) with lipid bilayers in the presence and absence of cholesterol were studied by means of DSC and ^{1}H NMR. In order to enhance the effect of counterions on water structure, two series of experiments were performed. In the first one a surfactant was added to the water phase while in the other one to the lipid phase (mixed film was formed). In the case of the first method the transitions were asymmetrical while in the second method nearly symmetrical. It is suggested that surfactant – rich and surfactant- poor domains are formed when surfactants are added to the water phase. The effects of particular surfactants were more pronounced when they were added to water phase in the case of liposomes formed from phosphatidylcholine only; the opposite happened when cholesterol was present. In the presence of cholesterol the water-ions interactions are probably less significant as they are in the absence of cholesterol. Probably, more important are cholesterol-surfactant interactions in which counterions are involved.
B. Rózycka-Roszak and H. Pruchnik, Z. Naturforsch. 2000, 55c – in press.
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3A-41
STRUCTURE OF PHOSPHATIDYLINOSITOLPHOSPHATE MONOLAYERS AT THE AIR/WATER INTERFACE

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The molecular organization of phosphatidylinositolphosphate (PIP) monolayers at aqueous surfaces was studied using Langmuir isotherms, fluorescence microscopy as well as x-ray and neutron reflectometry. PIPs represent a physiologically highly relevant class of membrane constituents involved in various cellular processes which has so far not much been characterized on the molecular level. Furthermore, PIPs are quite attractive in terms of the development of x-ray reflectometry since they provide a high electron density in their headgroup. We have investigated monolayers of synthetic phosphatidylinositol-3′-, 4′- and 5′-monophosphates on pure water and physiological buffers, both in pure systems and in mixtures with neutral phospholipids. Already the π-A isotherms show large differences between the various isomers. These have been confirmed and further investigated using fluorescence microscopy and reflectometry. X-ray results are dominated by the large electron densities of the headgroups in that the observed reflectivity at high momentum transfer is much larger than that of conventional phospholipid monolayers. Data evaluation uses the quasimolecular modeling techniques we have recently developed [1,2]. Neutron reflectivity measurements are in progress to work out the molecular organization of the lipid headgroups in great detail.

[1] M. Schalke et al., Biochim. Biophys. Acta 1464 (2000), 113.
[2] M. Schalke & M. Lösche, Adv. Colloid Interf. Sci., in press.

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3A-42
THERMODYNAMICS OF SOLUTE-MEMBRANE INTERACTIONS

Christa Trandum, Peter Westh

Numerous investigations have indicated that alcohol-induced perturbation of the physical properties of the cell membrane is an important aspect underlying alcohol intoxication. The thermodynamic property of most fundamental interest for an improved understanding of the energetics of this association process is the free energy. This quantity provides direct information about the net affinity of the membrane for a given alcohol. Further, evaluation of derivatives of the free energy function (e.g. enthalpy and entropy) may contribute significantly to the understanding of molecular interactions.

In the present work we have obtained partial molar enthalpies by use of isothermal titration calorimetry and determined chemical potentials by use of a newly developed vapour pressure equipment in order to evaluate molecular interactions between both unilamellar liposomes and short chain alcohols and erythrocyte gohsts and short chain alcohols at physiologically relevant concentrations. The alcohols investigated were ethanol, and 1-butanol.

The calorimetric data show that the interaction of the alcohols with membranes is endothermic but strongly dependent on both the acyl chain length of the alcohol and the composition of the membrane. The vapour pressure data reveal that for short chain alcohols (ethanol) the affinity of the membrane for water is higher than the affinity for the alcohol, whereas an enrichment of alcohol in the membrane is observed for the longer alcohol (butanol).

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3A-43
COMPUTER SIMULATIONS OF MODEL MEMBRANES

Maddalena Venturoli, Berend Smit

The role of the lateral pressure profile in biological membranes has been the topic of discussions in literature; the change in pressure profile is in fact a proposed mechanism by which variations in the lipid composition of membranes can influence the function of membrane proteins. An important aspect in these discussions is that there are no experimental methods to determine such pressure profiles. At present, we still have to rely on computer simulation or theory. However, molecular simulations in which the atomic details of the molecules forming the membrane are retained, have to start from a reliable initial configuration of the membrane, otherwise it would require far to much CPU time to observe the self-assembly of the membrane. It is also possible, when the properties of interest are due to a collective behavior of the molecules forming the membrane, to adopt a coarse-grained approach in which a less detailed model is used. In our simulations we have used such a coarse-grained model as well as an alternative simulation method, the dissipative particle dynamics technique, to simulate the self-assembly of a model membrane. The effects of changes in the chain length and stiffness of the surfactants on the membrane structure and on the pressure profile have been studied.

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3A-44
TRIFLUOPERAZINE INDUCED PHASE SEPARATION IN PHOSPHATIDYLCHOLINE BILAYERS AS DETECTED BY LAURDAN FLUORESCENCE

Olga Wesolowska, Andrzej B. Hendrich, Krystyna Michalak

Microcalorimetric studies performed in our laboratory have shown the possible phase separation induced by trifluoperazine (TFP) – a potent neuroleptic agent – in phosphatidylcholine (DPPC) bilayers. To investigate this possibility we have used Laurdan, the fluorescent probe that have different spectral properties in gel and liquid-crystalline lipid phases. Studying the dependencies of Laurdan generalised polarisation (GP) values on temperature and excitation wavelength (λ_{ex}) one can – respectively – follow the sample behaviour during lipid main phase transition and confirm/exclude coexistence of phases at a given temperature. In present study we show that TFP:DPPC (mole ratio 0.5) mixture has broader main phase transition temperature range as compared to pure DPPC. As indicated by positive GP(λ_{ex}) slope in temperatures below melting temperature (T_M) TFP:DPPC mixture has half gel/half crystalline liquid characteristics with both phases present in separate domains. At T_M both phases become homogenised (lack of GP on λ_{ex} dependence) and at $T > T_M$ the pure liquid crystalline phase is present (GP becomes descending function of λ_{ex}). We conclude that TFP induces phase separation in DPPC bilayers and propose the membrane domains to be regions enriched in/depleted of TFP or to be the result of different membrane binding of protonated and deprotonated TFP species.

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3A-45
EFFECTS OF HIGH FREQUENCY ELECTROMAGNETIC FIELDS ON PLANAR LIPID MEMBRANES

Andreas Wienand, Guenter Wrobel, Volkert Hansen, Guenther Boheim

Planar lipid membranes are exposed to electromagnetic fields of carrier frequency f_T = 900 MHz using a specially designed hollow waveguide setup. Upon HF-field exposure an excess current $I_{exc,bil}$ is observed in addition to the membrane current I_{dc}. For undoped planar lipid membranes $I_{exc,bil}$ consists of a permeation current component and a displacement current component described by the sum of three exponential terms. The ratio of the respective three current amplitudes depends on membrane capacitance, whereby the fast current component decreases with increasing membrane capacitance.
With Gramicidin-A single-channels a permeation current $I_{exc,gram}$ is observed during HF-field exposure, which reflects an increase of channel conductance G. The percentage change of G_{xW} is independent on G_{0W}. By varying α, which describes the angle between the membrane plane and the electric field component, changes of the conductance result.
Our working hypothesis is that interfacial membrane bound water seems to be the molecular acceptor for HF-field energy. Starting from this layer energy dissipation towards the local environment occurs.
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3A-46
FLUORESCENCE CORRELATION SPECTROSCOPY OF GIANT VESICLES ON OXIDIZED SILICON

Karsten Kottig, Peter Fromherz

The electrical coupling of a membrane in close contact to a semiconductor device and the self-organization of channel proteins are related to membrane intactness and fluidity.
We built an optical system for measuring the diffusion of membrane components by fluorescence correlation spectroscopy (FCS) and applied it to giant lipid vesicles in contact to a silicon-chip. The local measurement in the confocal setup leads to a separate view of the free membrane and the supported membrane. The reflecting silicon was taken into consideration and adapted to the focus calculation. The data were fitted with different diffusion models: normal, multicomponent and anomalous diffusion.
Giant vesicles were prepared from the lipids POPC and DOPME and a fluorescent dye by electroswelling on planar ITO/quartz electrodes. The vesicles adhered to the poly-lysine coated substrate.
Measurements in the free membrane show good agreement with the normal diffusion model. The lipid-like probes (TRITC-DHPE, Di-8-ANEPPS, $DiIC_{18}$) have a diffusion coefficient of $D = 6.2\ \mu m^2/s$ and the antibiotic gramicidin C of $D = 3.8\ \mu m^2/s$; both are similar to literature data for hydrated multibilayer systems at 25 °C. The normal diffusion model fails in the attached membrane. The diffusion process in the contact-membrane is slower by a factor 2–3 and the best description corresponds to anomalous diffusion model.
We studied the helical peptide LAH_4 which switches pH-dependent from an in plane to a transmembrane orientation. The change in diffusion by this mechanism can be monitored by FCS.

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3A-47
LIPOSOMES COATED LIPOSOMES. CHROMATOGRAPHY AND FLUORESCENCE STUDY

Zygmunt H. Zawada

Encapsulation of small liposomes in large liposomes permits, among other things, on separation them from contact with surrounding solution and receiving straight models of cells with separated different water phases. All methods based on hydratation of phospholipid film are suitable to this of aim, if water phase is a solution of liposomes. Especially, the DRV metod preparation of liposomes is used to enclosing single unilamellar vesicles with covalently bound protein in large liposomes.
The small vesicles L1 and L2 (~100 nm in diameter) were obtained modified reverse phase evaporation method (REV). The liposomes prepared from egg lecithin and cholesterol were labeled with fluorescence labels: NBD-PE and N-Rh-PE respectively. Solution of these liposomes was enclosed to the large liposomes (500-800 nm in diameter) prepared from DHPC and cholesterol by modified REV method. Non encapsulated L1 and L2 liposomes were separated by gel chromatography on SEPHACRYL 1000 column and from these data the efficiency of encapsulation was carried out. Independently, reducing sodium ditionite NBD-PE label from outer surface of liposomes the efficiency of encapsulation was caried out too. For encapsulated L1 and L2 liposomes were measured the changes of fluorescence and energy transfer from NBD-PE to N-Rh-PE.
One ascertained that efficiency of encapsulation L1 and L2 liposomes carries out 20–50%. Enclosed L1 and L2 liposomes do not surrender fusion with external DHPC/Chol membrane.

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3A-48
SIMULATING OIL-WATER-SURFACTANT SYSTEMS WITH DISSIPATIVE PARTICLE DYNAMICS

Jocelyne Vreede, Marieke Kranenburg, Berend Smit

Dissipative particle dynamics (DPD) is a simulation technique with a soft repulsive potential between particles. The particles are mesoscopic elements of the underlying liquid. Oil and water particles have a larger repulsive interaction toward each other than they have among themselves, hence a phase separation will occur. Surfactants, consisting of hydrophilic and lipophilic groups are constructed by connecting particles via harmonic springs. The surfactants in an oil-water system will form a monolayer at the interface of the oil and water phase, reducing the interfacial tension. Simulating monolayer systems is much alike simulating lipid bilayer systems.
The concentration and the shape of the surfactants influence the behaviour of the monolayer and the interfacial tension (ift). The ift decreases with increase of the surfactant concentration. The structure of the surfactant plays an important role in reducing the interfacial tension. The surfactant is better at reducing the ift for larger head groups. The shape of the tail has an effect as well.
Above certain surfactant concentrations micelles in the oil and the water phase have been observed, a critical micelle concentration. The existence of such a concentration has been observed experimentally. The shapes of the surfactants have an effect on the formation of micelles.

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3B-1
THE STRUCTURE OF A FUSOGENIC PEPTIDE SEQUENCE IN MEMBRANES IS STUDIED BY SOLID STATE ^{19}F-NMR

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The sea urchin fertilization protein "bindin" is used as a model to study protein-lipid interactions that lead to membrane fusion. A short, fully conserved 18-residue sequence "B18" represents the minimal functional part of the 24 kD protein. To determine the structure of this fusogenic peptide in lipid membranes, we synthesized a series of B18 analogues with a single leucine to 4F-phenylglycine (4F-Phg) substitution at different positions along the backbone. This non-natural side chain is a suitable label for solid state ^{19}F-NMR studies of polypeptides, since the ^{19}F-label is rigidly attached to the peptide backbone, and it provides an outstanding sensitivity. The introduction of 4F-Phg did not affect the secondary structure or fusogenic activity of the peptide significantly.
The local orientation and mobility of the labelled segments are accessible from NMR studies on oriented membrane samples. Since B18 can form either an α-helical structure or β-sheet fibrils, depending on the lipid environment, the incorporation of B18 had to be carefully optimized for the NMR analysis, and different strategies for peptide reconstitution were tested. The successful preparation of oriented NMR samples allowed the collection of a series of axially averaged ^{19}F-CSA tensors, each of which describes the alignment of the corresponding segment with respect to the membrane normal. The interpretation of the complete membrane-associated peptide structure is in progress.

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3B-2
BIOPHYSICAL ANALYSIS OF MEMBRANE ASSOCIATED POLYPEPTIDES BY SOLID-STATE NMR

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Solid-state NMR spectroscopy on oriented membranes provides important information on the structure, function, topology and dynamics of membrane-associated polypeptides. Antibiotic peptides, the Vpu viral polypeptide channel, signal sequences, bacteriorhodopsin as well as membrane active fragments of colicin A, B, N or E1 are studied in our laboratory by NMR spectroscopic and other biophysical techniques. Due to the anisotropic nature of nuclear interactions, valuable structural information including the secondary structure and the topology of polypeptides in oriented lipid bilayers is calculated from solid-state NMR spectra. We have used ^{15}N and ^{2}H solid-state NMR spectroscopy to differentiate between transmembrane and in-plane alignments of peptide α-helices. The design of model peptide antibiotics has allowed a detailed analysis of energetic contributions during polypeptide insertion into biological membranes. These include hydrophobic, polar and electrostatic interactions as well as mismatch between the hydrophobic length of the protein and the bilayer thickness. Studying the membrane topology of peptide antibiotics in a quantitative manner has resulted in new concepts for the prediction of membrane polypeptide topologies. The roles of histidines and lysines has been studies in considerable detail. Novel developments include solid-state NMR experiments aimed at understanding the regulatory mechanisms of polypeptide function on a structural level.

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3B-3
EFFECT OF LIPID ACYL-CHAIN LENGTH, TEMPERATURE, PRESSURE AND COMPOSITION ON THE STRUCTURE AND PHASE BEHAVIOR OF LIPID BILAYER/GRAMICIDIN MIXTURES

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Using FT-IR-, fluorescence-, ^{2}H-NMR-spectroscopy, DSC and SAXS, the effect of gramicidin D (GD) insertion on the structure and phase behavior of aqueous dispersions of the lipid bilayers DMPC, DPPC and DSPC has been studied, and a temperature range of 0–80 °C and a pressure range up to 15 kbar has been covered. Pressure has been applied to be able to tune the lipid chain length and to select different gel phases at constant thermal energy. At concentrations above 2 mol% GD, the structure of the temperature- and pressure-dependent lipid phases is significantly altered by the insertion of the polypeptide, and broad gel/fluid coexistence regions are induced. We have also seen that the lipid matrix has the ability to modulate the conformation of the inserted polypeptide. The balance between double-helical and helical dimer structures of GD depends significantly on the phospholipid hydrocarbon chain length and phase state. The larger the hydrophobic mismatch, the higher the population of double-helical conformers. By formation of broad gel-fluid coexistence regions the system has a further means of avoiding large hydrophobic mismatch. In these regions, wetting-like adsorption and a molecular sorting mechanism seem to be very likely. No pressure-induced unfolding of the polypeptide is observed up to pressures of 10 kbar.

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3B-4
SOLID STATE ^{19}F-NMR FOR STRUCTURAL STUDIES IN BIOMEMBRANES

Stephan L. Grage, Ulrich Dürr, Ralf W. Glaser, Douglas Young, Junfeng Wang, Tim A. Cross, Anne S. Ulrich

Solid state NMR is a powerful tool to measure structural parameters of polypeptides and proteins in biomembranes. To extend the low sensitivity and distance range of conventional isotope-labels (^{2}H, ^{13}C or ^{15}N), we introduce fluorine as an NMR reporter. In order to determine the orientation of a labelled segment with respect to the bilayer plane, it is first of all necessary to know the ^{19}F-chemical shift tensor and its alignment within the molecular frame. In order to use the advantages of fluorine for structural studies, we have systematically characterized the chemical shift tensors of many different fluorinated amino acids in their crystalline state.
In a subsequent step, these amino acids are being incorporated one by one into the known structure of the antimicrobial peptide gramicidin A. In this rigid transmembrane scaffold, the ^{19}F-NMR analysis provides information on the mobility and local geometry of the labelled side chains in the lipid environment. We illustrate the results on a series of gramicidin A peptides with different ^{19}F-labelled tryptophan sites. This knowledge can now be used in the study of unknown polypeptide structures.

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3B-5
LIPID DRIVEN AGGREGATION OF INTEGRAL MEMBRANE PEPTIDES STUDIED BY DIFFERENTIAL SCANNING CALORIMETRY AND ATOMIC FORCE MICROSCOPY

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The interaction between lipid molecules and membrane peptides takes place at the lipid-peptide interface. It is believed that the dominant part of the interfacial energy stems from a hydrophobic mismatch between lipids and peptides. Since the hydrophobic length of the lipids is a function of the temperature and is significantly altered in the lipid main transition regime, the interfacial free energy changes upon lipid melting. This leads to a change in the mixing properties of lipids and peptides. There are three limiting cases, no aggregation, peptides aggregating in either gel or the fluid phase, or in both lipid phases, which can be easily distinguished in the heat capacity traces.
From the specific heat capacity profiles of mixed systems like DMPC or DPPC with gramicidin A we conclude that peptide aggregation takes place in both lipid phases, where aggregation in the gel phase is more pronounced. The predictions were tested with atomic force microscopy. Small point like aggregates corresponding to about 40 protein molecules were observed in the fluid DMPC bilayer. In the gel DPPC membrane we observed big line-type gramicidin A aggregates, which suggests that there is a total demixing between the peptide and the gel DPPC phase.

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3B-6
A VIRAL PEPTIDE SWITCHES MEMBRANE MORPHOLOGY. NEW INSIGHT INTO RNA TRANSFECTION

Andreas Janshoff

The binding of virus capsides to cellular membranes is a key event in the transfection and life cycle of all kinds of viruses. Nodaviruses are simple non-enveloped RNA animal viruses composed of a single gene-product capsid protein that packages a bipartite single-stranded RNA genome. High resolution structural investigation of the Flock House nodavirus (FHV) and related viruses has led to the intriguing hypothesis that a C-terminal capsid protein cleavage product, called the γ-peptide in FHV, plays a pivotal membrane-permeablizing role that facilitates RNA/virion translocation.
In general, we were interested in the biophysical characterization of viral peptides with artificial model membranes. In particular, we have established that the N-terminal 21 residues of the 44-residue γ-peptide of the capsid protein do indeed bind to fluid phase membranes with high avidity as well as induce dramatic increases in liposomal bilayer permeability. Remarkably, synthetic γ_1 exerts a unique effect on neutral gel-phase lipids: treatment of dipalmitoyl phosphatidylcholine (DPPC) bilayers with low mole fractions of γ_1 results in a wide-spread change in morphology consistent with acyl chain interdigitation of gel-phase phospholipids. In contrast to saturated phospholipids, the γ-peptide acts vividly with unsaturated lipids by creating large defects in a carpet – like mechanism and forming globular structures eventually leading to a decomposing of the planar membrane.

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3B-7
EFFECTS OF HIGH FREQUENCY ELECTROMAGNETIC FIELDS ON ALAMETHICIN-F30 DOPED PLANAR LIPID MEMBRANES

Guenter Wrobel, Andreas Wienand, Volkert Hansen, Guenther Boheim

Planar lipid membranes doped with the channel forming α-helical peptaibol alamethicin-F30 are exposed to electromagnetic fields of frequency f_T = 900 MHz using a rectangular hollow waveguide setup.
Increasing HF-power P_W causes an increase in mean conductance G_ν for each channel level ν in single channel experiments. Additionally the mean channel level ν shifts to lower levels in combination with decreasing mean lifetimes τ_ν. The current changes registered at alamethicin-F30 multi-channel systems are caused by a combination of the HF-induced effects on single-channel states mentioned above. Varying the angle α between the direction of the E-field vector and the membrane plane alterations in ν, G_ν and τ_ν are similar to those observed for increasing HF-power.
Data are compared with those experiments carried out at different temperatures of the electrolyte solution.
The results could be interpreted that the molecular target which seems to interact with the applied HF-field is located in the membrane vicinity. The HF-induced effects seem to be caused by interactions of the HF-field with membrane bound water.
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3B-8
INTERACTION OF FUSOGENIC PEPTIDES WITH MICELLES AND PHOSPHOLIPIDS

Ilona Laczkó, Gábor K. Tóth, Elemér Vass, Miklós Hollósi

The elucidation of the mechanism of the translocation of hydrophilic compounds like antigenic peptides or oligonucleotides is an important step in evaluating their possible therapeautic applications. Penetratins as well as fusion peptides or lipopeptides have been considered as possible candidates to deliver molecules into the cytosol. To investigate their translocating ability we have analyzed the conformational behaviour and associative properties of a series of peptides by circular dichroism, Fourier transform infrared and fluorescence spectroscopies in tne presence of micelles and negatively charged liposomes. These spectroscopic studies have been undertaken on the following peptides: (i) the third helix of the Antennapedia homeodomain $pAntp_{43\text{-}58}\text{-}NH_2$ (ii) fragments from the immunodominant region of influenza virus hemagglutinin, $HA_{317\text{-}329}\text{-}NH_2$ and $HA_{317\text{-}341}\text{-}NH_2$ (iii) C-terminal O-palmitoyl and O-octanoyl derivatives of the latter peptides (iv) and also their analogues where Phe338 is replaced by Try. A conformational shift towards ordered secondary structures was found in the presence of micelles and liposomes depending on the sequence of peptides and the presence of C-terminal fatty acid moiety.

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3B-9
MOLECULAR DYNAMICS STUDY OF PEPTIDE-MEMBRANE INTERACTION

Céline Escrive, Michel La Guerre

Peptide-lipid interactions are undergoing more and more studies in order to understand the insertion and the folding of membrane proteins, the action of antibiotic peptides and the lysis of membranes by toxins.
By use of the molecular dynamics approach, it is possible to observe the orientation of peptides at the interface water/lipids. This orientation is highly dependant of several parameters : the secondary structure (either α-helix or β-sheet) is directly responsible of the depth of the insertion, but the spatial orientation of the peptide (parallel or perpendicular to the lipids acyl chains) is regulated either by the length of the peptide versus the width of the membrane or the number and position of the charged residues along the secondary structure, but also by the nature of the polar heads of the lipids.
This approach will be illustrated by simulations of the interaction of amphipathic peptides with phospholipids Langmuir films or bilayers. Peptides have been chosen either as ideally secondary amphipathic or as amphipathic di-block within natural toxins or a minimalist list L_iK_j but also among pulmonary surfactant proteins (mainly SP-C). Whereas lipids were chosen among phospholipids either in homogeneous layers (PC, PE or PG) or in mixed layers (PC/PG = 4/1). All simulations were performed during ~2 ns with all-atoms force-field (CVFF) using periodic boundary conditions.

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3B-10
SUPRAMOLECULAR STRUCTURE OF MAGAININ PORE AS SEEN BY MOLECULAR DYNAMICS SIMULATION

Krzysztof Murzyn, Marta Pasenkiewicz-Gierula

Magainins are short, cationic peptides isolated from the skin of *Xenopus Leavis*. They show both antibacterial and anticancer activity, but are not hemolytic. In the membrane environment, they form α-helices of distinct hydrophobic moment. In the physiological conditions, their total electrostatic charge is +4 e. It has been postulated that magainins disturb the structure of the bacterial cell membrane, subsequently causing cell lysis. Recently, on the basis of the in-plane neutron scattering data, a toroidal model of magainin pores in the plasma membrane has been proposed (Ludtke S. J., He K., Heller W. T., Harroun T. A., Yang L., Huang H.W., (1996), Biochemistry, 35, 13723-13728). In this report, results of molecular dynamics simulation of a magainin-2 amide pore in the model bacterial membrane are presented. The membrane consisted of 138 1-palmitoyl-2-oleoyl-*sn*-glycerol-3-phosphatidylethanolamine (POPE) and 46 1-palmitoyl-2-oleoyl-*sn*-glycerol-3-phosphatidyl-*rac*-glycerol (POPG) molecules (3:1 proportion). The lipid composition of the model membrane reflected that of a natural bacterial membrane, as the membrane contained both negatively charged (POPG) and neutral (POPE) lipids. In the simulation, a total time of 5 ns was covered. This allowed a meaningful analysis of the supramolecular structure of the magainin-2 pore in the membrane. The analysis enabled identification of factors responsible for the formations and stability of the structure.

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3B-11
INTERACTIONS BETWEEN ALAMETHICIN AND MODEL MEMBRANES VIEWED BY NEUTRON AND X-RAY SCATTERING

L. Perino-Gallice, I. Grillo, G. Fragneto

Alamethicin is a small antimicrobial peptide of 20 amino acids. It has been shown that this peptide exhibits a concentration dependent behavior as it passes from an adsorbed configuration on the lipid bilayer at low peptide concentrations, to an inserted one at high concentrations. Our goal is to understand which structural changes and interactions cause such a behavior. In order to study the interactions between this peptide and cell membranes by the way of neutron and X-ray scattering, we have modelled the highly complex system of the cell by simple lipid bilayers.
We are interested in two membrane models consisting of DMPC, a well known zwitterionic lipid which is in the fluid phase, the relevant biological one, at temperatures above 23 °C. The first model is formed by two lipid bilayers deposited on a substrate.Using specular and off-specular reflectivity it is possible to determine the position of the peptide in the bilayer. The second one is formed by lamellar phases in solution. SANS and SAXS experiments have been done recently for different peptide/lipid and lipid/water ratios and have shown an important effect of the peptide as the lowering of the bilayer thickness in presence of the peptide in the low peptide/lipid ratios.
Further data analysis by scattering length densities calculation and fitting procedures will consider three different possible models of insertion with the peptide dispersed in the water layer, adsorbed on the bilayer or inside the bilayer.
We will present the first results of this study.

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3B-12
AN OLIGOMERIC PORE STRUCTURE OF GRAMICIDIN S IN LIPID MEMBRANES IS REVEALED BY SOLID STATE NMR

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The alignment of the cyclic decapeptide gramicidin S (Val-Orn-Leu-DPhe-Pro)$_2$ in lipid bilayers was determined by solid state ^{19}F-NMR. The antimicrobial peptide ("GS") forms an antiparallel β-sheet with an amphipathic character, which destroys bacterial membranes. We replaced the two equivalent Leu residues by 4F-phenylglycine, which is well suited for structural studies, because the ^{19}F-labels protrude rigidly from the backbone and provide an outstanding NMR sensitivity. The orientation and dynamics of the ^{19}F-labelled segments in the membrane-bound peptide were determined by static NMR experiments using macroscopically oriented films on planar glass supports. The behavior of GS in DMPC bilayers is closely linked to the phase state of the lipid. At most temperatures the amphipathic β-sheet was found to be aligned flat on the membrane surface, as expected. Surprisingly, the alignment of GS is transiently flipped while passing through the lipid phase transition, which encompasses a considerably broad temperature range at high peptide-to-lipid ratios. Our ^{19}F-tensor analysis defines the alignment of the gramicidin molecule as *upright* within the bilayer. This structure is fully consistent with an oligomeric peptide pore in the membrane, arranged as a β-barrel and stabilized by intermolecular H-bonds. The hydrophilic opening would allow the passage of polar molecules and could explain the destruction of cellular membranes.

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3B-13
COMPETITION OF ION AND WATER FLUXES ACROSS GRAMICIDIN AND DESFORMYLGRAMICIDIN CHANNELS

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Water movement across the two peptide channels led to accumulation of solutes on one side of the membrane and depletion on the other. Pore permeable ions were pushed by water across the channel (solvent drag). Ion and water fluxes were assessed from the concentration distributions of both pore impermeable and permeable cations that were simultaneously measured by double barrelled microelectrodes in the immediate vicinity of a planar bilayer. From solvent drag experiments, approximately five water molecules were found to be transported by a single file process along with one ion through the gramicidin and desformylgramicidin channels. Their water conductivities differed by two orders of magnitude. With respect to the single channel hydraulic permeability coefficient of 1.1×10^{-12} cm^3 s^{-1}, desformylgramicidin may serve as a model for extremely permeable aquaporin water channel proteins (Aqp4 and AqpZ).

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3B-14
INTERACTION OF ANTIBACTERIAL PEPTIDE MAGAININ 2 WITH MULTILAMELLAR LIPID BILAYER SYSTEMS PROBED BY NEUTRON- AND X-RAY SCATTERING

Christian Münster, Burkhard Bechinger, Tim Salditt

The membrane-active 23-residue helical peptide magainin 2 belongs to a class of antibacterial (and fungicidal) peptides, which are part of the vertebrate immune system and interact directly with the lipid bilayer of the bacterial cell wall. Despite recent advances, many aspects of lipid-peptide interaction are to date not well understood. We have studied the bilayer structure, the elasticity and fluctuation behavior, as well as the lipid chain packing in model systems of multilamellar membranes (POPC, POPS, DMPC, DLPC, DOPC). For this purpose, highly aligned samples have been prepared on silicon and glass substrates and were investigated by synchrotron based x-ray and neutron scattering in a temperature and humidity controlled chamber. While the scattering length density profile was determined by reflectivity measurements, diffuse x-ray and neutron scattering was used to characterize the bilayer fluctuations and elasticity properties. The lateral membrane structure on molecular length scales was probed by synchrotron based grazing incidence x-ray diffraction. The results obtained for different lipids are discussed as a function of peptide concentration.

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3C-1
FLUCTUATIONS OF ACTIVE MEMBRANES

Jacques Pécréaux*, Patricia Bassereau*, Jacques Prost*, Hans-Günther Döbereiner*+

Theoretical calculations (1,2) predict that the non-equilibrium activity of pumps or ions channels incorporated in lipid bilayers changes both qualitatively and quantitatively the shape fluctuations of lipid membranes.

We have probed the modifications of the fluctuations of giant phospholipid vesicles after reconstitution and activation of bacteriorhodopsin (BR), a light activated proton pump. We have measured the excess area due to the shape fluctuations of the vesicles by aspiration in a micropipet (3). The activity of the protein was easily turned on and off by adjusting the wavelength of the light relative to the adsorption band of the protein. We have found that the shape fluctuations are highly increased when the membrane is active, in quantitative agreement with theoretical models. In addition, we have performed real time contour analysis of the active vesicles using videomicroscopy. Our results confirm the amplification of the fluctuations due to the activation of the BR.

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[3] J.-B. Manneville, P. Bassereau, D. Levy, J. Prost, *Phys. Rev. Lett.* 82 (1999) 4356.

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3C-2
OPTICAL TWEEZERS STUDY OF DIFFUSION OF PROTEINS IN MEMBRANES

Kirstine Berg-Sørensen, Lene B. Oddershede, Sonia Grego, Jakob Kisbye Dreyer, Simon F. Nørrelykke, Stanley Brown

We have built an optical tweezers equipment to be used to study diffusion of membrane proteins in living cells. We plan to study proteins in both eucaryotic and procaryotic membranes. Initially, we study the mobility of the λ-receptor in the outer membrane of *E. coli* bacteria. The λ-receptor has been modified such that it is biotinylated at an extracellular site, allowing for attachment of a streptavidin coated polystyrene bead that can be manipulated by the optical tweezers. In order to measure the diffusion coefficient of the λ-receptor, we monitor the stochastic motion of the bead trapped in a soft optical potential. This stochastic motion is determined by both the stochastic motion of the protein, and that of the bead. We present a simple theoretical model to separate the motion of the protein from that of the protein-bead complex, and use it to analyze our experimental results.

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3C-3
STUDY OF HYDROPHOBIC INTERACTIONS BETWEEN ACYLATED PROTEINS AND PHOSPHOLIPID BILAYERS USING BIACORE

Joël Chopineau,[1] Marie-Odile Roy[2], Martine Pugniere[1], Magali Jullien[2], Jean-Claude Mani[3]

The purpose of this work was to quantify the effect of acyl chain length on hydrophobic interactions between acylated proteins and membranes. The binding of an artificially acylated model protein toward electrically neutral phospholipids has been studied by surface plasmon resonance, using BIACORE. Kinetic rates for the binding of bovine pancreatic ribonuclease A (Rnase A), monoacylated on its N-terminal lysine with fatty acids of 10, 12, 14, 16 or 18 carbon atoms, to hydrophobic sensor chips have been measured. In contrast to unmodified ribonuclease, acylated Rnases A bind to the sensor chip and the association level increases with the acyl chain length with a maximum for C16. Reproducible kinetics were obtained which did not fit a 1:1 Langmuir model but rather a two-step binding profile.

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3C-4
LOCALIZATION OF β-AMYLOID PEPTIDE IN MODEL MEMBRANES BY NEUTRON DIFFRACTION

Silvia Dante, Thomas Hauss, Norbert A. Dencher

Although its mechanism of action in the neurodegenerative process has not been yet established, the β-amyloid (βA) peptides play an important role in the pathogenesis of Alzheimer's disease (AD). βA isolated from the senile plaques of AD patients consists predominantly of 39–42 amino acid residues; in addition, shorter fragments of βA have been proved to be toxic to neuronal cells, and they have been proposed to activate the process of neuronal dysfunction.
We have investigated the interaction of the βA fragment 25–35 with model membranes by neutron diffraction. Very well oriented lipid membranes were obtained by spraying with an airbrush a chloroform solution of different lipids on a quartz support. Selectively deuterated β-amyloid at the amino acid in position 34 was synthesized for this purpose. The neutron diffraction spectra of the lipid mixtures containing labeled and unlabeled peptide allow the reconstruction of the scattering length density profile in direction perpendicular to the membrane planes, *via* Fourier synthesis. Comparison of the two profiles shows unequivocally the position of the label and therefore allows localizing the deutereted part of the peptide in the membranes. For two different lipid mixtures the localization of βA(25–35) in the region of the alkyl chains was possible.
Further results concerning the interaction of the peptide with Langmuir monolayers of the same lipid mixtures are presented as well.

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3C-5
THE MAJOR MEMBRANE ANCHORING DOMAIN OF TALIN HAS FUSOGENIC PROPERTIES

S. Doerhoefer*, E.-I. Pecheur#, W.H. Goldmann°, D. Hoekstra#, G. Isenberg*

Secondary structure predictions has lead to the identification of the major membrane anchoring domains within the 47 kDa talin head fragment spanning aminoacid residues 21–39 and 385–406. Corresponding synthetic peptides bind to POPC/POPG lipid vesicles synergistically and insert into the hydrophobic phase with an partition coefficient of 10^6 M^{-1} as determined by isothermic titration calorimetry and the monolayer expansion technique (A. Seelig, Li Blatter, Frentzel & Isenberg, J. Biol. Chem. 2000 in press). As measured by CD-spectroscopy peptide 385–406 refolds in the presence of liposomes from random coil and β-sheet conformation into an amphipathic α-helix, prior to an oblique insertion at an angle of 18°. We have applied resonance energy transfer (RET) assays using NBD- and rhodamine labeled lipids to analyze for fusogenic properties by lipid mixing assays. In addition, we used liposomes containing 6-carboxyfluorescein to measure contents leakage. Both assays demonstrate that the major membrane anchoring domain of talin is highly fusogenic. This first example of an actin binding protein exhibiting fusogenic properties could be of importance for understanding membrane targeting and motile events in the leading front of cells.

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3C-6
EFFECT OF HEME IRON VALENCE STATE ON THE ASSOCIATION AND CONFORMATION OF CYTOCHROME *C* WITH MEMBRANE INTERFACES

Adelaide Faljoni-Alário, Iseli L. Nantes, Maria R. Zucchi, Otaciro R. Nascimento

Recently cytochrome *c* is mentioned as an important mediator in the events of cellular oxidative stress and apoptosis . In order to investigate the influence of the charged interfaces on the conformation of cytochrome *c*, the circular dichroic (CD) and magnetic circular dichroic (MCD) behavior of ferri and ferrous citochrome *c* in homogeneous media, phosphatidilcoline/ phosphatidilethanolamine/ cardiolipine (PC/PE/CL) or dicetylphosphate (DCP) liposomes were obtained in the wavelength region 300–600 and 200–320 nm. EPR spectra demonstrate that the association of cytochrome c with membranes promotes alterations of the crystalline field symmetry and spin state of the heme Fe^{3+}. The studies also include the effect of inorganic phosphate (Pi), NaCl and $CaCl_2$. MCD and CD results shows that the interaction of both ferrous and ferric cytochrome *c* with charged interfaces promotes conformational changes in the α-helix content, terciary structure and heme iron spin state. Moreover the association of cytochrome *c* with different liposomes is sensitive to the heme iron valence state. The more effective association with membranes occurs with ferrous cytochrome *c*. DCP liposomes, as negative charged membrane model, promoted the more sharp conformational modification in the cytochrome *c* structure. The lipid/protein association is impaired in crescentof ionic strength.

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3C-7
SPECTROSCOPIC CHARACTERIZATION OF ANTIBIOTIC Y IN AQUEOUS AND HETEROGENEOUS ENVIRONMENT

Krzysztof Polewski, Piotr Golinski

Antibiotic Y (AY) is a secondary metabolite extracted from fungus *Fusarium avenaceum*, a recognized pathogen in plants. Absorbance and fluorescence methods were used to describe AY in homogeneous and heterogeneous environment.
Absorbance maximum depends on the dielectric constant of the solvent, concentration of AY and its ionization state. At low concentration in emission spectrum prevails peak at 450 nm, at increasing concentration new peak with maximum at 515 nm appears. The absorbance and fluorescence data indicate that this peak may be assigned to AY dimer. In pure water we observe existence of the both forms with the fluorescence intensity is 50 times lower compared to methanol. At low pH about 90% of emission originates from dimer. Increasing pH shifts the maximum to 450 nm form with simultaneous decrease in fluorescence intensity.
To elucidate the character of AY binding to membrane, its fluorescence in non-polar media and micelles were studied. AY fluorescence in solvents of lower polarity is characterized by an enhancement in intensity and blue shift of the monomer form and red shift of the dimer form. In anionic micelle SDS, sodium dodecyl sulphate, 10 fold in fluorescence intensity increase compared to water is observed. In cationic micelle TTABr, tetradodecyl trimethyl ammonium bromide, only two-fold, compared to water, intensity increase is observed. Concentration studies with detergents suggets that only monomeric form is inserted into micellar core.

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3C-8
MECHANISM OF INTEGRAL MEMBRANE PROTEIN SOLUBILIZATION: A CALORIMETRIC AND KINETIC STUDY

Ernst Grell, Eginhard Schick, Erwin Lewitzki

The molecular aspects of the solubilization of Na,K-ATPase membrane fragments with different polyoxyethylene monoalkylether detergents have been investigated. It was possible to differentiate between endothermic detergent monomer binding to the lipid, endothermic disintegration of the fragments, exothermic detergent binding to the protein and an endothermic transition of the micellar phase, attributed to the dissociation of Na,K-ATPase dimers. The corresponding thermodynamic parameters have been determined. All processes are influenced by the length of the alkyl- and polar polyoxyethylene-chain as well as by the composition of the medium. The results allow to develop a molecular interpretation which makes understandable why only a few members of this detergent class can provide full enzymatic activity in the solubilized state of the protein. Kinetic stopped-flow studies provided evidence for at least three processes: A fast, not time-resolvable pre-equilibrium due to detergent demicellization and binding of detergent monomers to the membrane fragments (< 1 ms) is followed by a slower process in the ms time range, which is dependent on detergent concentration. This surprisingly fast, but still time-resolvable process is assigned to membrane fragment disintegration. An even slower process was found in the time range of seconds. Based on these thermodynamic and dynamic properties, a mechanism of the transition to the protein's micellar phase is derived.

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3C-9
INTERACTION OF LIPOPOLYSACCHARIDE (LPS) AND LPS-BINDING PROTEIN (LBP) WITH PLANAR MEMBRANES

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Reconstituted planar membranes are a common tool to characterize the effects of pore-forming molecules on membranes. Here, we show that this method can also be utilized to investigate non pore-forming proteins playing a role in signal transduction. We investigated the mechanism of interaction of the lipopolysaccharide (LPS)-binding protein, LBP, with symmetric and asymmetric planar bilayers composed of different lipids applying voltage-clamp, potential, and capacitance measurements. Binding of LBP to bilayers with a negative surface charge density of the outer leaflet could be deduced from changes of the innermembrane potential difference of the membranes. We observed binding of anti-LBP antibodies and LPS or its lipid A moiety to the intercalated LBP on both sides of the bilayer. The effects resulting from an interaction of anti-LBP antiserum with membrane-bound LBP depend on the side of addition of the antiserum, indicating a directed transmembrane intercalation of LBP into the membrane. Adding LPS on the same side as LBP, the binding of these two components leads to a change of the conformation or orientation of LBP in the membrane. Thus, the discussed "shuttle" function of soluble LBP is rather unlikely to occur, whereas a membrane-associated interaction is more likely.

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3C-10
Ca^{2+}-MYRISTOYL SWITCH AND MEMBRANE BINDING OF ACYLATED NEUROCALCINS

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Neurocalcins are brain-specific proteins which are cognates to recoverin. They are *N*-terminus myristoylated *in vivo* and present a Ca^{2+}-myristoyl switch which functions upon membrane binding. In order to emphasize the role of myristate among other fatty acids in this mechanism, we use reversed micelles to chemically acylate non-myristoylated neurocalcin at its *N*-terminus with fatty acids of different lengths (from C12 to C16). The ability to specifically attach fatty acids at the *N*-terminus allows us to examine the membrane-binding properties of these proteins. Two different experimental setups, lipidic vesicles or hybrid-supported monolayers, reveal that the Ca^{2+}-myristoyl switch can accommodate other lipid moieties and is not strictly specific to myristate.

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3C-11
RECONSTITUTION OF MEMBRANE PROTEINS IN GIANT UNILAMELLAR VESICLES

Nicoletta Kahya, Eve-Isabelle Pecheur-Huet, Wim P. de Boeij, Dick Hoekstra, Douwe A. Wiersma

A novel procedure was developed to reconstitute proteins into Giant Unilamellar Vesicles (GUVs, diam. 10-100 μm). The approach relies on the ability to fuse Large Unilamellar Vesicles (LUVs, diam. 100 nm), into which proteins can be (more) readily reconstituted, with GUVs. By employing assays based on lipid and contents mixing, we demonstrate that peptide-induced membrane fusion occurs between LUVs and GUVs. To monitor contents mixing in this system, a new assay had to be devised. Importantly, no leakage occurs during LUV-GUV fusion, implying that overall membrane integrity is maintained. The microscopic structure and the lateral dynamics of lipids in the fusion products were analyzed by Fluorescence Correlation Spectroscopy. We next reconstituted Bacteriorhodopsin (BR) from Halobacterium Halobium into GUVs, using the procedure as described above. BR was first reconstituted into LUVs. The presence of the bulky protein did not affect the LUV-GUV fusion, as verified by lipid and content mixing. Data are presented which demonstrate that the structural and functional properties after reconstitution into GUVs are not distinguishable from those determined in LUVs, prior to transfer into GUVs, implying that proteins can be reliably reconstituted into GUVs according to the methodology as presented here. This procedure thus offers the exciting possibility to study the dynamics of membrane proteins in a close-to-native environment by means of optical microspectroscopy.

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3C-12
STRUCTURAL CHANGES OF COLICIN A EXAMINED BY EPR SPECTROSCOPY

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The study of structural elements of proteins and their structural changes is the key to a deeper understanding of protein function. The area of application of well-known methods of structure determination like NMR and crystallographic methods is restricted with respect to size or type of the protein. EPR in combination with site-directed spinlabeling (SDL) is emerging as a powerful method to get specific information about secondary and tertiary structure, polarity, mobility and intramolecular distances at selected sites. Furthermore changes of these parameters can be observed time dependently. This method can successfully be applied to study pore forming processes of channels or protein folding. We will present first results on spin labeled colicin A, a ion channel forming protein toxin produced by E. coli. This protein has a water-soluble form but it is able to build a voltage gated ion channel in lipid vesicles accompanied by a major change of structure. Our aim is to reveal structural elements of the membrane bound form and to observe changes after/during membrane insertion. X-Band and W-Band EPR results on nitroxide spin labels bound to helix nine performed in the presence and absence of lipid vesicles show dramatic environmental changes in the vicinity of the binding sites.
To perform time dependent measurements we built a stopped flow apparatus on the basis of the BRUKER dielectric resonator. Details of the construction and first applications to the folding reaction of cytochrome c are presented.

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3C-13
STRUCTURAL MODIFICATIONS IN CYTOCHROME C ASSOCIATED WITH SDS MICELLES. A MCD AND EPR ANALYSIS

Iseli L. Nantes, Maria R. Zucchi, Paulo R. M. Yunes-Filho, Rodrigo R. Pessoa, Otaciro R. Nascimento, Adelaide Faljoni-Alario

This work characterizes the interaction of ferric and ferrous cytochrome *c* with negatively charged SDS micelles. The ferrous cytochrome *c* UV-Vis spectra obtained in the presence of SDS micelles, pH 7.4 reveals a Soret band blue shift and the desappearence of the ferrous cytochrome *c* characteristic band at 549 nm. Soret band blue shift was also observed with ferric cytochrome *c* associated to SDS micelles. Magnetic Circular Dichroism spectra of SDS bound ferrous and ferric cytochrome *c* suggest that the association with this negativelly charged micelles change the heme iron spin state from 0 to 2 and from 1/2 to 5/2 and modified 1/2 respectively. EPR spectra of SDS bound ferric cytochrome *c* corroborated the spin state change in ferric cytochrome *c*. In order to discriminate effect of the negative charge on the micelle interface from that produced by the acidic microenvironment at the micelle surface of the SDS micelles, the MCD spectra of ferric and ferrous cytochrome *c* in pH range from 1.5 to 13 were obtained. The results indicate that the conformational and spin state changes produced by the interaction of cytochrome c with SDS micelles cannot be attributed to pH in the microenvironment. Probably other important fact in this protein/micelles interaction is the insertion of the SDS chain in the cytochrome c hydrophobic core.

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3C-14
EPR STUDY OF LIPID SPIN LABEL INTERACTION WITH CYTOCHROME *C* TO UNDERSTAND CYTOCHROME *C*/MEMBRANES ASSOCIATION

Otaciro R. Nascimento, Maria R. Zucchi, Iseli L. Nantes, Adelaide Faljoni-Alário

This work characterizes the interaction of different phosphatidylcholine spin labels with cytochrome *c* in ferric and ferrous form. It was used the following spin labels: 16PC, 12PC, 10PC, 7PC and 5PC. EPR measurements were made in X-band at three different temperatures: 300 K, 77 K and 2 K. The results help us to understand the hydrophobic interaction resulting from the insertion of the acyl chain in cytochrome *c* hydrophobic channel near the heme crevice, as proposed by Rytömaa and Kinnunen (Rytömaa, M.; Kinnunen, P. K. (1995). *J. Biol. Chem.* 270, 3197-3202). We are shown in previous work that the cytochrome *c*/membranes hydrophobic interaction is modulated by the electrostatic interaction that is dependent from the charge and type of polar head group of the used lipids. In the presence of zwitterionic phospholipids only saturated acyl chains were able to induce spin form changes in cytochrome *c*. Analysis of the line shape of EPR spectra of these spin labels are indicating that the interaction depends of the position of nitroxide group in the acyl chain and the valence and spin state of the heme iron. A molecular modeling of this interaction are in progress.
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3C-15
MOLECULAR MECHANICS STUDIES OF A VIRAL COAT PROTEIN IN A PHOSPHOLIPID BILAYER

Denys Bashtovyy, Marcus Hemminga, Derek Marsh, Tibor Páli

Molecular structures of the major coat protein of the M13 bacteriophage, determined recently in detergent micelles, have been modified by replacing certain residues with spin-labelled analogues. These residues were selected on the basis of recent site-directed spin labelling EPR measurements of distance and order parameter data for the membranous assembly of the coat protein. A single layer solvation shell of dioleoylphosphatidylcholine lipids was built around the protein. The main criteria for identifying promising protein structure candidates for the membranous state, out of the 300 single residue mutant models, were the lack of steric conflicts with the phospholipid bilayer, good match of positions of spin-labelled residues along the membrane normal between the model and experimental distance data, and a good match between the sequence profiles of experimental local order parameter and a structural restriction parameter of the spin-labelled residues obtained from the model. A unique structure has been selected which satisfies these criteria and is also in agreement with further experimental data. It is also a good starting point for full-scale molecular dynamics simulations and for the design of further site-specific spectroscopic experiments. The structural parameter can be used in other site-directed spin-labelling studies.

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3C-16
REVERSIBEL INSERTION AND PORE FORMATION DRIVEN BY PROTEIN ADSORPTION TO SURFACES

Martin L. Zuckermann, Thomas Schlötzer, Thomas Heimburg

Membrane adsorbed proteins form a surface gas. The lateral pressure of this gas has a considerable effect on the adsorption isotherm. This pressure depends on shape and state of aggregation of the ligands, but also on the nature of other species that compete for available surface. In particular the adsorbed concentration is much lower than that found in the case of simple Langmuir adsorption to specific binding sites which is often used to describe binding to membranes. We propose that the lateral pressure can become high enough to promote reversible insertion of certain peripheral proteins (for example cytochrome c) directly into the bilayer. To this purpose we present both experimental evidence from binding studies and EPR data for adsorption induced insertion of cytochrome c into anionic lipid membranes. We provide a model based on the scaled particle theory for reversible protein insertion induced by interfacial adsorption. Furthermore we extend our theory to reversible pore formation (for peptides like melittin) based on a similar mechanism. The effect of a second adsorbing species on pore formation is also considered. A second species is shown to promote the insertion of the first species into the membrane.

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3C-17
THERMODYNAMICS OF A LIPID MEMBRANE WITH ADSORBED PROTEINS AND CHOLESTEROL

Hergen Schultze, Reiner Kree

We investigate the thermodynamic properties of phospholipid membranes close to the main transition including cholesterol or adsorbed small proteins. The lipid membrane is modeled as a lattice gas and the small proteins are supposed to cover not more than one lattice site. The protein degrees of freedom can be eliminated without approximations. The resulting Hamiltonian is treated in cluster mean field approximations and so we get an analytic expression of the free energy. We calculate the specific heat as a function of temperature and fraction of cholesterol or proteins respectively and compare it with results of experiments and Monte-Carlo simulations as taken from the literature. The analytic theory matches qualitatively with experiments and simulations. We argue that quantitative differences are due to the size of the proteins. The nearest neighbour correlations show a lipid driven cluster behaviour. The present approach can easily be extended to more complex systems.

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3C-18
INTERACTION OF Raf-C1 WITH LIPID MEMBRANES INVESTIGATED BY QUARTZ CRYSTAL MICROBALANCE

Claudia Steinem, Andreas Eing, Claudia Bex, Christoph Block, Hans-Joachim Galla

Raf-1 is a protooncogenic Ser/Thr kinase that plays an essential role in signal transducing processes from the cell surface to the nucleus via activation of a MAP-kinase module by a GTP switch. It has been shown, that although activation of Raf-C1-Kinase is concomitant with its recruitment to the membrane induced by Ras, a simple association between Raf-C1 and Ras is not sufficient for its activation. In analogy to protein kinase C, in which a zinc containing cysteine rich domain is believed to play a pivotal role in the activation of the enzyme by binding to acidic phospholipids – phosphatidylserine and diacylglycerol – it is proposed that the cysteine rich domain of Raf-C1 behaves in a similar fashion.
Different fusion proteins composed of a His-tag, the Ras-binding-, cysteine-rich-domain and maltose-binding-protein have been constructed bearing mutations at positions K144A/R164A and K144A/L160A/R164A. The interaction of the wild type and mutants with membranes was investigated using solid supported lipid bilayers immobilized on gold electrodes of 5 MHz quartz crystals. The experiments revealed that the interaction of the proteins with lipid membranes is predominately electrostatic. Comparison of the binding of the two mutants with that of the wild type Raf-C1 showed that the kinetics are only slightly different, though the mutants both exhibit a decreased Raf activation as deduced from reporter gene assay.

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3C-19
DIFFERENT MODES OF ANNEXIN I BINDING TO PHOSPHOLIPID VESICLES IN THE ABSENCE AND PRESENCE OF CALCIUM

Olaf Zschörnig, Frank Opitz, Klaus Arnold

Annexin I belongs to a class of Ca^{2+}-binding proteins for which an involvement in exocytosis and in the coagulation processes are discussed. All these functions are related to the ability of annexins to bind to acidic phospholipids. Annexin I strongly binds to phosphatidylserine (PS) and phosphatidic acid (PA) large unilamellar vesicles (LUV) at acidic pH, at neutral pH only weak binding to PA and no binding to PS occurs. Addition of 40 μM Ca^{2+} leads to a strong binding to vesicles made of each lipid also at neutral pH. This tight binding of annexin I induces dehydration of the vesicle surface and a decrease of the lateral diffusion within the bilayer. Both effects are much greater at pH 4 than at pH 7.4. Annexin I promotes the Ca^{2+}-induced fusion of PA LUV by diminishing the Ca^{2+} threshold concentration at pH 7.4, whereas fusion of PS LUV is not affected. On the other hand, the permeability of small molecules encapsulated in PS and PA LUV is strongly modulated by annexin I and Ca^{2+} concentrations. It is concluded, that at neutral pH annexin I seems to bind two PA vesicles simultaneously however only one PS vesicle at the same time. At pH 4 annexin I induces fusion and leakage of either vesicles without Ca^{2+}. It is concluded, that in the absence of Ca^{2+} annexin I penetrates into the phospholipid bilayer at pH 4 contrary to the known location at the vesicle surface at neutral pH.

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3C-20
STRUCTURAL MODIFICATIONS ON FERRIC CYTOCHROME *C* INDUCED BY MEMBRANES INTERACTIONS: A CW-EPR STUDY OF HEME IRON

Maria R. Zucchi, Iseli L. Nantes, Adelaide Faljoni-Alário, Otaciro R. Nascimento

This work characterizes the influence of different phospholipid acyl chain and polar head groups in the cytochrome *c* with lipid membranes. The results are in agreement with two types of cytochrome *c*/phospholipids: interactions: electrostatic and a putative hydrophobic interaction resulting from the insertion of the acyl chain in cytochrome *c* hydrophobic channel. The hydrophobic interaction is modulated by the electrostatic interaction that is dependent from the charge and type of polar head group. The strong interaction of negative polar head group with cytochrome *c* favors the insertion of saturated and unsaturated acyl chain in its hydrophobic channel and leads to a spin state change from low to high spin and/or modified low spin forms In the presence of zwitterionic phospholipids only saturated acyl chains were able to induce spin form changes in cytochrome *c*. A strong and specific interaction of cytochrome *c* with bovine brain phosphatidylserine was observed. A characterization of the EPR spectrum of each spin species are presented and a partition distribution as a function of lipid/protein ratio are indicated. These systematic results helps to understand the apoptotic role and the oxidase/peroxidase activity of cytochrome *c* as associated with negatively charged liposomes. *Supported by CNPq, FAPESP and FAEP-UMC, Brazil.*

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3C-21
SIMULATION OF 7TM PROTEINS IN MEMBRANE ENVIRONMENT

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Theoretical structure prediction of membrane proteins remains a challenge in biophysical research. Using the GROMACS molecular dynamics package and force field membrane patches[1] of suitable size were constructed and equilibrated as a basis for the study of integral membrane proteins. For a 7TM vertebrate olfactory receptor of the rhodopsin class, OR17-40, a trial structure was taken from a GPCR database of Vriend[2]. In simulations the development of the model structure, a pure helix bundle with the loops omitted, towards an expected equilibrium structure in its natural environment was examined monitoring tilts, rotations, displacements, and deformation of the helices. The Bacteriorhodopsin trimer was reconstructed from the 1999 1.9 Å X-ray structure of Belrhali et al.[3]. After completing the termini the protein was inserted into a membrane patch and equilibrated. The resulting dynamic model is a promising basis to study properties that cannot directly be derived from the X-ray structure, e.g. water positions and dynamics in cavities, conformational changes of side chains and H-bonding patterns. Of particular interest are conformational changes in tertiary structure during the photocycle, and experimentally measured effects of site directed mutagenesis.

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3C-22
ISOLATION AND CHARACTERISATION OF A CHANNEL FORMING PROTEIN OF NOCARDIA FARCINICA

Franziska G. Rieß, Klaus P. Schaal, Roland Benz

A channel-forming protein was identified in cell wall extracts of the gram-positive, strictly aerobic bacterium *Nocardia farcinica*. The cell wall porin was purified to homogeneity and had an apparent molecular mass of about 87 kDa on tricine-containing SDS-PAGE. When the 87 kDa protein was boiled for a longer time in sodium dodecylsulfate (SDS) it dissociated into two subunits with molecular masses of about 19 and 23 kDa. The 87 kDa form of the protein was able to increase the specific conductance of artificial lipid bilayer membranes from phosphatidylcholine (PC) phosphatidylserine (PS) mixtures by the formation of ion-permeable channels. The channels had on average a single-channel conductance of 3.0 nS in 1 M KCl, 10 mM Tris-HCl, pH 8, and were found to be cation-selective. Asymmetric addition of the cell wall porin to lipid bilayer membranes resulted in an asymmetric voltage-dependence. The analysis of the single-channel conductance data in different salt solutions using the Renkin correction factor and the effect of negative charges on channel conductance suggested that the diameter of the cell wall porin is about 1.4 to 1.6 nm. Channel-forming properties of the cell wall porin of *N. farcinica* were compared with those of mycobacteria and corynebacteria. The cell wall porins of these members of the order *Actinomycetales* share common features because they form large and water-filled channels that contain negative point charges.

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3D-1
IMAGING AND DIFFUSION OF INDIVIDUAL FLUORESCENT FUSION-PROTEINS IN CELL MEMBRANES

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L-type calcium channels are voltage-gated, multi-subunit proteins involved in the regulation of calcium ion influx into muscle cells. To investigate the diffusion, conformational state and stoichiometry of these proteins in relation to their function, one has to study these channels on the single molecule level. Here we present results obtained by two complementary techniques: wide-field imaging and fluorescence correlation spectroscopy. We first describe the signal and mobility of the eYFP in cells and on membranes. To observe the calcium channels, the α_1 subunit is fused to the enhanced Yellow Fluorescent Protein (eYFP). Using wide field imaging, the diffusion of individual eYFP calcium channel fusion proteins was observed in the membranes of living cells. The analysis of a multitude of trajectories yielded a diffusion constant for the channels in cell membranes. With FCS we were able to measure diffusion of eYFP in buffer and in the membrane and the cytosol of living cells. The experiments shown clearly demonstrate the possiblity of studies on protein dynamics at the single molecule level with the use of GFP and are the first done on individual proteins (GFP) in a cell with wide-field epi-illuminescence

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3D-2
FLUORIMETRIC STUDIES OF STRESS INDUCED CHANGES OF THE LIPID ORDER PARAMETER IN VARIOUS REGIONS OF BLOOD CELLS PLASMA MEMBRANE BILAYER

Adrian Căplănusi, Gyöngyvér Katona, Diana Ionescu, Ervin Tanos, Laszlo Katona, Eugen Trutia, Eva Katona

Selecting appropriate probes, DPH (5 μM) and TMA-DPH (1.5 μM), we used continuous wave steady-state fluorimetry to monitor stress induced fluidity/lipid packing density changes in the core and in the polar headgroup regions of human megakaryocytic and/or lymphoid cells plasma membrane bilayer.
Decrease induced in the plasma membrane lipid packing density was reversible in 20–30 min following mild heat shock (5 °C rise of the suspension temperature for 10–30 min) and appeared to be irreversible in 40–60 min in case of severe heat shock (10 °C temperature rise for 15–60 min).
Effects induced by the inhibition of both aerobic and anaerobic metabolic pathways (2.5 mM NaCN and 2-deoxyglucose substitution for glucose) were more substantial, but even partial chemical ischemia (glucose deprivation) caused significant decrease of the lipid packing density. Partial recovery in lipid order was observed after withdrawal of the metabolic inhibitors.
Propranolol, a cardiovascular drug known to induce membrane-bound calcium release, caused decrease in the lipid order parameter in the polar headgroup region, while low-level laser irradiation with red (685 nm) and/or infrared light (830 nm) reversibly reduced the average lipid packing density in both the headgroup region and in the core of the plasma membrane bilayer.

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3D-3
STUDIES ON THE HEMOLYSIS OF HUMAN ERYTHROCYTES INDUCED BY RADIATION

A. Hategan, D. Martin, A. Popescu, C. Oproiu

The effects of 5 MeV accelerated electrons (0 °C) and of 2.45 GHz microwaves (−196 °C) at 0-850 W power levels, as well as preliminary results of the combined treatment, on the osmotic fragility of human erythrocyte membranes are presented. The changes in the properties of the erythrocyte membranes were estimated by measuring the radiation induced haemoglobin release from the erythrocytes and the osmotic fragility of the membranes, determined by postirradiation induced osmotic stress. We obtained no hemolysis induced by accelerated electrons in the range 0–400 Gy, whereas the microwave irradiated erythrocytes showed in the ranges (1–2 min) and (400–500 W) values of very small hemolysis, down to 50% from the control. The osmotic stress experiments indicated a significant increase in osmotic fragility for 200–400 Gy electron doses, whereas the 100 Gy irradiated sample showed a hemolysis down to 35% from the control. Similarly, the microwave irradiated erythrocytes showed values down to 60% from the control for (1 min, 850W). Both radiations induced at definite parameters values of very small hemolysis, suggesting a stabilisation of the membranes and an increase in osmotic resistance. Our preliminary results on simultaneous irradiation of the frozen erythrocytes seem to indicate a significant contribution of microwaves in hemolysis evolution, while the successive irradiation procedure did not allow so far a clear interpretation, further studies being necessary to elucidate the membrane molecular mechanisms induced.

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3D-4
IN VITRO STUDIES OF CHEMICAL HYPOXIA/ISCHEMIA EFFECTS ON EARLY SIGNAL TRANSDUCTION EVENTS, AS SEEN IN VARIOUS HUMAN BLOOD CELLS

Gyöngyvér Katona, Adrian Căplănusi, Adrian Onu, Cristiana Matache, Maria Stefănescu, Eugen Trutia, Eva Katona

Wavelength-domain steady-state fluorimetry in association with electrophoresis/immunoblotting were exploited to investigate early biophysical/biochemical events involved in cellular signal transduction. Using appropriate molecular probes (Fura2-AM, 1.2 μM) to monitor calcium signalling and polyclonal antiphosphotyrosine antibodies to detect tyrosine-phosphorylated proteins, we studied characteristics of thrombin and/or antigen receptor activation in human megakaryocytic, myeloerythroid and/or lymphoid cells, as well as consequences of chemical hypoxia/ischemia therein.
We compare resting calcium levels and calcium signals induced by serine protease activated receptor stimulation with various thrombin doses (50 pM-500 nM), as well as effects of partial (induced by oxidative phosphorylation block by cyanide or glucose deprivation alone) and quasitotal chemical ischemia (induced by the inhibition of both aerobic and anaerobic metabolic pathways) in various human blood cells. Cross-modulation of thrombin and antigen receptor signalling in Jurkat T cells, and protective effects during chemical ischemia of low concentration of thrombin, as well as of prior mild ischemic insults applied to cultured cells are documented.
The relation of these findings to posssible in vivo mechanisms of ischemic damage and induced ischemic tolerance is discussed.

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3D-5
CYCLIC LIPO-DEPSIPEPETIDES INDUCED FLUIDITY CHANGES IN LIPID MOIETY OF RED BLOOD CELLS. A SPIN LABEL SDUDY

Zsófia Szabó, Pál Gróf, Katalin Blaskó

It is well known, that the plant pathogen cyclic lipo-depsipeptides, produced by *pseudomonas syringae pv. syringae*, change cationic transport processes in different cell membranes .Importance of the cyclic lipo-depsipeptides relys on its fungicid, plant-pathogenic effects. Molecular level studies with radioactive isotopes have proven that its effect is a consequence of the pore formation, which presumably involves formation an ensembly of five/six molecules of the given lipo-depsipeptide molecules . In earlier studies, from our laboratory, it has been shown, that the time course and the extent of the inactivation of pores depends on the given derivative of a cyclo lipo-depsipeptide and on the temperature. To understand the molecular level changes provoked by the cyclic lipo-depsipeptides, we have undertaken a EPR study, to detect the membrane fluidity in native and treated red blood cells.
The methods of the EPR spectroscopy allows detection of the membrane fluidity at different depth of the membrane. Spin labeled fatty acid derivatives can monitor the cell membrane either close to the hydrophilic head, or at the middle- and the end of the hydrocarbone chains.
Our results show that syringopeptine cause an increase in the membrane fluidity, both measured close to the hydrophilic head and at the middle of the hydrocarbone chain; the extent of this altered fluidity was found to be greater at the middle than close to the hydrophil head region.

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3D-6
STATIONARY AND DYNAMIC DIELECTRO-DEFORMATIONS OF ERYTHROCYTE MEMBRANE

Vadim L. Kononenko, Jaunius K. Shimkus

The elongation of erythrocytes in low-conductivity isotonic medium under high-frequency (1-2 MHz) electric field, termed dielectrodeformations (DD), is studied theoretically and experimentally. The theory based on ellipsoidal approximation for cell shape considers field-induced bending deformations of lipid bilayer and shear deformations of underlying membrane skeleton. Depending on possible inter-relations between these deformations in erythrocyte or in lipid vesicle membrane, various deformation patterns are analyzed. The influence of mechanical, electrical, and geometrical parameters of erythrocyte on various parts of its stationary elongation curves, field-jump response curves, and field-amplitude-harmonic-modulation response curves is elucidated. The saturation of stationary cell elongation, and monotonic decrease of its viscoelastic response time at high field strengths are explained and calculated, in accordance with experiment. The saturation part of elongation curve is shown to be the measure of cell (vesicle) volume, while the field-dependent elongation is very sensitive to internal/external conductivity ratio. The theory fit to experimental data measured and available in literature gave field-dependent relaxation time of erythrocyte DD, and temperature dependent values of erythrocyte volume and cytoplasm conductivity in the range −15 °C to 25 °C. The conductivity is substantially smaller then the physiologically normal value, due to the ions efflux from the cell into low-conductivity medium.

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3D-7
MEMBRANE MECHANICAL PROPERTIES AND RED BLOOD CELL DEFORMABILITY

Drago Kuzman, Marjan Gros, Sonja Vrhovec, Sasa Svetina, Bostjan Zeks

It is known that in the blood flow the shape of red blood cell (RBC) is changed. The ability to undergo deformation during passage through the microvasculature is critical for the RBC to optimally perform its function of oxygen delivery. A deformation response of the RBC to fluid forces is a complex phenomenon that depends on a number of different cell characteristics, which include membrane material properties, cell geometry and cytoplasmic viscosity.
We present experimental study of a possible influence of various chemical agents on a RBC deformability. To quantify a change of deformability caused by chemical modification of the cell membrane we measured several cell parameters before and after modification: mean cell volume, mean cell surface, cytoplasmic viscosity and the elongation of RBCs subjected into shear flow in a transparent cone-plate rheoscope. Measured data were analysed by a simple mathematical model of the RBC deformation in a shear flow. As a result we are able to estimate the effect of the modification of the cell membrane on the ratio of the cell volume to the cell surface area, on the cytoplasmic viscosity and on the membrane material properties which are quantified by relevant elastic moduli. Relative importance of the contribution of the local and nonlocal membrane bending and of the membrane skeleton shear elasticity is evaluated.

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3D-8
THE ROLE OF CELL DEFORMABILITY IN THE ELECTROPORATION INDUCED MEMBRANE PERMEABILITY

Heiko Mussauer, Vladimir Sukhorukov, Ulrich Zimmermann

Membrane charging and electroporation caused by high-intensity electric field pulses are preceded and accompanied by transient electrodeformation forces (caused by the Maxwell stress) which affect the degree of the plasma membrane permeabilization in response to a breakdown pulse. In low-conductivity media the transient stretching force on cells in the field direction assumes its maximum value. This facilitates electroinjection of xenomolecules into mammalian cells and also cell electrofusion. We studied here the electroporation of human erythrocytes (RBCs) whose deformability was modified by the depletion of the extracellular calcium. The interaction of RBIs with the cheating agent DTPA led to the following, obviously interrelated phenomena: 1) DTPA protected RBCs against electrohemolysis; 2) The inhibition of electrohemolysis was observed only in low-conductivity solutions; 3) The uptake of DTPA by RBCs was relatively low; 4) DTPA reduced markedly the deformability of cells, as revealed by the electrodeformation experiments using AC electric fields. The data indicate that stiffer cells are more resistant to electric field exposure. The observed effects of DTPA might have an origin in molecular changes of the bilayer or in the membrane-coupled cytoskeleton, induced by alternations of the ionic equilibrium (e.g. Ca^{2+} sequestration) in the vicinity of the cell membrane.

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3D-9
NANOMETER-SCALE HETEROGENEITY OF THE FIBROBLAST CELL MEMBRANE

Arnd Pralle, J.K.Heinrich Hörber, Kai Simons, Ernst-Ludwig Florin

The kinetics of the initial events in cell signaling, such as receptor clustering, and in endocytosis are influenced by the lateral mobility of proteins in the plasma membrane. The mobility is determined by the lateral heterogeneity, expected to be in the several hundred nanometer range, and local viscosity of the membrane, but details are largely unknown.
Here we present for the first time quantitative images of lateral interaction potentials and membrane viscosity with nanometer spatial resolution. Typical structures found are a few hundred nanometer apart and not continuous, and have energy barriers higher than the experimental assessable energy (six times the thermal energy). In areas of free Brownian motion, the lateral mobility agrees with the one measured in synthetic lipid bilayers and derived theoretically.
We applied a novel type of near-field microscopy, which uses a single membrane protein or macromolecular complex, such as a glycosphingolipid-raft as a local probe. On fibroblasts, the obstacles are spaced such that single rafts diffuse over the cell surface, while clustered rafts are more confined, which might be important for cell signaling.

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3D-10
TIME-RESOLVED FLUORESCENCE SPECTROSCOPY OF INTRACELLULAR CHANGES IN ION CONCENTRATION DURING INDUCED APOPTOSIS WITH STAUROSPORINE IN EL4-CELLS

Bernd Saeume

The aim of our study was to improve the understanding of the role of intracellular ions like Ca^{2+}, Na^+, K^+ in the process of apoptotic cell death in EL4-cells. The primary factor that controls volume regulation in most cells is the Na^+/K^+-ATPase with a netto flux of K^+ across the plasmamembran. Cell shrinkage is a major characteristic of apoptosis. $[Ca^{2+}]_i$ appears to be a major link and signalling event.
Fluorescent ion sensitive indicators were used to observe changes in $[Ca^{2+}]_i$ (Fura-2), $[K^+]_i$ (PBFI) and $[Na^{2+}]_i$ (SBFI). The EL4-cells were immobilized in small threads of agarose. Apoptosis was induced by 500 µM Staurosporine. The maximum rate of apoptotic cells, ca. 80%, determined by Annexin V-Fluorescein labelling, were reached after 6–8h. The Perfusion with Dulbecco's modified eagle medium (10% CO_2) permit stable control conditions in cuvette up to 24h with normal growth of the cells. Changes in cell volume were measured with laser scanning microscopy by R. Jessel in our Department.
Our data showed a correlated increase of $[Na^+]_i$ and decrease of $[K^+]_i$. The dynamics in changes of $[Na^+]_i$ and $[K^+]_i$ corresponded to changes in cell volume. For $[Ca^{2+}]_i$ we found a fast increase. Measurements with extracellular applied Ca^{2+}-changes with Ca-chelators like EGTA should elucidate if the $[Ca^{2+}]_i$-elevation is due to Ca^{2+}-store depletation.

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3D-11
SINGLE PARTICLE TRACKING OF A G PROTEIN COUPLED RECEPTOR ON LIVING CELLS REVEALS LARGE TIME SCALE CONFINED DIFFUSION

Frédéric Daumas, Stéphanie Ducasse, Ma Corbani, André Lopez, Laurence Salome

Single particle tracking is a powerful tool to relate the organization and dynamics of the membrane constituants to the functions accomplished at the plasma membrane. We adress this question in the case of the µ opioid receptor which belongs to the large family of the G protein coupled receptors involved with other partners (G protein and effector) in a signal transduction pathway.
NRK fibroblast cells were stably transfected to express the receptor coupled to a T7-Tag at its N-terminus which allows for its specific labelling by colloidal gold coupled to a monoclonal anti T7-Tag antibody. The lateral movements of the particles (40 nm diameter) were followed at 40 ms time resolution during 2 min with a precision in the particle position determination of 15 nm. Approximately 40% of the receptors show pure confined diffusion restricted to $\approx$ 600 nm domains. The behavior of an other 40% of the receptors is well described by the summation of an equivalent pure confined diffusion and a slow random diffusion. The microscopic diffusion coefficients of the receptors found in these two populations are larger than those of the last 20% which present a simple slow random diffusion behaviour. These results are in favour of the existence of barriers to the diffusion of this membrane receptor, they give also a clear indication of their dynamic character.

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3D-12
CHANGES IN THE PLASMA MEMBRANE STRUCTURE OF YEAST CANDIDA UTILIS INDUCED BY ACRIFLAVINE

Ezzatollah Keyhani

Acryflavine (Acr) has been used for mitochondrial mutagenesis in yeast. In this paper we report that, besides changes in mitochondria structure, Acr produced severe alterations in the plasma membrane (PM) of Candida utilis hitherto unreported for any PM. C. utilis was cultured in the presence of various concentrations of Acr (2 to 14 µg/ml). Freeze fracture (FF) and thin section of the cells were done according to [1] and [2]. FF of untreated yeast cells exhibited numerous linear depressions (grooves) in the PF face which were elevations in the EF face. Acr treated yeast PM showed polymorphic alterations. In some cells, the PM showed only a length extension of the linear grooves forming a reticulum at their surface. Other cells exhibited a deep depression all over the PM surface. However, no aggregation of intramembrane particles was found. Thin section of permanganate fixed cells allowed for the determination of the depth and other dimensions of the damages. These varied between simple, roughly circular, plasma membrane depressions 75 to 500 nm deep, to depressions 2.8 µm wide and 2.6 µm deep. In all cases the depression area was filled with cell wall material. Thus besides being a mitochondrial mutagen, Acr also produced large scale alterations of the PM, much more important than those reported for ethidium bromide [3,4].
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3E-1
CROSS-LINKING VESICLES BY INTEGRIN/RGD INTERACTION – A MODEL SYSTEM FOR THE STUDY OF CELL ADHESION

M. Bärmann, B. Hu, Z. Guttenberg, J. Böhm, E. Sackmann

Cell adhesion is governed by a complex interplay of adhesive and repulsive forces. The interaction of integrins with extracellular ligands is a major factor of determining the specificity and strength of adhesion. Integrin $\alpha_{IIb}\beta_3$ was prepared from human blood platelets and reconstituted into phospholipid vesicles. The RGD-containing cyclic hexapeptide, c[Arg-Gly-Asp-D-Phe-Lys-Gly-] has been shown to be highly specific for integrin $\alpha_{IIb}\beta_3$ with a relatively high K_D of 1.5 μM when bound to surfaces through a 2.2 nm spacer. The cyclic compound was coupled to a dimyristoylthioglyceryl anchor and reconstituted in phospholipid vesicles. In a mixture of the two types of vesicles, the integrin molecules on the one side, and the cRGD-lipid molecules on the other, are free to move in the 2-dimensional fluid of phospholipid membranes so as to optimize the intermolecular array of their binding complexes. It is shown by fluorescence microscopy and cryo electron microscopy that integrin reconstituted into phospholipid vesicles binds to vesicles decorated with the cRGD-lipopeptide, forming regularly spaced bridges between the two kinds of vesicles. Thus, adhesion is accomplished by multivalent binding overcoming the high K_D. With the novel lipopeptide one can study of the self-organization of integrin clusters and focal adhesion complexes. Also, it allows the measurement of binding strength to RGD either of isolated and reconstituted integrin, or of whole cells.

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3E-2
FORCE MEASUREMENTS AT SINGLE FOCAL ADHESIONS USING ELASTIC MICRO-PATTERNED SUBSTRATES

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We have developed a novel method for the quantitative measurement of the force applied by individual focal adhesions to the substrate. The method combines the micro-patterning of elastomer substrates with fluorescence imaging of the focal adhesions by GFP-tagged adhesion molecules. The forces are extracted from the distortions of the regular pattern using elasticity theory. We were able to measure, for the first time, the force exerted at a single focal adhesion, and show that its magnitude and direction correlate with the dimension and orientation of the focal adhesion. By blocking acto-myosin contractility, using certain drugs, we were able to monitor the time dependence of the disruption of adhesion and the relaxation of the cellular contractile force. The results bring evidence for correlation between force and structure, at the level of a single focal adhesion.

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3E-3
THE TEMPERATURE DEPENDENCE OF HUMAN BLOOD PLATELET AGGREGATION

Vladimir P. Berest, Sergiy V. Gatash

Despite large number of papers concerning the effect of temperature on platelet aggregation the mechanism explaining platelet hyperaggregation at room temperatures is not clear. By means of light scattering, UV- and dielectric spectroscopy methods we have studied the influence of temperature in range from 4 to 40 °C on platelet aggregation and structural changes of platelet membrane and blood plasma proteins. It has been shown, that maximal aggregation induced by such physiological agonists as thrombin, epinephrine and ADP was observed at 18–22 °C. These agonists provide common fibrinogen dependent pathway of platelet aggregation. For non physiological agonist ristocetin aggregation decrease with rise in temperature. Structural transitions in platelet plasma membrane and cytoskeletal proteins occur at 18–20 and 30–32 °C. Also at 20 °C conformational transition, which is accompanied by hydration change, takes place in fibrinogen molecule known as a main cofactor of platelet aggregation. We suppose that the increase of platelet aggregation at temperatures 4–20 °C is defined by natural Arrhenius rate constant increase, and platelet aggregation decrease at temperatures above 20 °C is due to the conformational changes observed in platelet membrane and cytoskeletal proteins and in blood plasma cofactor (fibrinogen) at temperatures about 20 °C.

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3E-4
DISTRIBUTION OF THE CELL ADHESION MOLECULE LFA-1 PROBED BY ATOMIC FORCE MICROSCOPY

A. Cambi, F. De Lange, R. Huijbens, Y. van Kooyk, C. Figdor

Lymphocyte function-associated molecule 1 (LFA-1; CD11a/CD18) is a member of the β_2 group of integrins and it is exclusively expressed by leukocytes. LFA-1 is involved in a broad range of adhesive interactions and signaling events in the immune system. It mediates adhesion by binding to one of its three cellular ligands: ICAM-1, -2 and -3. Although extensively studied, the precise mechanism that controls LFA-1-mediated ligand binding remains elusive. Models propose two distinct mechanisms in LFA-1-mediated adhesion: alteration in the binding affinity, resulting from a conformational change, and avidity alterations, caused by clustering of LFA-1 molecules on the cell surface. To get more insight in the surface distribution of LFA-1 and its organization, we employed confocal microscopy, electron microscopy and also atomic force microscopy (AFM). The localization of LFA-1 on the plasma membrane of transfected mouse fibroblasts (L-cell) was detected by AFM, using antibody labeled colloidal gold beads. Employing 10 nm beads (with or without silver enhancement) we were able to quantitate the number of LFA-1 molecules on cells as well as on ventral plasma membranes of cells grown on different substrates (fibronectin, ICAM-1, -2, -3). Our results demonstrate that colloidal gold beads are extremely useful to visualize individual LFA-1 molecules on cells. We aim to employ this technique to get insight on the dynamic behavior of LFA-1 on single living cells.

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3E-5
COMBINED MEASUREMENT OF CELLULAR ADHESION AND ACIDIFICATION ON A SILICON SENSORCHIP

R. Ehret, W. Baumann, M. Brischwein, I. Freund, M. Lehmann, S. Drechsler, B. Wolf

Impedance measurements on cellular systems with Interdigitated Electrode Structures (IDES) have shown to be an effective way for on-line monitoring of cellular behavior [1, 2]. This sensor signal is influenced by changes in number, growth and morphological behavior and is mainly due to insulating effects of the cell membranes, which force the electrical current to bypass them. Additionally, the cell membrane may act as a diffusion barrier to metabolites excluded from the cells. Studies with cells growing directly on Ion Sensitive Field Effect Transistors (ISFETs) suggested a decreased pH in the volume between the silicon substrate and the cell membrane compared to the bulk solution [3]. This pH-shift showed to be dependant on the adhesive properties of the cells. Therefore, combined experiments of the cellular adhesion and acidification with a silicon sensor chip with both IDES and ISFET sensors were performed to elucidate the cellular behavior.

[1] Ehret R. et al. (1998): Medical & Biological Engineering & Computing 36, 365–370
[2] Ehret R. et al. (1997): Biosensors & Bioelectronics, 12 1, 29–41
[3] Baumann W. et al. (1999): Sensors & Actuators B 55, 77–89

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3E-6
EVALUATION OF DIFFERENT MICROSCOPY METHODS IN STUDYING CELL ADHESION ON SOLID SURFACES

Yoriko Iwanaga*, Peter Geggier†, Günter Fuhr†, Peter Fromherz*

According to the classical measurement with interference reflection microscopy (IRM), the cells are found to approach the substrate down to around 10 nm at focal adhesion contacts. We have compared IRM pictures with three other microscopic techniques: cyan fluorescence protein (CFP) tagging of vinculin to localize focal contacts in situ, fluorescence interference contrast (FLIC) microscopy to determine the absolute distance between the lower membrane and a substrate of oxidized silicon and total internal reflection aqueous fluorescence (TIRAF) microscopy to obtain a relative topography of the cell-substrate separation on a glass.
The dark patches in IRM images known as focal adhesion contacts matched the expression pattern of CFP-vinculin. However, FLIC and TIRAF microscopy showed that fibroblasts cultured on fibronectin maintain a rather homogeneous gap of 50 nm between the lower membrane and the substrate. We did not find focal adhesion contacts with a reduced cell-substrate gap. Based on the distance maps of FLIC microscopy and relative distance maps from TIRAF images, we simulated optical multilayer systems consisting of glass, medium, lower membrane, cytoplasm and upper membrane to describe the IRM images by varying optical parameters of the model.

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3E-7
THE INVESTIGATION OF CELL ADHESION PATTERNS WITH TIME RESOLVED TOTAL INTERNAL REFLECTION AQUEOUS FLUORESCENCE (TIRAF) MICROSCOPY

Peter Geggier, Magnus Jäger, Günter Fuhr

The establishment of regular cell adhesion patterns arising during the approach of the lower cell membrane to the substrate is still unclear. Experiments have been done with Interference Reflection Microscopy (IRM) but image interpretation of this technique is in part ambiguous and the vertical z-resolution in the range from 0 to 30 nm is low. In order to study pattern formation and dynamic behaviour of the adhesion topography on solid surfaces we have developed a time resolved TIRAF microscope. It possesses a high vertical z-resolution in the range from 0 to 150 nm and a lateral resolution close to the classical limit of 237 nm.
Long term measurements (up to 11 h) on fibroblasts with a time resolution from 1 to 5 min demonstrate the mechanics of pattern growth. Here, a nearly homogeneous topography in the beginning is transformed to a more structured pattern composed of elementary adhesion clusters (round, linear, branched and ring clusters). Interactions between these substructures can result in a realignment of adhesion contacts. In fibroblasts cultured over 24 h we have found highly active lamellipodia-like structures existing in the gap between the lower cell membrane and the substrate.
Short term measurements over several minutes with a time resolution of about 1 s demonstrate oscillatory behaviour of single adhesion clusters. The duration of these lateral fluctuations is in the order of 15 s.

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3E-8
ULTRAWEAK SUGAR INTERACTIONS FOR TRANSIENT CELL ADHESION

Christine Gourier, Frédéric Pincet, Tugdual Le Bouar, Eric Perez, Yongmin Zhang, Jacques Esnault, Jean-Maurice Mallet, Pierre Sinay

All living organisms are characterised by the presence of glycoproteins and glycosphingolipids on the cytoplasmic membranes. Their carbohydrate chains have firmly been recognized as interaction sites in cell adhesion processes. Such events are commonly attributed to lectine or lectine like protein and the corresponding specific carbohydrate ligands. It is only recently that some carbohydrate-carbohydrate interactions found in biological processes have been suspected to be specific. One of these carbohydrates, the $Lewis^X$ determinant, has been shown to be involved in murine embryogenesis. In vitro experiments suggested that a Ca^{-+}-mediated homotypic interaction between the $Lewis^X$ determinants may drive cell adhesion. We have confirmed the existence of this specific interaction in a physicochemically well defined system and in conditions similar to the natural environement of the glycolipid, i.e. lipid bilayers, by reporting direct quantitative measurements. The adhesion between geant vesicules functionnalised with $Lewis^X$ was obtained by micromanipulation and contact angle measurements. The specific calcium mediated interaction is found to be well below the thermal energy. Sufficient to promote cell-cell adhesion, this adhesion is ultralow and is therefore difficult to measure. Such small interactions explain why the concept of specific interactions between carbohydrates is often neglected.

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3E-9
CELL ATTACHMENT STUDIED BY FLUORESCENCE INTERFERENCE AND GFP-TAGGING

Yoriko Iwanaga, Peter Fromherz

We have investigated cell adhesion of primary rat hippocampus neurons and mouse fibroblasts cultured on a silicon chip. The cell-substrate distance was measured using fluorescence interference contrast (FLIC) microscopy[1-3]. To localise biochemically defined focal adhesion plaques, we have fused the green fluorescence protein GFP to the N-terminus of vinculin. The actual positions where the cells anchor to the extracellular matrix protein were marked by fusing GFP to the N-terminus of integrin ß1 in its extracellular domain. A topographical image of cell-substrate distance obtained from FLIC microscopy was compared with the fluorescence image of GFP-tagged proteins using confocal microscopy.
In fibroblast cells, vinculin was recruited into strong stripes when cultured on fibronectin-coated substrate. These aggregations induced membrane raffling parallel to the stripes but were not exactly at the position of close adhesion. In the neuronal primary culture, distance measurement showed mostly smooth attachment of cell membrane to the substrate (laminin:100nm, fibronectin:50nm). Accordingly,vinculin expression and recruitment were at lower level. The expression of integrin ß1 subunit showed the anchorage sites as dots in various sizes. These point contacts did not induce close adhesion of the membrane to substrate in neuronal culture.

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[2] D. Braun and P. Fromherz (1997) Appl. Phys. A 65:341.
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3E-10
LFA-1 DISTRIBUTION FROM A NEAR-FIELD POINT OF VIEW

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The integrin LFA-1 (lymphocyte function-associated molecule-1) is a transmembrane protein expressed on the cell membrane and serves as a mediator between the extracellular environment and the cytoskeleton, e.g. in cell adhesion and cell-cell communication processes. It is well known to play a crucial role in many signaling mechanisms in immunology. LFA-1 mediated adhesion has been proposed to involve a conformational change in the protein (affinity regulation), as well as clustering of many LFA-1 molecules at adhesion-contact sites (avidity). Here, we apply near-field scanning optical microscopy (NSOM) to study LFA-1 distribution in a mouse fibroblast cell-line exogenously expressing a human-LFA-1*GFP fusion protein. We obtained ~70nm resolution optical images of the cell surface showing the signature of single Green Fluorescent Protein (GFP) fluorescence, i.e. dynamic on-off behavior, characteristic photon counts and distinct polarization characteristics (see Garcia-Parajo et al. 1999, *Cytometry 36*, 239-246). By labeling LFA-1 with fluorescent antibodies and using multiple-color excitation schemes we show that the GFP signal indeed represents single LFA-1 molecules. Finally, LFA-1 distribution characteristics, obtained from image analyses (Fourier filtering, 2-D autocorrelation functions), will be presented.

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3E-11
THE GLYCOLIPID INTERACTION AS A MODEL FOR CELL ADHESION

Barbara Lorz, Christian Gege, Erich Sackmann

To study the physical properties of the cell-tissue interaction a model system is established consisting of DMPC giant vesicles and solid supported bilayers. We focus on lock and key interactions of glycolipids which have a natural concentration of 5% in animal plasma membranes. Two kinds of glycolipids have been synthesized which are called Sialyl-Lewis X (sLeX) and Lewis X (LeX). Their functional headgroups consist of three cyclic sugars. The Sialyl-Lewis X contains an additional charged sialic acid. These headgroups are linked to two alkyl chains (length 16 C) with a lactose spacer. Due to these chains they can easily be embedded into a matrix consisting of DMPC and cholesterol. LeX is a self-recognizing molecule while the charged sLeX requires a crosslinker to bind to itself - the lectin. It is also possible to bind the sLeX to surface grafted selectin proteins. The adhesion of these vesicles is observed by reflection interference contrast microscopy (RICM).
After the characterization of the binding properties of the above described systems the influence of a nonequilibrium is shown. For this purpose a hydrodynamic flow field from the side is used to mimick the shear field caused by the blood flow in the vessel. The reaction of the system to the shear stress is characterized by RICM.

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3E-12
MEASURING LIGAND-RECEPTOR UNBINDING FORCES WITH MAGNETIC BEADS

Stefanie Marx, Zeno Guttenberg, Robin Bruinsma, Erich Sackmann

A method to measure the forces between receptor-ligand pairs in adhesion domains is presented. It relies on the micro-interferometric analysis of adhering vesicles subjected to an external force. To this end a magnetic bead is attached to the vesicle membrane and exerted to an inhomogeneous magnetic field. By observing the resulting force dependent deformation of the vesicle adhesion area the forces acting on receptor-ligand pairs can be deduced from the classical elasticity theory.
We investigate the binding strengths between solubilized solid supported integrin receptors $\alpha_{IIb}\beta_3$ of blood platelets and cyclic hexapeptide ligands containing arginine-glycine-aspartate. The hexapeptide ligand is imbedded in the vesicle and recognized selectively by the integrin receptor. The integrin receptors are immobilized via a biofunctional chain on single gold nanobeads in order to restrict the area and thereby the number of receptors mediating the adhesion. We find that the unbinding of the hexapeptide integrin bonds takes place at force levels far below those found in comparable single-molecule studies. We suggest interpreting our findings in terms of the relative effectiveness of torque over traction in fracturing ligand-receptor bonds.

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3E-13
SPECIFIC ADHESION: FROM MACROSCOPIC SURFACES TO SINGLE BONDS

Frédéric Pincet, Eric Perez

We present a general framework enabling the study of molecular recognition forces and energies. Surfaces bearing recognition molecules adhere in a so specific way that even a very small modification made in a key part of the molecule will substantially change the surface forces and adhesion. Two kinds of molecular recognitions will be investigated : complementary bases of DNA and oligosaccharides.
Mechanical measurements on lipids functionnalized with nucleosides or oligosaccharides allow to obtain weak binding energies as low as 0.2 k_BT. These results can be confirmed by balancing the non-covalent bond with a controlled electrostatic repulsion. We have done such measurements performed between lipid bilayers with a Surface Forces Apparatus and with vesicles. The known self-assembly properties of nucleosides ("π-stacking") can influence the bidimensional arrangement of layers made of these lipids. X-ray diffraction at grazing incidence on liquid-expanded monolayers of these lipids shows that a translational order can take place although monolayers in this state are known to be bidimensional liquids and therefore affect the resulting surface interaction.

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3E-14
SWELLING BEHAVIOR AND ELASTIC PROPERTIES OF POLY(2-OXAZOLINE) BRUSHES GRAFTED ON SOLID SURFACES

Florian Rehfeldt, Lorena Pagnoni, Motomu Tanaka, Rainer Jordan

The grafting of biocompatible polymers onto solid surfaces attracts attention for biological and clinical applications because they can prevent nonspecific adsorption and denaturing of proteins. In the present work, poly(2-oxazoline) with terminal surface coupling silanol groups and lipophilic alkyl moieties (lipopolymers) have been synthesized. The influence of the polymer side chain, polymer chain length and effect of the balance between the hydrophilic polymer chain and the lipophilic alkyl moiety on the morphology of the stratified systems were studied.
These polymers were grafted onto thermally oxidized silicon wafers and the swelling behavior was systematically studied by ellipsometry. The thickness of the grafted films could be measured as a function of the relative humidity of the air. The forces operating within the film can be analyzed in terms of disjoining pressure, which relates to the change in the chemical potential of water. Viscoelastic properties of the polymer film were studied by applying a colloidal probe technique based on latex beads. By analyzing the vertical Brownian motion of the beads with reflection interference contrast microscopy (RICM), the interfacial interaction potential could be determined.

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3E-15
SWELLING AND PHASE BEHAVIOR OF GLYCOLIPIDS

Matthias Schneider, Motomu Tanaka, Gerald Mathe, Christian Gege, Richard R. Schmidt, Erich Sackmann

There is a growing interest on oligosaccarides as a mediator in cell-cell recognition or interaction with toxins and viruses. Furthermore they can provide a soft "cushion" between tissue-forming cells because of their significant swelling behavior. Although such phenomena have been widely studied, only little is known about the interaction mechanism on a molecular-level.
In the present study, lipids covalently attached to lactose oligomers (the number of lactose units, N = 1, 2, 3) were synthesized as simple model molecules. From calorimetry (DSC) and Langmuir isotherms of lipid monolayers, we were able to derive thermodynamic and structural parameters. In order to understand the forces acting between headgroups, swelling behavior of the lactose oligomers was studied. The glycolipid monolayer was transferred onto hydrophilic surfaces of thermally oxidized silicon wafers, then thickness of the saccharide layers was determined as a function of disjoining pressure by using ellipsometry under controlled relative humidity.
For lipids with short head groups, the isotherms are very similar to those of phospholipids. As the head group length increases, the entropic interaction between head groups can be observed. Although this lipids behave like phospholipids at the air/water interface, they swell like lipopolymers, which has been interpreted by using a self consitent field approach.

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3E-16
ELASTIC INTERACTIONS OF CELLS

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Adhering and locomoting cells can exert forces in the nano-Newton range at sites of focal adhesion. Forces in biological systems often have structural functions (like remodeling of the connective tissue by fibroblasts), but there is also evidence for a role in signal transduction (e.g. during development). We study theoretically the elastic interactions of typical cellular force patterns which have been measured with the elastic substrate method. We consider interactions mediated both by elastic substrates and by three-dimensional gels as models for basal laminae and (reconstituted) tissues, respectively. Our analysis includes predictions for structure formation of a small number of cells and the effect of boundary conditions (that is tissue shape).

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3E-17
KINETICS OF CELL SPREADING MONITORED BY ELECTRIC CELL-SUBSTRATE IMPEDANCE SENSING

Joachim Wegener, Charles R. Keese, Ivar Giaever

Electric cell-substrate impedance sensing (ECIS) is a novel experimental technique to monitor the attachment and spreading of mammalian cells on artificial surfaces in a highly quantitative and computer-controlled manner. The method is based on measuring changes in the electrical impedance of small gold film electrodes (d = 250 μm) deposited on a culture dish by means of photolithography and used as growth substrate.
Based on experimental data and theoretical considerations we demonstrate that impedance readings above a threshold frequency of 10 kHz return a direct measure for the degree of cell spreading on the electrode surface. Interpretation of ECIS data was validated by means of established microscopic techniques.
The excellent time resolution of the ECIS device allows an in-depth analysis of cell-spreading kinetics. From ECIS data we extracted both the time necessary to achieve half-maximum cell spreading and the apparent spreading rate for various experimental conditions. For instance, we compared the attachment and spreading of epithelial MDCK cells on different protein coatings and investigated the impact of divalent cations on spreading kinetics. We quantified the inhibitory effect of soluble peptides that mimic the recognition sequence of fibronectin (RGDS). We also applied the ECIS technique to monitor the detachment of confluent fibroblastic cell layers by means of these peptides.

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3E-18
GIANT LIPID VESICLES ON SILICON PROBE STERIC REPULSION BY LAMININ IN CELL ADHESION

Günther Zeck, Peter Fromherz

In cell adhesion a cleft of 110 nm separates a surface coated with the protein laminin from neuronal membranes [1]. To decide wether the repulsion is due to a specific interaction with membrane receptors or to an unspecific physical effect of laminin, we studied the adhesion of giant lipid vesicles.
Using fluorescence interference-contrats microscopy we found an average distance of 160 nm on laminin as compared to 50 nm on albumin. The enhanced distance on laminin indicates an additional repulsion. We can exclude a larger range of the undulation force, which is responsible for the 50 nm on albumin [2], from evaluation of the profile at the adhesion rim.
We propose that in contact to electrolyte the adsorbed laminin forms a diluted layer of dangling molecules with an average thickness of 110 nm. The surface of that cushion restricts the undulations of the membrane and gives rise to a renormalized repulsive force.
We suggest that the steric effect of laminin is effective also in cell adhesion: a few molecules interact specific with membrane receptors, whereas all unbound molecules keep the membrane apart from the substrate.
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3E-19
INVESTIGATIONS OF CELL TRACES USING AN ATOMIC FORCE / INTERFERENCE REFLECTION MICROSCOPE

Heiko Zimmermann, Ines Westphal, Ulrike Rehn, Günter Fuhr

Since the first detailed description of the highly ordered material released by migrating adherent cells on artificial surfaces [Fuhr et al. (1998) *Biol. Chem.* 379:1161], this research topic has become of special interest for biophysics of cell migration and the characterisation of artificial surfaces. We present studies of these submicrometre structures with different methods using, particularly, non-contact atomic force microscopy combined with interference reflection microscopy. This combination allows fast orientation and high-resolution *in situ* mapping of topological and topographical features and the adhesion patterns of these traces. Typical features are characteristic branches with clearly defined angles and patches with sufficient material for microanalysis in the future [Zimmermann et al. (1999) *Eur. Biophys. J.* 28:516]. The topological and topographical maps of traces depend on cell type, physiological status and motion of the donor cell and the surface of the substrate. Common biophysical models of cell migration do not predict or even comprehensively explain the phenomenon of these membrane enveloped releases, which contain cytoskeletal components. The aim of our work is, therefore, (i) to integrate thus into existing biophysical models and (ii) to establish the conditions for biomedical and biotechnological applications, e.g. single cell diagnostics and surface characterisation of implants.

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4A-1
POSSIBLE IMPLICATON OF SURFACE POTENTIAL IN CONFORMATIONAL STATES OF 7-HELICAL PROTEINS

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A sharp transition between a low and high temperature conformational state was observed for the EF-loop in the 7-helical transmembrane protein bacteriorhodopsin (bR). This conformational change was detected by time-resolved fluorescence depolarisation and site-directed labeling in position 160 using the thiol-reactive fluorescent dye iodoacetamidofluorescein. The changes in the amplitude of the loop rotational correlation time with the temperature indicate the existence of two states. Therefore, the conformational change is considered to follow a two state mechanism and was analyzed accordingly. The transition temperature increases with increasing salt concentration, suggesting a surface potential effect. A negative surface potential was shown previously for bR (Alexiev *et al.*, 1994, Biochemistry 33, 298-306). Furthermore, in the 7-helical G-protein coupled receptor rhodopsin a negative surface potential was detected for the cytoplasmic surface using wt and single cysteine substitution mutants of rhodopsin in the cytoplasmic loops. A detailed investigation of the surface potential and its relation to loop conformational changes in rhodopsin will be presented.

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4A-2
RETINAL AFFECTS THE PROTEIN-LIPID INTERACTION IN BACTERIORHODOPSIN-LIPID VESICLES

Krzysztof Bryl, Kazuo Yoshihara

Bacteriorhodopsin (BR) is a retinal-opsin complex present in the purple membrane of *Halobacterium salinarum*. Retinal affects the structure of BR. BR has a strong influence on the phase transition of the various lipid species in which it was reconstituted. However, the problem whether retinal influences the protein-lipid interaction is open.

The aim of our study was to clarify the influence of the retinal molecules on the protein-lipid interaction reflected by phase transition of BR-lipid systems. The phase transition of unbleached (or bleached) BR-lipid vesicles was monitored by fluorescence and phase properties of 1-acyl-2-[8-(2-anthroyl)-octanoyl]-*sn*-glycero-3-phosphocholine probe.

Retinal removal led to slight changes of phase characteristics and down-shift of the main lipid phase transition temperature. The temperatures corresponding to the beginning and ending of the gel/liquid phase transition were also changed demonstrating that retinal bleaching facilitated lipid transition into liquid phase. The results indicate that removal of retinal alters the protein-lipid interaction. It is suggested that this alteration might be related to the change in the lipid molecular packing.

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4A-3
PHOTOCURRENTS GENERATED BY BACTERIORHODOPSIN ADSORBED ON LIPID MONOLAYERS AND THIOL/LIPID BILAYERS SUPPORTED BY MERCURY

A. Dolfi, F.T. Buoninsegni, M.R. Moncelli , R. Guidelli

Bacteriorhodopsin (bR) is a light-driven proton pump present in the purple membrane (PM). Upon light excitation, bR undergoes a cyclic sequence of conformational transitions and protonation/deprotonation steps, leading to the translocation of a proton from the cytoplasm to the extracellular medium. PM fragments are readily adsorbed on lipid monolayers as well as octadecanethiol/lipid bilayers supported by a hanging mercury drop electrode (HMDE). While PM adsorption on bilayers causes a decrease in the differential capacity C of the interphase, which measures the extent of adsorption, that on monolayers causes a slight increase in C that can be reduced by consecutive potential scans.

Following the adsorption, the proton pump is activated by shining the mercury drop with a He-Ne laser ($\lambda = 543.5$ nm) and the transient capacitive current generated by the protein is measured under short-circuit conditions and in the absence of photoartefacts. Illuminating PM adsorbed on a lipid monolayer causes a light-on photocurrent transient, while interrupting illumination causes a light-off current transient. The light-on transient attains a stationary value that first increases linearly from +30 to −200 mV / SCE, and then decreases toward more negative potentials. The light-on and light-off current peaks of PM adsorbed on thiol/lipid bilayers are highly reproducible. The light-on current transient shows a vanishingly small stationary current. The dependence of the photocurrents on pH is investigated under different conditions.

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4A-4
COMPARING THE FLUORESCENT STATES OF NATIVE AND NON-ISOMERIZING BACTERIORHODOPSIN

S. Haacke, S. Vinzani, S. Schenkl, M. Chergui, M. Ottolenghi, S. Ruhman, M. Sheves

We have studied the picosecond fluorescence of native bacteriorhodopsin (wt-bR) and of bR with retinal analogues sterically hindered from isomerization by a five-membered carbon ring around the critical $C_{13}=C_{14}$ bond. The retinal analogs are thus blocked in the *all-trans* (bR5.12) or alternatively *13-cis* configuration (bR5.13). Previous femtosecond absorption pump-probe experiments have shown a great similarity in the spectral properties of the excited state of both analogs with the first intermediate I_{460} of wt-bR. Our fluorescence study performed using a streak camera with 3 ps time resolution confirms an excited state lifetime of several ps for the analogs and shows the absence of dynamic spectral shifts on this timescale. In addition, we show for the first time that the fluorescence spectra are almost identical to those of wt-bR. From the comparison of the spectra of excited state absorption, stimulated and spontaneous emission one obtains a strong similarity of the excited states of the retinal analogs and of I_{460}. This is further confirmation for the fact that I_{460} does not fulfill torsional motion but evolves along other degrees of freedom, such as double bond stretching and relaxation involving the protein environment. Important conclusions can be drawn for the shape of the potential energy curves along these "non-reactive" coordinates.

Additional femtosecond experiments using fluorescence up-conversion are in progress.

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4A-5
FUNCTIONAL DYNAMICS IN THE PURPLE MEMBRANE: COMPARISON BETWEEN THE WILD TYPE AND A MUTANT MEMBRANE

U. Lehnert, V. Réat, D. Oesterhelt, G. Zaccai

Bacteriorhodopsin (BR) is the only protein in the Purple Membrane (PM) of *Halobacterium salinarum*. After absorption of a photon by its chromophore, retinal, BR passes via several sequential photointermediates and finally returns to its initial state. Light induced structural changes occurring during the photocycle and the appearance and decay of late photointermediates are highly hydration dependent [1,2]. By the means of incoherent neutron scattering, dynamical transitions in wild type PM and their hydration dependence have been studied extensively [3]. A striking correlation appeared between these fast, pico to nanosecond, thermal motions and the slower kinetic constants of the photocycle and conformational changes. We are now investigating the motions of a mutant of BR: D96G/F171C/F219L. This mutation destabilises the wild-type structure to generate almost the full extent of the main photocycle conformational change in the dark, with minimal additional light-induced changes [4]. We observed a significant difference between the thermal motions of the wild-type and mutant membranes for the hydrated samples. In contrast to this, the dry membranes showed similar dynamical behaviour.

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4A-6
UNRAVELING CONFORMATIONAL CHANGES OF BACTERIORHODOPSIN USING PAIRS OF SPIN LABELS

Nicole Radzwill, Klaus Gerwert, Heinz-Jürgen Steinhoff

The study of interspin distances in proteins with two nitroxide spin labels attached to specific sites is a new approach to obtain structural information on proteins in solution or in the membrane with a time resolution in the ms-time range.
This method has been applied to site- directed spin labeled bacteriorhodopsin (BR). Distances between pairs of spin labels attached to the cytoplasmic loops CD and EF or to the cytoplasmic end of helix G were determined after freezing the protein in different intermediates, distance changes prove conformational changes occurring during the photocycle. Spectra recorded at room temperature contain additional information about the interspin distances and the mobility of the nitroxide side chains. Kinetic difference spectra between the BR ground state and the photoexcited states are composed of changes of the tertiary interaction and variations of the dipolar interaction. Comparison of the kinetics of the transient EPR-signals measured at fixed magnetic field positions with the kinetics of the photocycle determined in the visible spectrum assigns the structural changes to the photocycle intermediates.
The analysis of the conformational change of BR reveals that a transient opening of the proton channel occurs, which is characterized by an outward tilt of the cytoplasmic part of helix F in the range of 0.2 nm during the M to N transition. The cytoplasmic end of helix G moves closer to the CD loop at the same time.

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4A-7
OPTIMIZATION OF BACTERIORHODOPSIN FILMS FOR HOLOGRAPHIC APPLICATIONS

A. Seitz, N. Hampp

Since the discovery of bacteriorhodopsin a lot of applications have been proposed. Among them optical application play an important role. For optical recording a high light sensitivity, a high contrast ratio and a high contrast decay time is required. All these parameters are coupled to the M-lifetime of bacteriorhodopsin films. Increasing the M-life-time leads to a proportional reduction of the light intensity required for the optical writing process. The M-lifetime of BR-WT and BR-D96N can be tuned by changing the pH-value resp. the proton avaibility in the matrix of the film, and in addition the temperature or the water content in the film.
At low humidities the proton transport steps linked to the photocycle are limiting the over-all kinetics. A new, the so called proton diffusion limited two-state model (PDL2 model) for bacteriorhodopsin is presented. It allows to model mathematically the optical excita-tion and thermal relaxation processes for high as well as low humidities in bacterio-rhod-op-sin films. BR-WT and BR-D96N Films are compared in dependence on the pH-value and the relative humidity at 20 °C. At low light levels of 100 $\mu W/cm^2$ only BR-Films containing BR-D96N can be used for recording. In a typical application where a high light-sensitivity is a key issue it is demonstrated to what high extent the water content in the films affects their suitability for recording at low light levels.

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4A-8
UNRAVELING PHOTOEXCITED CONFORMATIONAL CHANGES OF BACTERIORHODOPSIN BY TIME RESOLVED EPR SPECTROSCOPY

Heinz-Jürgen Steinhoff, Thomas Rink, Nicole Radzwill, Matthias Pfeiffer#, Dieter Oesterhelt#, Klaus Gerwert

By means of time resolved electron paramagnetic resonance (EPR) spectroscopy the structure and photoexcited structural changes of site-directed spin labeled bacteriorhodopsin are studied. A complete set of cysteine mutants of the C-D loop, positions 100 to 107, and of the E-F loop including the first α-helical turns of helices E and F, positions 154 to 171, was modified with a methanethiosulfonate spin label. The analysis of the EPR spectra reveals a single turn in the E-F loop. The spectral changes which were observed during the photocycle are consistent with a small movement of helix C and a prominent outward tilt of helix F. Double spin labeling of residues located at the cytoplasmic ends of helices C, F or G show that this tilt of helix F is accompanied by an inward movement of helix G. The kinetic analysis of the transient EPR data, FTIR and absorbance changes in the visible spectrum reveals the structural rearrangement to occur during the lifetime of the M intermediate. Prominent rearrangements of nitroxide side chains in the vicinity of D96 take place during the M to N transition. All structural changes reverse with the recovery of the BR initial state.

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4A-9
TIME-RESOLVED FTIR SPECTROSCOPIC STUDIES ON THE PROTON PATHWAY OF BACTERIORHODOPSIN

Christian Zscherp[#]*, Ramona Schlesinger[#], Joachim Heberle[#]

The photoreactions of wild-type and E194Q mutant bacteriorhodopsin (BR) have been investigated at various pH values by time-resolved step-scan FTIR difference spectroscopy using the attenuated total reflection technique. Analysis of the pH dependence of the signal at 1741 cm^{-1} reveals a drop of the pK_a of D96 from > 12 in the ground state to 7.1 in the N state . This quantifies the role of D96 as the internal proton donor of the Schiff base.At the end of the photoreaction of the E194Q mutant an intermediate state can be detected in which D85 is deprotonated but another carboxylate is transiently protonated. We assign the corresponding difference bands to protonation changes of D212 by using the quadruple mutant E9Q/E74Q/E194Q/E204Q. Since we have previously detected a small difference band at the same wavenumber also in wild-type BR , it is proposed that protonation of D212 represents an intermediate step during proton transfer from D85 to the proton release group in the final stage of the reaction cycle. The difference spectra of E194Q at high pH are comparable with the N-BR difference spectra of the wild type with the remarkable exception that D85 is deprotonated. Since the retinal configuration is not perturbed by the E194Q mutation it is concluded that there is no interaction of D85 with the retinal in the N state.

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4A-10
A HIGH-FIELD EPR STUDY OF THE CONFORMATION AND CONFOR-MATIONAL CHANGES OF SITE-DIRECTED SPIN LABELED BACTERIORHODOPSIN

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Electron paramagnetic resonance (EPR) spectroscopy in combination with site-directed spin labeling (SDSL) allows to obtain structural information of membrane proteins or proteins which cannot be cristallized. The present report shows 95 GHz (W-band) EPR spectroscopy data on site-directed spin labeled bacteriorhodopsin (BR), an integral membrane protein which acts as a light driven proton pump. Spectra recorded at room temperature allow a detailed analysis of the anisotropy of the spin label motion. Because of the complexity of the nitroxides motion molecular dynamics simulations were necessary to calculate EPR spectra and extract motional and magnetic parameters. Further on the sensitivity of EPR spectra towards the environmental polarity facilitates to characterize the behavior of the hydrophobic barrier of the BR proton channel. Therefore the values of the hyperfine tensor element A_{zz} and g_{xx} were determined using the second derivative of the experimental spectra recorded at T = 170K. Beyond this the values of A_{zz} and g_{xx} allowed to classify the environment of the spin labeled side chain either as protic or aprotic. W-band EPR spectroscopy offers the opportunity for a detailed characterization of spin-label movement. In addition to X-band it is a powerful tool for determining the details of the protein structure and for studying structural changes in proteins in the ms-time range.

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4B-1
TOPOLOGY OF P680 IN PHOTOSYSTEM II: FINE ANALYSIS OF DIFFERENCE SPECTRA BY POLARIZED SPECTROSCOPY

Ralf Ahlbrink, Wolfgang Junge

Water oxidation in photosystem II (PS II) is driven by a photochemical reaction center, to which the catalytic center, a Mn-Y_Z entity, is attached.
The innermost chlorophyll components, denoted by P_{680}, are at the basis of the extremely oxidising power of PS II. The monomeric versus oligomeric properties of P_{680}^+ are under debate. The optical difference spectra at room and cryogenic temperature are known (1), but their deconvolution in terms of Gaussians is inherently ambiguous.
Previously, we demonstrated the anisotropy of the antennae system of PS II by polarized laser flash spectroscopy (2). We attempted a fine analysis of P_{680} difference spectra at 293 and 78 K.

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4B-2
CHARACTERIZATION OF THE FLUORESCENCE QUENCHING OF CHLOROPHYLL *A* BY 1,4 BENZOQUINONE USING A NONLINEAR ANALYSIS

Doina M. Gazdaru

The results of the fluorescence quenching of chlorophyll *a* by substituted quinones in a series of aliphatic, long-chain hydrocarbons, cyclic aliphatic hydrocarbons and organic solvents have been presented in many studies. Frequently, positive deviations from the Stern – Volmer equation are observed when the extent of quenching is large. This work presents the values of quenching parameters of chlorophyll *a* - 1,4-benzoquinone system, in ethanol solution, using a nonlinear analysis. Intensities of the chlorophyll *a* fluorescence were corrected in the manner described by Beddard *et al.* The samples were excited in red and blue spectral range. For the 650 nm excitation wavelength the dynamic quenching constant is about 93 M^{-1} and the static constant is about 24 M^{-1}. That means a quenching constant of 1.9×10^{10} M^{-1} s^{-1} (obtained with a fluorescence lifetime of 4.72 nsec in the absence of the quencher) and an *active radius* (for the volume considered as *active element* surrounding the fluorophore molecule) of 21 Å. The results for the 430 nm exciting wavelength are the following: 129 M^{-1}, 46 M^{-1}, 2.73×10^{10} M^{-1} s^{-1} and 26 Å, respectively.
Therefore two different ways for this quenching process have been proposed. The first way involves the formation of ground-state complexes and the second one is related to the probability of benzoquinone-to-chlorophyll encounters.

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4B-3
PH-DEPENDENT RETARDATION OF PROTON TRANSFER TO Q_B BY TRANSIENT METAL IONS IN BACTERIAL REACTION CENTER

László Gerencsér, Péter Maróti

Zn^{2+} [1,2] and Cd^{2+} [2] bind stoichiometrically to the reaction center (RC) of photosynthetic bacteria and inhibit the proton transfer to the secondary quinone Q_B. Using structural data [3], a dominant proton pathway from the surface to Q_B (P3) was identified [2,4]. The inhibition showed marked pH-dependence peaked at neutral pH and became less striking in the surrounding pH ranges. The dissociation constant (K_D) of Cd^{2+} was pH-dependent: 0.2 μM at pH 8.5 and 2.5 μM at pH 6.3. Ni^{2+} had less binding affinity ($K_D = 1.0$ μM) but larger inhibition of proton transfer rate ($k = 10\ s^{-1}$) than Cd^{2+} ($K_D = 0.5$ μM and $k = 30\ s^{-1}$, respectively) at pH 7.9. The metal ions slowed down the kinetics and increased slightly the stoichiometry of the first proton binding at pH 8.5. The inhibition was largely recovered by addition of weak acids (*e.g.* azide). Continuous turnover measurements on inhibited RC [5] indicated, that the rate limiting step was the second proton uptake (pH > 6.2). The results are interpreted by changes of the proton donor properties of the protonatable groups at the entrance of P3 (His, Asp and structural water molecules) upon complex formation with the transient metal ion.

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4B-4
INVESTIGATION OF THE IRON SUBSTITUTED MN-CLUSTER OF PHOTOSYSTEM II

Klaus Grantner, Michael Reiner, Olga Iakovleva, Wolfram Meyer-Klaucke[1], Boris Semin[2], Fritz Parak

Most of the oxygen molecules of our atmosphere are produced by the water splitting complex of Photosystem II (PSII) of higher plants. The necessary charge accumulation occurs in a tetranuclear manganese cluster. The mechanism of the water splitting is still discussed controversial. The structure of PSII is not known. To get more information about the active center of the water splitting enzyme Mn depleted PSII membrane fragments have been reconstituted in part with iron [1]. (PSII-4Mn)-membrane fragments were treated with Fe^{2+} under illumination. Mössbauer spectroscopy gave strong evidence that on the Mn binding sites 3 high-spin Fe^{3+} ions were bound building a trinuclear cluster with a total spin of S = 1/2. Another iron-ion binds as Fe^{2+} low-spin at Ca- sites. Sequence analogies of the water splitting enzyme with proteins having a two iron center were used to construct a structural model. Fe- K_α EXAFS has been used to test this model. Distances of 3.37Å were obtained between the specific irons. This values are compared with Mn – Mn distances determined by XAS studies [2].

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4B-5
CHIRAL MACROAGGREGATES OF LHCII IN GREEN LEAVES DETECTED BY CIRCULAR POLARIZATION OF CHLOROPHYLL LUMINESCENCE (CPL)

Eugene Gussakovsky, Yosepha Shahak

LHCII particles form chiral macroaggregates of LHCII in chloroplasts under certain physiological conditions. In isolated chloroplasts they were studied by circular dichroism. However this technique is not convenient for studies of green leaves. We have recently shown that chiral macroaggregates could be detected in isolated chloroplasts by CPL. Obviously, this method is potentially more convenient for green leaf research, and in the present study was applied to intact leaves of pea, mango and coffee plants. The coincidence of the CPL spectra shape and intensities for pea leaves and chloroplasts indicated that the CPL signal indeed reflected chiral macroaggregates of LHCII particles in leaves. The photoinhibitory illumination did not significantly change the chiral macroaggregates content in green leaves, while their photosynthetic efficiency is markedly reduced. No change of CPL was found during a total chlorophyll fluorescence change at the illumination. Evidently, chiral macroaggregates remain unchanged during the photochemical equilibration. It is suggested that chiral macroaggregates might serve as a stable structural template for a proper functioning of the photosynthetic apparatus in green pea leaves. The CPL technique allows non-destructively detecting structural changes of the LHCII chiral macroaggregates in green leaves under natural and stress conditions of plants.

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4B-6
PHOTOPHYSICS OF INDIVIDUAL REACTION CENTER CONTAINING PHOTOSYNTHETIC UNITS

F. Jelezko, C. Tietz, U. Gerken, E. Thews, S. Schuler, A. Wechsler, J. Wrachtrup

Single molecule spectroscopy has been used to investigate the photophysics of the reaction center containing Photosystem I from the cyanobacterium *Synechococcus elongatus*. Room-temperature bleaching dynamics indicates that low-energy Chl pool is a focalizing element on the energy transfer pathway from the bulk antenna to the reaction center. Spectroscopic study shows that two subgroups of pigments present in the red-most Chl pool differ not only by their spectral position but also by the the coupling efficiency with the protein environment. Based on our study and recently available hole-burning and structural data we believe that the red-most state is a Chl dimer located in the vicinity of the special pair. Our results demonstrate that efficient fluorescence quenching by the special pair is not a dramatic obstacle to detect a single reaction center at work.

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4B-7
EFFECTS OF ELECTRON DONORS ON THE A_T-BAND OF THERMOLUMINESCENCE (TL) IN MN-DEPLETED PS II

M.Yu. Kultisheva[1], E.R. Lovyagina[1], A.M. Kuznetsov[2], M.K. Solntsev[2], I.I. Ivanov[1], B.K. Semin[1]

Mn-depleted photosystem II (PSII) oxidize electron donors like Mn(II), Fe(II), diphenylcarbazide, NH2OH. Donation of electrons by Mn(II) and Fe(II) [Semin B.K., Parak F. (1997) FEBS Lett. 400 259-262] takes place probably via Mn-binding site. Extraction of Mn from PS II inhibits the main bands of TL and induces new band (AT) at −200C. Formation of AT-band is due to charge recombination of QA- with oxidized His on the donor side of PS II. Participation of this His in the coordination of Mn-cluster was suggested. In present work the influence of Mn and Fe as well as DPC and NH2OH at the AT-band have been studied with purpose of investigation of this His in the coordination of Mn/Fe. We found that total inhibition of AT-band by Mn(II) and Fe(II) takes place after preliminary incubation of Mn-depleted PS II under light with cations. Disappearance of AT-band was observed at concentration of cations, which block the electron transport from DPC to DCIP. DPC and NH2OH diminish the AT-band more effectively than Mn(II) and Fe(II).The role of redox-active His in the binding of Mn and Fe is discussed.

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4B-8
THE INFLUENCE OF SOLVENT DYNAMICS ON ELECTRON TRANSFER IN BACTERIAL REACTION CENTERS

Andreas Labahn, Ralf Schmid

The photosynthetic reaction center from Rhodobacter sphaeroides is an integral membrane protein-pigment complex which converts light into chemical energy. Following the absorption of a photon, an electron is transferred from the excited primary donor D to the primary ubiquinone QA. In vitro, in the absence of an exogenous reductant and the secondary ubiquinone QB, charge recombination occurs with the rate constant kAD.

The rate kAD increased from 8 s-1 to 38 s-1 when the temperature was varied in the range from 293 K to 77 K in agreement with previously data (e.g. [1,2]). To unravel the factors governing this effect, we measured the temperature dependence of kAD in different solvents with glass temperatures ranging from 129 K to 181 K. Our data demonstrate that the large increase of kAD upon lowering the temperature is correlated to the glass transition of the solvent. These findings indicate a strong coupling of the protein relaxation to the dynamics of the surrounding medium. The data can be described with an electron transfer model assuming a decrease in the reorganization energy coupled to an increase in the free energy difference. Additional results will be presented to elucidate the molecular basis of the observed temperature profile of kAD in different solvents.

[1] J.M. Ortega et al., Biochemistry 35, (1996), 3354. [2] B.H. McMahon et al. Biophys. J. 74, (1998), 2567.

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4B-9
TIME-RESOLVED ENTHALPY MEASUREMENTS OF LIGHT INDUCED REACTIONS BY OPTICAL INTERFEROMETRY

H.H. Leyser, W. Doster

The exchange of energy between structural and reactive degrees of freedom is a basic feature of biomolecular processes. We present a new method that allows to measure the enthalpy and volume relaxation of light-induced reactions. The method is based on a measurement of the optical pathlength of the aqueous sample which changes with temperature as a result of thermal expansion. By recording the interference pattern produced by the sample and the reference path in a Michelson interferometer as a function of time, we detect temperature changes with sub-Millikelvin resolution on a microsecond to second time scale. This range is difficult to explore by other time-resolving thermal methods. We studied the kinetics of ligand binding to myoglobin and the P+Q- charge recombination of photosynthetic reaction centers by flash photolysis using a combined optical and thermal detection. The enthalpic relaxation in myoglobin follows the kinetics of ligand binding whereas the charge recombination in the reaction centers of Rb. sphaeroides involves a two-step thermal process which may correlate with changes in the optical spectrum seen at 420 and 633 nm. The reaction volume for CO-binding to myoglobin is found to be negative, while it is positive for oxygen binding. Potential applications to other light induced reactions are discussed.

T. Kleinert, W. Doster, H. Leyser, W. Petry, V. Schwarz, and M. Settles, Biochemistry 37: 717-733 (1998)

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4B-10
PROTON UPTAKE AND PROTEIN RELAXATION IN REACTION CENTER OF PHOTOSYNTHETIC BACTERIA MONITORED BY DELAYED FLUORESCENCE OF THE DIMER

Péter Maróti, László Rinyu, Kinga Turzó, Gábor Laczkó

Efficient conversion of light energy into electrochemical energy takes place in reaction center (RC) protein of photosynthetic bacteria: the absorbed energy of a flash of light induces charge separation (the yield is close to one) followed by a series of electron transfer reactions coupled to uptake of H^+ ions. The protein accommodates to the redox changes of the cofactors in a wide (from picosecond to second) time scale in forms of structural, energetic and functional changes. We used delayed fluorescence (arising from back reactions generating the excited state of the bacteriochlorophyll dimer) as a quantitative probe of the time-dependent energetics of the charge separated states of the RC in photosynthetic purple bacteria *Rhodobacter sphaeroides*. The free energy states relative to that of the excited dimer was determined by comprehensive measurement of the intensities of the prompt and delayed light emission. The proton uptake by the RC contributed to the solvation of the semiquinone in the protein environment. The nature of quinone in the acceptor complex of the RC determined the pH profile of the free energy levels of the charge separated states. The relaxation behavior in the millisecond time range was studied by use of an electromechanical shutter. Preliminary data will be presented in shorter time scale by using electronic gating of the photomultiplier.

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4B-11
CHARATERIZATION OF Q_A AND Q_B BINDING SITES IN PHOTOSYSTEM II BY FTIR-SPECTROSCOPY

Jens Niklas, Matthias Rögner, Johan Lugtenburg, Klaus Gerwert

FTIR-spectroscopy allows the investigation of interactions between cofactors and protein environment. The quinone binding sites in photosystem II are analyzed. Mutants in the binding sites of *Synechocystis*' photosystem II are investigated. Analysis of the Q_B binding site provides information about mechanisms of herbicide binding.
With the obtained information a structured model of the quinone binding sites is possible.

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4B-12
RESPONSE OF THE WATER-PROTEIN MEDIA ON THE ION-RADICAL PAIR FORMATION IN THE RC OF PURPLE BACTERIA

Vladimir Z. Paschenko, Peter P. Knox, Andrew B. Rubin

Cofactors of electron transfer in bacterial reaction centre are: a) dimer of bacteriochlorophyll molecules P_1P_2; b) monomeric molecules of bacteriochlorophyll (BCl) and bacteriopheophytin (BPh) and c) quinone acceptor Q_A. These cofactors are connected to the proteins by means of hydrogen bonds. After light excitation of a dimer P a charge transfer (CT) state $\{P_1^+P_2^-\}^*$ occures. As a result of the electron transfer (charge separation process) from this CT state to the BPh molecule the ion-radical pair P^+BPh^- is formed during ~3 ps. The next step is the electron transfer to quinone acceptor Q_A within ~200 ps. Nonequlibrium dipoles $P_1^+P_2^-$; P^+BPh^- and $P^+Q_A^-$ relax to the pseudoequilibrium states as a result of solvation processes.
In this study a dynamic of water-protein surrounding of cofactors caused by the formation of the electric dipole in RC is studied experimentally and analysed on the base of simple mechanical models. It is shown that the deformation of hydrogen bonds as a result of proton displacement in the electric field of central dipole (charge) takes place during ~100 fs. In this process the energy ~0.05 eV is stored. The results of this study are considered in the frame of the regulatory role of protein molecules in the charge separation and electron transfer reactions in photosynthesis.
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4B-13
STEP-SCAN FTIR SPECTROSCOPY OF THE $Q_A^-Q_B \rightarrow Q_AQ_B^-$ TRANSITION IN *RB. SPHAEROIDES* REACTION CENTRES

André Remy, Robin Rammelsberg, Harald Chorongiewski, Klaus Gerwert

Step-scan Fourier transform infrared spectra of the $Q_A^-Q_B \rightarrow Q_AQ_B^-$ transition in *Rhodobacter sphaeroides* R26 reaction centres with a 30 ns time resolution are shown. A global fit analysis yields three exponentials with half-times of 500 ns, 80 µs and 2.1 ms in agreement with IR spectroscopic studies at single wavenumbers, in the UV/VIS and in the near IR.
The use of a recently constructed cyclic sample changer allows to average eight samples during the same measurement. By this new technique the transition can be investigated in detail in the mid-infrared region (1900–1200 cm^{-1}). For example, the protonation of glutamic acid L212 can be followed kinetically as well as protonation reactions of at least two further amino acids (1735 cm^{-1}, 1707 cm^{-1}). The establishment of the step-scan technique provides a new approach to elucidate the molecular mechanism of this transition.

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4B-14
ASSIGNMENT OF UBIQUINONE-10 METHOXY VIBRATIONS IN THE REACTION CENTRE OF *RB. SPHAEROIDES* R26

André Remy, Ronald Brudler, Johan Lugtenburg, Huub J.M. de Groot, Klaus Gerwert

Using native and site-specifically labelled 1-^{13}C-, 2-^{13}C-, 3-^{13}C-, 4-^{13}C-, 5-^{13}C-, and 6-^{13}C-UQ_{10} reconstituted at either the Q_A or Q_B binding site of *Rhodobacter sphaeroides* R26 reaction centres and FTIR difference spectroscopy the influence of the methoxy substituents of UQ_{10} on the cofactor protein interaction is investigated. By this means two IR bands at 1288 cm^{-1} and at 1264 cm^{-1} are assigned to the (ring-)C-O vibrations of the methoxy groups. These bands do not shift in frequency at the Q_A and Q_B binding sites, respectively, compared to unbound UQ_{10}. It can be ruled out that different conformations of the methoxy groups at different binding sites significantly contribute to the extraordinary downshift of the 4-C=O stretching vibration of UQ_{10} at the Q_A site and thereby to the tuning of UQ_{10} to its specific functions at the different binding sites. Therefore the π-stacking due to interactions with aromatic amino acids seems to be more favorable.

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4B-15
FE(II) BLOCKS THE DONATION OF ELECTRONS BY MN(II) TO Y_Z^+ THROUGH THE HIGH-AFFINITY MN-BINDING SITE

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The donation of electrons by Mn(II) and Fe(II) to Y_Z^+ in Mn-depleted photosystem II (PSII) membranes has been studied by flash-induced fluorescence measurements. The K_m value (0.72 μM) for electron donation to Y_Z^+ by added Mn(II) indicates that the reaction takes place through the high-affinity (HA) Mn-binding site on PSII. Added Fe(II) donates electrons to Y_Z^+ with the same efficiency as Mn(II), since the concentrations of Fe(II) and Mn(II) that saturate the reaction are the same, 5 μM. However, electron donation by Fe(II) shows cooperativity at lower concentrations. Following a short incubation of the membranes with 5 μM Fe(II) in the light, added Mn(II) cannot be photooxidized by Y_Z^+ any longer. This result suggests that Fe(II) binds irreversibly to the HA Mn-binding site in the light and blocks the subsequent binding of added Mn(II) and eleñtron donation to Y_Z^+. We have used Fe(II) to block the HA Mn-binding site and are investigating the capability of other known PSII donors such as diphenylcarbazide and hydroxylamine to reduce Y_Z^+ under the same conditions.

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4B-16
RELATED HETEROGENEITIES OF THE ELECTRON AND PROTON TRANSFER KINETICS ASSOCIATED WITH THE FORMATION OF Q_B IN REACTION CENTERS

Julia Tandori[1], Pierre Sebban[2], Laura Baciou[2]

In the reaction center (RC), light is initially converted into chemical free energy through the formation of the semiquinone form of the secondary quinone electron acceptor Q_B. This photochemical step is associated with a substoechiometric proton uptake from the bulk phase. The previously detected heterogeneity of the first electron transfer from Q_A (first quinone electron acceptor) to Q_B was investigated here in the isolated native RCs from *Rb. sphaeroides*. The fast (~80 μs) and the slow (~300 μs) components and their relative amplitudes have been studied by flash induced absorption change spectroscopy at 750 nm. The rate constant of the fast component is pH independent between pH 7 and 10. In contrast, the rate constant of the slow component of electron transfer steeply decreases above pH 9. The relative amplitudes of both rate constants are pH dependent but display a different pH pattern. The kinetics of proton uptake associated with the first flash has been measured by using pH sensitive dyes at 582 nm. They are also biphasic. The fast and slow rate constant values are close to those measured for the electron transfer process and display similar pH dependence patterns. Our data suggest for electron and proton transfers, the existence of a fast phase kinetically limited by a shared gating process, maybe triggered by the formation of Q_A^-. The slow components could in both cases reflect protein rearrangements involving protonation events.
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4B-17
SINGLE MOLECULE SPECTROSCOPY ON PHOTOSYNTHETIC ANTENNA COMPLEXES

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Single molecule spectroscopy was applied to unravel the energy transfer pathway in supramolecular aggregates carrying important function in photosynthesis. Spectroscopic properties of the peripheral plant antenna LHC II and bacterial light-harvesting complex LH 2 are analyzed. The excitonic pigment-pigment interactions govern the excitation dynamics in the densely packed B850 chlorophyll ring of the bacterial antenna. Single molecule polarization study shows that the natural disorder may influence on the energy equilibration process. The major plant antenna LHCII representing the next step of the evolution process cannot be characterized like a highly symmetric structure. Interpigment energy flow between the red-most pigments in this species occurs by the Foerster mechanism. High resolution fluorescence emission and excitation spectra of the individual LHC II trimers indicate that at low temperature the excitation energy is localized on the red most Chl*a* pool absorbing at 680 nm. The spectral lines of single Chl*a* molecules absorbing at 675 nm are broadened because of resonance energy transfer towards the red-most pigment. The polarization sensitive study of the bleaching dynamics reveals that intersubunit coupling in the LHC II trimer is weak and intramonomer energy equilibration leads to energy transfer towards the PS II core complex in nature.

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4C-1
DIRECT ELECTROCHEMISTRY OF THE UBIQUINONE POOL INCORPORATED IN A SUPPORTED BILAYER. KINETIC COUPLING WITH A MEMBRANE ENZYME

Damien Marchal, Vanessa Proux-Delrouyre, Jean-Marc Laval, Jacques Moiroux, Christian Bourdillon

In the membrane-bound electron transfer chains, the electron carriers like ubiquinones can move only in a two-dimensional manner. The quantitative characterization of the kinetics of membrane bound enzymes coupled to the electron carriers involved in these highly organized spatial environments is rarely achieved. The aim of the work is to demonstrate that transient electrochemical techniques offer an efficient approach to such a type of problem in heterogeneous enzymology.
The first step was the development of a convenient biomimetic structure. Our strategy involved successive self-assembly of the different components in an especially designed electrode we name the "microporous electrode". The fluidity of the bilayer was found similar to classical supported bilayers and the lateral diffusion coefficients of several isoprenic quinones incorporated in the bilayer were measured by chronocoulometry without labeling of the molecules. The next step was the incorporation of a peripheral enzyme (pyruvate oxidase) in the structure. The successful electrocatalytic coupling between the different components was studied by means of cyclic voltammetry. A thorough quantitative analysis of the observed catalytic currents gave access the enzyme kinetic constants operating in a two-dimensional environment.

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4C-2
FLUORESCENCE AND FUNCTIONAL ACTIVITY OF CHLOROPLASTS IN ACID AND ALKALINE ZONES OF *CHARA*

Alexander A. Bulychev, Alexander A. Cherkashin, Andrew B. Rubin, Wim J. Vredenberg, Vladimir S. Zykov, Stefan C. Müller

The relationship between pH banding in *Chara* cells and photosynthetic activity of chloroplasts located in cell regions adjacent to acid and alkaline bands was investigated. The combined use of pH microelectrodes and pulse-amplitude modulation (PAM) microfluorimetry enabled parallel measurements of longitudinal pH profiles and chlorophyll fluorescence yield in acid and alkaline zones of individual *Chara* cells. The scanning with a pH-microelectrode along the cell length revealed the light-dependent pH pattern, i.e., alternating acid and alkaline bands with pH difference as large as 2-3 pH units. In parallel measurements of chlorophyll fluorescence yield under actinic light, it was found that the effective photochemical yield of photosystem II is substantially higher in acid than in alkaline zones. The longitudinal profile of current at the open Pt microelectrode, presumably related to O_2 content in various regions of *Chara* cells, was antiparallel to the pH profile. The results clearly show that the banding pattern is not confined solely to the plasmalemma but is also manifest in alternating photosynthetic performance of the underlying chloroplast layer. Apparently, the acid regions enriched with CO_2 ensure sufficient flow of this substrate to the Calvin cycle reactions, thus promoting photosynthesis, whereas the alkaline zones devoid of CO_2 promote dissipative losses of absorbed solar energy in chloroplasts.

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4C-3
THE ROLE OF METAL SITE CONFORMATION IN PLASTOCYANIN BINDING TO PHOTOSYSTEM I

Eva Danielsen, Lars Elkjær Jørgensen, Henrik Vibe Scheller, Rogert Bauer, Lars Hemmingsen, Morten Jannik Bjerrum, Örjan Hansson

The technique of perturbed angular correlation of γ-rays (PAC) can be used to study the structure of the metal site in Cd or Ag substituted proteins, the binding of a small protein to a larger protein as well as the time constant of the structural relaxation of the Ag-substituted protein after the decay of ^{111}Ag to ^{111}Cd. All these features are important for the presented study of the binding of plastocyanin to photosystem I, where Ag and Cd are used as models for native Cu(I) and Cu(II), respectively. A dissociation constant of 5 μM was found for Ag-plastocyanin, whereas the dissociation constant was at least 24 times higher for Cd-plastocyanin. The structural study indicates a planar configuration of one cysteine and two histidines where one of the coordinating histidines seems to have an in-plane flexibility and a different position in the Ag protein than in the Cd protein. The decay of Ag to Cd causes a relaxation of the position of this histidine to the position in the Cd protein within 20 ns. Binding of Ag-plastocyanin to photosystem I stabilized the Ag metal site structure so that no relaxation was observed on a time scale of 100 ns. The study thus suggests that the metal site structure is involved in regulating how the dissociation constant for plastocyanin depends on the charge of the metal ion.

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4C-4
ACTIVITY AND SPECTRAL STUDIES OF THE EFFECT OF UREA ON PURIFIED CYTOCHROME C OXIDASE

Jacqueline Keyhani, Ezzatollah Keyhani

EPR and absorption spectra as well as oxygen uptake of purified beef heart cytochrome c oxidase (CCO) were measured after treatment of the enzyme with increasing urea concentrations at room temperature for 30 min. In up to 2 M urea, EPR and oxidized or reduced absorption spectra of CCO remained unaltered while oxygen uptake in the presence of 6 μM cytochrome c and 5 mM ascorbate was twice that of the control. In higher urea concentrations (up to 8 M), the low spin heme signal ($g=3$) and the copper signal ($g=2.01$) decreased progressively to 40% and 15% of the control value, respectively. The oxidized absorbance spectrum of CCO showed a 2 nm blue shift in the α band and red shifts in the γ band of 5 to 6 nm. The reduced absorbance spectrum of CCO showed a 2.5 nm blue shift in the α band and 5.5 to 8 nm blue shifts in the γ band. Oxygen uptake diminished to 60% of the control in 8 M urea. Urea treated CCO that was extensively dialysed against phosphate buffer to remove the urea did not recover its original properties. Oxygen uptake with 6 μM cytochrome c and 5 mM ascorbate as substrates was 110% of the control value for 1 M urea and then declined progressively to 25% of the control for 8 M urea. Neither absorption nor EPR spectra were restored to those of the untreated enzyme. Data suggested that low urea concentrations caused minor structural alterations favoring the enzymatic activity while higher concentrations were disruptive. Removal of urea by dialysis did not restore the enzyme original configuration.

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4C-5
EXTERNAL NADH OXIDATION AND PROTON MOTIVE FORCE GENERATION IN RAT LIVER MITOCHONDRIA

Victor V. Lemeshko

Various authors have postulated that external NADH can be directly oxidized by rat liver mitochondria. But the rate of the intermembrane electron transport in the intact mitochondria is known to be extremely low. The inner membrane potential has been recently demonstrated to depend on external NADH oxidation in the presence of exogenous cytochrome *c*.
In our work, the energy-dependent mitochondria swelling in the presence of potassium acetate and valinomycin was measured as the main criterion of the proton motive force generation by complexes III + IV, complex III or complex IV of the respiratory chain. Under external NADH oxidation, well-detected mitochondria swelling and membrane potential generation were observed after the outer membrane rupture caused by hypotonic treatment, by the energy dependent swelling-contraction cycle or by the inner membrane permeability transition of mitochondria.
Presented data suggest that external NADH oxidation can support the inner membrane potential only in mitochondria with damaged or permeabilized for cytochrome *c* outer membrane.

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4C-6
ACCOUNTING FOR ENVIRONMENTAL EFFECTS IN AB INITIO CALCULATIONS OF PROTON TRANSFER BARRIERS

Markus A. Lill, Michael C. Hutter, Volkhard Helms

The proton transfer between imidazole and water was studied by quantum chemical calculations in the presence of further ligand water molecules. In particular, we investigated the effect of the position of secondary waters relative to the proton transfer system. It is shown that the energy surface of transfer can be well reproduced when these waters are replaced by point charges. We found that at close distance the charges need to be enhanced to account for induced polarisation. As a further simplification, the environmental effects of these secon-dary waters on the proton transfer barriers can be described analytically by the electrostatic interaction of fitted point charges placed at the position of the ligand waters using the Mulliken charges of imidazole and the primary water.

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4C-7
CALCULATING REDOX POTENTIAL IN NATIVE AND ARTIFICIAL CYTOCHROME B

Dragan Popovic, Ernst-Walter Knapp

De novo-design of artificial metallo-proteins for bio-electronic applications is now possible using modern techniques of peptide synthesis. One can vary the protein sequence on a large scale, thus providing an ideal test ground to study the factors on which electron transport processes in proteins depend on. The aim of this project is to rationalize the design and to understand the functions of artificial metallo-proteins, which support electron transport.
The artificial protein that we study in detail imitates structure and function of cytochrome b (cyt b), which contains two hemes as cofactors. Its structure consists in an anti-parallel four helix-bundle and a cyclic decapeptide as a template. As in the native cyt b, the model protein conserves the interior packing of the hemes by hydrophobic residues. The other residues are hydrophilic or serve to stabilize the helices.
The atomic coordinates of artificial cyt b were generated by modelling techniques and energy minimized by using the program CHARMM. The shifts of the redox potential of the hemes and the protonation states of titratible residues in artificial, as well in native cyt b are determined by calculating the electrostatic energies, solving the Poisson Boltzmann equation numerically on a grid with finite difference method and using subsequently a Monte-Carlo titration. The results are compared with available experimental data.

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4C-8
SINGLE-ELECTRON REDUCTION OF THE OXIDIZED STATE IS COUPLED TO PROTON UPTAKE VIA THE K-PATHWAY IN *PARACOCCUS DENITRIFICANS* CYTOCHROME *C* OXIDASE

Maarten Ruitenberg, Aimo Kannt, Ernst Bamberg, Bernd Ludwig, Hartmut Michel, Klaus Fendler

The reductive part of the catalytic cycle of cytochrome *c* oxidase from *Paracoccus denitrificans* was examined using time-resolved potential measurements on black lipid membranes (BLM). Proteoliposomes were adsorbed to the BLM and $Ru^{II}(2,2'\text{-bipyridyl})_3^{2+}$ was used as photoreductant to measure flash-induced membrane potential generation. Single-electron reduction of the oxidized wildtype cytochrome *c* oxidase reveals two phases of membrane potential generation ($\tau_1 \approx 20\mu s$; $\tau_2 \approx 175\mu s$) at pH 7.4. The fast phase is not sensitive to cyanide and is assigned to electron transfer from Cu_A to heme *a*. The slower phase is completely inhibited by cyanide and shows a kinetic deuterium isotope effect by a factor of 2-3. While two enzyme variants mutated in the so-called D-pathway of proton transfer (D124N, E278Q) show the same time constants and relative amplitudes as the wildtype enzyme, in the K-pathway variant K354M τ_2 is increased to 900 μs. This result suggests uptake of a proton through the K-pathway during the transition from the oxidized to the one-electron reduced state.

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4C-9
MODELLING OF PROTON TRANSFER: CONFORMATIONAL AND PROTONATION SUBSTATES IN GREEN FLUORESCENT PROTEIN (GFP)

Christina Scharnagl, Robert Raupp-Kossmann, Sighart F. Fischer

Due to its intrinsic fluorophore, GFP has widespread use as a label in molecular biology. Spectroscopic studies revealed the prominent role of protonation of the bifunctional *p*-hydroxybenzylidene-imidazolidinone for the photophysics. So far, the discussion of the equilibria involved rests on indirect evidence from tertiary structure analysis and quantum chemical calculations *in vacuo*. We present the theoretical study of the energetics of the relevant proton configurations in solvent and protein.
Aqueous pK_a's were evaluated using a semiempirical continuum-solvation model and scaling for the specific compound classes (phenol-OH; imino-N). Modifications of the protonation free energies in the protein were calculated with an extended multi-conformation continuum electrostatics method and statistical analysis of proton binding coupled to orientational fluctuations of side chains and water molecules.
Our results confirm the ground state phenol/phenolate equilibrium. Photoexcitation strengthens the acidic as well as the basic group, thus driving proton transfer and zwitterion formation. This phototautomerization is a process not yet discussed for the photophysics of GFP. Protonation equilibria of the fluorophore and internal proton acceptors are coupled. This proton dynamics is correlated with conformational fluctuations of the hydrogen-bonded network and provides the explanation for the experimentally found multi-step titration and dispersive decay kinetics.

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4C-10
LATERAL PROTON DIFFUSION RATES ALONG LANGMUIR MONOLAYERS

Christopher J. Slevin, Patrick R. Unwin

A key step in bioenergetic processes in cell membranes is the movement of protons between source and sink sites, and a potentially efficient pathway for proton transfer is lateral diffusion along the cell membrane.
We have measured proton in-plane lateral diffusion rates in Langmuir monolayers as a function of surface coverage using a new approach involving scanning electrochemical microscopy (SECM). A monolayer spread at the air/water interface is deprotonated locally by reducing protons in solution to hydrogen at an ultramicroelectrode (UME) probe. This creates a proton diffusion gradient both in solution and at the interface, and the transport-limited current flowing at the UME provides a measure of the rates of diffusion in these two environments. Measurements on stearic acid monolayers show that the in-plane lateral proton diffusion coefficient is less than that in bulk solution, and depends critically on the physical state of the monolayer.
Protons may also be injected locally at the monolayer surface by oxidising water at the UME. By switching the potential at the UME, electrogenerated protons may then be collected by reduction, and the measured current response enables proton transport to be quantified. SECM is now being combined with fluorescence microscopy, where fluorescent probe molecules enable diffusion rates to be measured optically following concentration changes induced locally at the monolayer surface by the UME.

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4C-11
THE INCREASE OF CHLOROPHYLL FLUORESCENCE YIELD (F_0) IN DARK CHLORELLA CELLS ORIGINATES FROM CHLORORESPIRATION

Yurii Chemeris, Andrei Rubin

The rate of chlororespiration increased by 10-11-fold during dark incubation of Chlorella at supraoptimal temperatures (40-41 °C) or in the presence of exogenous glucose. The effect was accompanied by the 2-2,5-fold F_0 increase which was completely reversed by the subsequent cell illumination. The dark F_0 rise may be due to the accumulation of RC PSII with Q_A, reduced by electron flow from plastoquinone (PQ), the latter being reduced via the chlororespiration electron transport chain. However in the fluorescence decay after dark incubation the yield of intermediate component ($t = 380$ ps) remained constant while the component with $t = 2,5$ ns (typical of RC PSII with singly reduced Q_A) we replaced by the decay component with $t = 7$ ns. As it was shown by [F.J.E. van Mieghem et al., 1992, BBA, 1100, 198-206] this long living component in the decay kinetics is characteristic of RC PSII with doubly reduced and protonated Q_A (Q_AH_2). The absence of S_2-state of oxygen evolving system in Chlorella cells after prolonged dark incubation results in the lack of Q_A^- reoxidation through recombination processes thus enhancing the observed F_0 fluorescence rise.

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4D-1
THE CADA ATPASE FROM *LISTERIA MONOCYTOGENES*

Nathalie Bal, Elisabeth Mintz, Chen Chou Wu, Florent Guillain, Patrice Catty

The CadA ATPase from *Listeria monocytogenes* is a P1-ATPase. Belonging to the P-type ATPases family, the P1-ATPases widespread from bacteria to human are involved in the transport of metals such as copper, zinc or cadmium. In addition to displaying all the consensus sequences of P-type ATPases, P1-ATPases exhibit specific sequence signatures : CXXC in a cytoplasmic aminoterminal domain of the protein and CPC in its transmembrane part. The CXXC motif is found in other proteins of metal pathways, like the mercuric reductase MerA or the copper chaperones Atx1 and HAH1.
We have expressed the *Listeria monocytogenes* CadA ATPase in the Baculovirus/Sf9 system. A SDS-PAGE analysis of a membrane fraction of Sf9 cells reveals an additional band of the size expected for CadA. In this fraction, ATP hydrolysis is cadmium-dependent; zinc, cobalt and copper being less efficient substrates. We evidenced a transient phosphoenzyme intermediate which only occurs in the presence of cadmium, is acid stable, sensitive to hydroxylamine and absent in the D398A mutant. Treatment of the protein by iodoacetamide completely abolished phosphorylation, underlying the role of cysteines in the enzyme mechanism. CadA is phosphorylated from inorganic phosphate, only in the absence of cadmium. This phosphorylation is enhanced by dimethylsulfoxide.
From these data, CadA enzymatically behaves like P2-ATPases. However, further investigations are needed to understand how the metal crosses the ATPase, from one side to the other side of the membrane.

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4D-2
MECHANISM AND SUBSTRATE BINDING SITE OF THE CA^{2+}-ATPASE STUDIED BY INFRARED SPECTROSCOPY

Andreas Barth, Frithjof von Germar

Infrared absorbance changes of the sarcoplasmic reticulum Ca^{2+}-ATPase associated with the catalytic cycle were followed in real time using rapid scan FTIR spectroscopy. This technique senses in a single experiment the conformation of the polypeptide backbone, the environment of individual amino acid side chains and the structure of the molecular groups that are chemically involved in catalytic reactions.
The extent of secondary structure change seems to be small and similar for the 4 partial reactions investigated. This suggests that ion transport is mediated by key conformational changes in small flexible regions of the protein.
Parallel conformational changes occur when Ca^{2+} is released from the phosphorylated and the unphosphorylated ATPase. The simplest explanation for this observation is the assumption of only one pair of binding sites for Ca^{2+} on the ATPase.
The nucleotide binding site has been mapped with several derivatives of ATP. Interactions between protein and nucleotide were found for the γ-phosphate, the 3'-OH group and the amino group of the adenine moiety. No interaction was detected for the 2'-OH group.
An isotope exchange experiment has enabled the selective observation of the phosphate group of the E_2-P intermediate. It is concluded that the phosphate group is in an asymmetric hydrophobic environment with its geometry probably altered. The catalytic ion Mg^{2+} restricts its conformational freedom most likely by direct binding.

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4D-3
CHARGE TRANSLOCATION OF THE F_0F_1-ATP-SYNTHASE FROM *ILYOBACTER TARTARICUS*

C. Burzik, P. Dimroth, E. Bamberg, K. Fendler

F_0F_1-ATPases catalyze the synthesis or the hydrolysis of ATP. Depending on their mode of action they are driven by an electrochemical ion gradient or build up an ion gradient. The ion translocation and the coupling of ion translocation and chemical reactions are unclear. Only little information about kinetic properties of these proteins is available.
The charge translocation of the sodium translocating ATPase from *Ilyobacter tartaricus* is characterized in both modes, ATP synthesis and hydrolysis, by time resolved current measurements with focus on electrogenic steps. In this way ion transport or conformational transitions of ATPases can be detected.
The BLM (black lipid membrane) and SSM (solid supported membrane) techniques were used which are based on the adsorption of proteoliposmes on planar lipid membranes (bilayers). Protein pumping currents are measured after rapid substrate activation (ADP or ATP). The substrate concentration jump is generated by release of ADP (or ATP) from caged ADP (or ATP) within milliseconds after a laser flash or by rapid solution exchange.
The influence of an applied potential or concentration gradient and kinetic models are discussed. For ATP hydrolysis an ion dependent electrogenic step of the reaction cycle of 30 s^{-1} (H^+) and 70 s^{-1} (Na^+) was resolved describing probably the occlusion of the transported ion.

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4D-4
INTERACTION OF AMPHIPOLS WITH SARCOPLASMIC RETICULUM CA^{2+}-ATPASE

P. Champeil*, T. Menguy, C. Tribet, J.-L. Popot, M. le Maire

Amphipols are short-chain amphipathic polymers designed to keep membrane proteins soluble in aqueous solutions. We have evaluated the effects of the interaction of amphipols with sarcoplasmic reticulum Ca^{2+}-ATPase either in a membrane-bound or a soluble form. If the addition of amphipols to detergent-solubilized ATPase was followed by removal of detergent, soluble complexes formed, but these complexes retained poor ATPase activity, were not very stable upon long incubation periods, and at high concentrations they experienced aggregation. Nevertheless, adding excess detergent to diluted detergent-free ATPase/amphipol complexes incubated for short periods immediately restored full activity to these complexes, showing that amphipol had protected solubilized ATPase from the rapid and irreversible inactivation that otherwise follows detergent removal. Amphipols also protected solubilized ATPase from the rapid and irreversible inactivation observed in detergent solutions if the ATPase Ca^{2+} binding sites remain vacant. Moreover, in the presence of Ca^{2+}, amphipol/detergent mixtures stabilized concentrated ATPase against inactivation and aggregation, whether in the presence or absence of lipids, for much longer periods of time (days) than detergent alone. Our observations suggest that mixtures of amphipols and detergents are promising media for handling solubilized Ca^{2+}-ATPase under conditions that would otherwise lead to its irreversible denaturation and/or aggregation.

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4D-5
INITIAL STEPS OF THE INTERACTION OF SR CA^{2+}-ATPASE WITH CA^{2+}

Birte Juul, Jesper V. Møller

As an extension of our investigations of the sarcoplasmic reticulum Ca^{2+}-ATPase with the proteolytic enzyme, proteinase K (Juul et al., 1995, J Biol Chem 270:20123-34), we have produced a preparation of the ATPase consisting of mainly 2 fragments: an 83 kDa C-terminal fragment (p83C) and the corresponding 28 kDa N-terminal fragment (p28N). Functional analysis of the cleaved enzyme (p28N/p83C) shows that it binds Ca^{2+} with the same affinity as intact Ca^{2+}-ATPase. Phosphorylation with [(-^{32}P]-ATP takes place at lower free Ca^{2+} concentrations than for the native enzyme, but after reaction with ATP p28N/p83C cannot dephosphorylate nor can the cleaved preparation in the presence of EGTA become phosphorylated with $^{32}P_i$. Thus the cleaved enzyme appears to be locked in an E_1 or E_1P conformation. Testing with a panel of sequence specific antibodies shows that the N-terminal region of L6-7, the cytosolic loop between transmembrane segments 6 and 7, becomes specifically exposed by the proteinase K treatment. Furthermore, CrATP is not capable of inducing occlusion of bound Ca^{2+} as in the intact enzyme. Previously, we have obtained evidence that the L6-7 region is involved in the initial interaction of Ca^{2+}-ATPase with Ca^{2+} (Menguy et al., 1998, J Biol Chem 273:6619-31). The present findings suggest that the L6-7 loop also has a gate function and is opened as a result of conformational changes caused by the proteolytic cleavage in the N-terminal domain.

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4D-6
THE EFFECT OF NITRIC OXIDE ON NA^+-CA^{2+} EXCHANGE CURRENT IN SECRETORY CELLS

Volodimir Manko, Olga Larina, Miron Yu. Klevets

The influence of nitric oxide (NO) on functioning of salivary gland secretory cell membrane Na^+-Ca^{2+} exchanger in *Chironomus plumosus L.* larva was investigated.The Na^+-Ca^{2+} exchange current ($I_{Na(Ca)}$) was registered by means of voltage-clamp method We found: effect of GTN (100 μM) and SNP (100 μM) leads to increase of $I_{Na(Ca)}$ amplitude by (25.54 ± 6.68) and (14.14 ± 3.24)%, respectively. But because $I_{Na(Ca)}$ amplitude is known to increase in conditions of Ca^{2+}-pump functional activity depression , the effect of NO may be conditioned by not only its direct influence on Na^+-Ca^{2+} exchanger but on Ca^{2+}-pump too. So we also investigated the effect of GTN and SNP in presence of Ca^{2+}-pump blockator, eosin Y (10 μM). We found : GTN and SNP in conditions of blockaded Ca^{2+}-pump also evoke the increase of $I_{Na(Ca)}$ amplitude by (13.03 ± 2.84) and (21.55 ± 5.16)%.Changes of $I_{Na(Ca)}$ amplitude were irreversible in all four cases.So results demonstrate: salivary gland secretory cell membrane Na^+-Ca^{2+} exchanger in *Chironomus plumosus L.* larva is really the target for NO. Changes of $I_{Na(Ca)}$ amplitude evoked by effect of NO donors may be explained by influence of NO on SH-groups of Na^+-Ca^{2+} exchanger molecule because its oxidation by heavy metal ions also leads to increase of $I_{Na(Ca)}$ amplitude.

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4D-7
THE RATE DETERMINING STEP OF THE Na^+, K^+-ATPASE

Christian Lüpfert, E. Bamberg, Ronald J. Clarke

The Na^+, K^+-ATPase is one of the major energy consumers and occurs in almost all animal cells. For the hydrolysis of each molecule ATP it transports three Na^+ out of and two K^+ into the cell. The concentration gradients thus generated across the cell membrane have numerous important physiological functions, e.g. in the maintenance of the resting potential in nerve cells and as a secondary source of energy in the reabsorption of nutrients in the kidney. A model for the Na^+, K^+-ATPase mechanism is the Albers-Post cycle. This cyclic sequence of partial reactions considers two conformations of the enzyme, E_1 and E_2, and their respective phosphorylation state. A detailed analysis of this model and the determination of the individual rate constants can be achieved by the application of rapid transient kinetic methods.
We have investigated the partial reactions of the Na^+, K^+-ATPase via the stopped-flow technique using the fluorescent probe RH421. This sensitive solvatochromic and electrochromic dye together with the fast experimental technique enabled us to determine all important rate constants.
The slowest step is found between E_2K_2 and E_1Na_3 with ATP facilitating the transition. This rate-determining step is probably regulated by the cellular metabolism and was therefore investigated in more detail. The kinetic data obtained suggest that the Na^+ binding precedes the conformational change. The influence of the buffer composition on the conformational equilibrium has also been investigated.

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4D-8
REGULATION OF THE NA,K-ATPASE PUMP DURING CELL CYCLE OF ACTIVATED HUMAN LYMPHOCYTES

Irina I. Marakhova, Faina V. Toropova

Functional expression of the Na,K-ATPase pump as determined by ouabain-sensitive Rb influxes has been investigated in phytohemagglutinin (PHA)-activated human lymphocytes. A rapid rise in ouabain-sensitive Rb influx in response to PHA is found to be followed by a long-term increase in pump activity, which is associated with cell cycle progression and blast transformation. Unlike the early pump activation the late enhanced pump activity is not a result of elevated cell Na content, it is protein synthesis-dependent and is abolished by inhibitors of transcription. As quantified by specific 3H-ouabain binding there is a two-fold increase in the Na,K-ATPase density per cell during later cell cycle progression and brefeldin A blocked this pump upregulation. An immunosuppressant cyclosporin A (CsA) inhibits the long-term enhancement of the Na,K-ATPase pump when present during competence induction by phorbol ester and ionomycin, being without effect on the increased pump activity during the interleukin-2-induced progression phase. It is concluded that in mitogen-activated lymphocytes (1) the long-term control of ion pump is mediated by regulating the pump numbers in the plasma membrane via translational and tanscriptional mechanisms, and (2) the cell cycle-associated upregulation of the pump is related to a CsA-sensitive signalling pathway.
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4D-9
ON THE NA^+,K^+ PUMP IN FLUCTUATING MEMBRANE POTENTIAL

Monica Neagu, Adrian Neagu

The interplay between active transport and fluctuations of the membrane potential has been proposed in the literature as a mechanism underlying the high electrosensitivity of some living cells, via stochastic resonance. The present work investigates the usefulness of noise in the activity of the Na^+,K^+ pump. Random gating processes in the neighboring ion channels cause local fluctuations of the electric potential. They are modeled by a Markovian symmetric dichotomic noise, added to the membrane potential. The noise-averaged pump current is calculated for a general rectangular voltage signal and the model parameters of the effective enzyme cycle are determined to fit experimental results. Then, the amount of translocated charge is calculated, and studied as a function of noise intensity. Signal and noise characteristics are identified at which fluctuations enhance pump activity. The biological impact of this phenomenon seems to be absent in physiological conditions for it occurs at high noise amplitudes (over 100 mV), which are unlikely to appear due to ion channels. However, under well-defined conditions, externally applied dichotomic noise may sensibly increase the quantity of transported charge.

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4D-10
THE *ESCHERICHIA COLI* F_0F_1 ATP SYNTHASE UNDER FERMENTATION: CYSB21ALA REPLACEMENT PERTURBS K^+ UPTAKE AND H_2 PRODUCTION

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E. coli, fermenting glucose, carries out H^+-K^+-exchange and produces H_2. These are respectively accomplished through F_0F_1 and the TrkA system and by formate hydrogenlyase. An interaction of F_0F_1 with TrkA and FHL is proposed in which reducing equivalents from formate are required for energy transfer from F_0F_1 to TrkA through a disulfide-dithiol interchange. This suggest that some cysteine of F_0F_1 may be involved. It was shown that (1) H^+-K^+-exchange and H_2 production were inhibited by *N,N'*-dicyclohexylcarbodiimide (DCCD) in whole cells; (2) DL-dithiothreitol is able to restore H^+-K^+-exchange and H_2 production in the presence of a protonophore; (3) the amount of accessible thiol groups of membrane vesicles is increased 1.8-fold by ATP, and this ATP-dependent increase is inhibited by DCCD. These were observed with wild-type and a strain with cysteine-less F_1 and absent in *atp* strain with deleted F_0F_1 and a strain with cysteine-less F_0 (Cysb21Ala): a different mode in K^+-uptake was suggested. In spite of significant H^+ pumping, H^+ efflux through F_0F_1 and its turnover number were 1.6-fold less in the latest strain. The results indicate that cysteine residue in F_0 b subunit is required for H^+-K^+ uptake and H_2 production.

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4D-11
THE HIGH-AFFINITY CATION BINDING SITE OF THE SR Ca^{2+}-ATPASE STUDIED BY FTIR SPECTROSCOPY

Carola Ulbrich, Agnes Troullier, Dietrich Kuschmitz, Yves Dupont, Klaus Gerwert

The high-affinity cation binding to the Ca^{2+}-ATPase of sarcoplasmic reticulum is investigated by time-resolved Fourier Transform Infrared (FTIR) difference spectroscopy at -8 °C (1). To start the protein reaction the divalent cation is released by UV laser flash photolysis from DM-Nitrophen, a photosensitive EDTA analog (2). Spectral bands induced by cation binding appear in the difference spectra and are absent in the presence of the inhibitor thapsigargin. Some could arise from amino acids that are directly involved in the cation chelation whereas others are due to conformational changes.

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4E-2
CHARACTERIZATION OF THE ORGANIC CATION TRANSPORTER 2 FROM RAT IN GIANT MEMBRANE PATCHES

Thomas Budiman, Ernst Bamberg, Hermann Koepsell, Georg Nagel

The polyspecific cation transporter rOCT2, which is mainly expressed in renal tubules, was studied after heterologous expression in *Xenopus laevis* oocytes. Excised giant patches of the plasma membrane were prepared in inside-out configuration and patch current was tested before and after perfusion with solutions containing substrates. It could be shown that electrogenic transport of choline, tetramethyl ammonium (TMA), and tetraethyl ammonium (TEA) is facilitated in both directions whereas tetrabutyl ammonuim (TBA) and quinine elicited no change in patch current, when tested alone. Transport of choline, TMA, and TEA in both directions can be inhibited, however, by applying TMA or quinine from the cytoplasmic side of the plasma membrane. The $K_{0.5}$ constants (at the cytoplasmic side) for choline, TMA, and TEA efflux as well as the inhibiting constant K_I for quinine (applied from the cytoplasmic side) were evaluated. These are rather similar to $K_{0.5}$ and K_I constants previously determined at the extracellular side. For current-voltage relations short voltage jumps were applied. In the observed voltage range (−60 to +60 mV) transport is voltage dependent.

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4E-1
TRANSPORT OF FLUORESCENT ANIONS BY MULTIDRUG RESISTANCE-ASSOCIATED PROTEIN

Blazej Rychlik, Adam Sokal, Slawomir Chrul, Grzegorz Bartosz

Multidrug resistance-associated protein (MRP) is a member of a family of ABC transporters functioning as xenobiotic export pumps. We compared several fluorescent anions: fluorescein, 5-(and-6)-carboxyfluorescein, calcein, 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF) and 2′,7′-bis-(3-carboxypropyl)-5-(and-6)-carboxyfluorescein (BCPCF) as possible substrates for MRP in erythrocytes. Studies of transport kinetics in whole cells (using fluorogenic esters of the anions) and accumulation of the anions in inside-out vesicles, of transport inhibition by vanadate, fluoride, probenecid and other compounds, and by metabolic depletion demonstrated that BCECF and BCPCF are the best substrates for MRP, better than calcein, while fluorescein is not a MRP substrate. Basing on these results we propose a functional cytofluorimetric test for the presence of MRP based on BCECF transport. This test clearly differentiates HL60 cells and HL60Adr cells overexpressing MRP1.

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4E-3
EVIDENCE FOR A ROLE OF HELIX IV IN CONNECTING CATION- AND SUGAR-BINDING SITES OF *E. COLI* MELIBIOSE PERMEASE

Emmanuelle Cordat, Gérard Leblanc, Isabelle Mus-Veteau

In order to improve the structural organization model of melibiose permease, we assessed the individual contributions of the N-terminal tryptophans to the transporter fluorescence variations induced by the binding of cations and β-configured sugars, by replacement of the six N-terminal tryptophans by phenylalanines and the study of the signal changes. Only two mutations, W116F located in helix IV and W128F located in the cytoplasmic loop 4-5, impair permease activity. The intrinsic fluorescence spectroscopy analysis of the other mutants suggests that W54, located in helix II, W116 and W128 are mostly responsible for the cation-induced fluorescence variations. These tryptophans, W116 and W128, would also be responsible for the β-galactoside-induced fluorescence changes observed in the N-terminal domain of the transporter. The implication of W116 and W128 in both the cation- and β-galactoside-induced fluorescence variations led us to investigate in detail the effects of their mutations on the functional properties of the permease. The results obtained suggest that these two tryptophans play a critical role in the mechanism of Na^+/sugar symport. Taken together the results presented in this paper and previous results suggest a fundamental role of helix IV in connecting cation- and sugar-binding sites of the melibiose permease.

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4E-4
INVESTIGATION OF SH-GROUPS ROLE OF Na^+-Ca^{2+} EXCHANGER PROTEIN MOLECULE IN ITS FUNCTIONING

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The intracellular loop f of Na^+-Ca^{2+} exchanger contains high-density regions with SH-groups. Availability of SH-groups in Na^+-Ca^{2+} exchanger of secretory cells membrane in *Chironomus* larvae's salivary gland we have assumed on the base of changes in $I_{Na/Ca}$ at the alkalisation of external solution. In order to confirm availability of SH-groups we studed influence of its protector dithiothreitol (DTT) and blocator parachlormercuribenzoate (PHMB) on Na^+-Ca^{2+} exchange. Ca^{2+} content in cells we measured using Arsenazo III, protein content in incubate as a secretion indicator - by Lowry method. We have analyzed changes in glands Ca^{2+} content and secretion in hyposodium medium (35 mM NaCl), since at these conditions the exchanger transports Ca^{2+} into the cells. At 0.1; 0.5; 1; 5 μM DTT increase Ca^{2+} content. Increasing of Ca^{2+} content at DTT influence could be evoked by stimulation Na^+-Ca^{2+} exchange. Addition of 1 μM PHMB to medium caused increase in Ca^{2+} content and secretion, but 2.5; 5 and 10 μM PHMB decreased Ca^{2+} content. We suppose, PHMB suppress Na^+-Ca^{2+} exchange and, probably, Ca^{2+}-pump. In order to test it to medium with PHMB inhibitor of Ca^{2+}-pump - eozyn Y (5 μM) was added. At these conditions PHMB evoked increase in Ca^{2+} content and secretion: at 1, 2.5, 5 μM, at 10 μM – proofs decreased. Different effects of 1 μM PHMB + eozyn Y and 1 μM PHMB caused by PHMB influence on Ca^{2+}-pump but PHMB in higher concentrations suppresses of Na^+-Ca^{2+} exchange.

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4E-5
PROBING FLAVONOID BINDING SITE ON THE MULTIDRUG TRANSPORTER P-GLYCOPROTEIN

Jacques Ferté, Alexia Garrigues, Manuel Garrigos, Stéphane Orlowski

P-glycoprotein (Pgp) is a membrane ATPase which interacts with a wide variety of drugs, some being transported across the plasma membrane. Among Pgp-interacting compounds, flavonoids are unique, possessing multiple phenolic groups. It has been proposed that their binding site on Pgp overlaps both the steroid- and the ATP-binding sites.

We have characterized the influence of five flavonoids on the enzymatic activity of Pgp. Because the lipid environment has been shown to affect Pgp ATPase activity, Pgp-containing native plasma membrane vesicles prepared from resistant DC-3F/ADX cells were used.

Acacetin stimulates Pgp ATPase activity (1.6-fold at 16 μM). No stimulation was seen for the other flavonoids (quercetin, kaempferol, genistein, apigenin), which progressively inhibit ATPase activity above ~10 μM. In any case, the Michaelis constant for ATP hydrolysis remained unchanged.

Verapamil and progesterone stimulate Pgp ATPase activity (1.7-fold at 20 and 60 μM, respectively). Each of the tested flavonoids abolished the stimulation by either compound, in a non-competitive manner. As a whole, these results suggest that flavonoids, verapamil and progesterone bind at distinct sites on Pgp, and that flavonoids do not compete for ATP at the ATP-binding site. The presence of several distinct binding sites could account for Pgp broad substrate specificity.

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4E-6
MULTIDRUG TRANSPORTER P-GLYCOPROTEIN AND CYTOCHROME P450-3A SHARING COMMON SUBSTRATES HAVE DIFFERENT MULTI-SPECIFIC RECOGNITION MECHANISMS

Alexia Garrigues, Marcel Delaforge, Jacques Ferté, Manuel Garrigos, Stéphane Orlowski

P-glycoprotein (Pgp) is an active plasma membrane transporter responsible for the efflux of numerous potentially cytotoxic amphiphilic compounds. Pgp can recognize many structurally different cytotoxic molecules which are also recognized by cytochrome P450-3A (cytP450-3A) isoform. The respective characteristics of multiple recognition of these two enzymes are here studied using a series of amphiphilic molecules, all with a cyclic peptidic moiety. Interaction of these molecules with cytP450-3A are studied on dexamethasone-treated rat liver microsomes. Measurements of Pgp ATPase activity in presence of the same molecules are performed on Pgp-rich native membrane vesicles prepared from multidrug resistant cells. All the tested molecules recognized by Pgp are also recognized by cytP450-3A. To determine if there is a common recognition mechanism between these two enzymes, we have analysed the mutual relationships between the same molecules taken by pair. No correlation is observed between Pgp and cytochrome P450-3A. This result indicates functional and structural differences between their multi-specific sites. Furthermore, we observe that metabolites of two pharmacologically important compounds, bromocriptine and cyclosporin A, activate Pgp ATPase activity. These results suggest that cytP450-3A metabolites can be Pgp transport substrates. Thus, cytP450-3A and Pgp can interact simultaneously or/and sequentially with their substrates.

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4E-7
PH-DEPENDENT STRUCTURAL CHANGES OF NHAA NA^+/H^+ ANTIPORTER OF *ESCHERICHIA COLI* INVESTIGATED BY INFRARED SPECTROSCOPY

Oliver Klein, Miro Venturi, Etana Padan, Hartmut Michel, Werner Mäntele, Christian Zscherp

NhaA (388 amino acids) is a Na^+/H^+ antiporter from *E. coli* which is important for adaptation to alkaline pH and high salinity. Between pH 7 and 8 the activity of NhaA increases over 3 orders of magnitude. The protein is susceptible to proteolytic digestion at the pH range where it is activated (1). This led to the hypothesis that the change in activity is associated with a conformational change.

We were able to verify the hypothesis and to characterise this structural change. Using ATR/FTIR difference spectroscopy, we have observed pH-dependent changes of the absorption of the amide I band (1600-1700 cm^{-1}). The magnitude of these changes was found to be about one percent of the total absorption of the amide I band. Furthermore, the difference spectra show that α-helices are involved in the pH-dependent conformational change. Frequency component analysis of the amide I band of the absorbance spectrum suggests that the major secondary structure element of NhaA is the α-helix. This is in agreement with models from biochemical (1) and electron cryo-microscopical (2) studies which found 12 transmembrane helices. However, a significant amount of typical β-sheet absorption was observed as well. β-sheet might be located in the loops connecting the helices like in the B-C loop of bacteriorhodopsin.

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4E-8
PROTEIN TRANSLOCATION ACROSS THE MITO-CHONDRIAL OUTER MEMBRANE: STRUCTURE AND CHANNEL PROPERTIES OF THE PROTEIN TRANSLOCASE

Uwe Ahting, Markus Dembowski, Klaus-Peter Künkele, Reiner Hegerl, Dieter Typke, Frank Nargang, Michel Thieffry, Walter Neupert, Stephan Nussberger

Transport of nuclear-encoded proteins into mitochondria is catalyzed by distinct protein translocation machineries in the outer and inner mitochondrial membranes. At the outer membrane, a multicomponent protein complex (TOM complex) performs and coordinates a series of reactions, which include preprotein recognition, unfolding, insertion and translocation. We have purified the TOM complex from *Neurospora crassa,* determined its subunit stoichiometry, analyzed its function, and determined its three-dimensional structure using electron tomography and image analysis techniques (Künkele *et al.*, 1998, *Cell* 93, 1009-1019, Ahting *et al.*, 1999, *J. Cell Biol.* 147, 959-968). Using electrophysiology methods we show that the TOM complex contains a cation-selective high conductance channel that can be blocked by signal sequence peptides. Fluorescence correlation spectroscopy allowed to determine the apparent affinity of preproteins to isolated TOM complex. Upon reconstitution into liposomes, the TOM complex mediates integration of proteins into and across the lipid bilayer. Electron tomography and image analysis reveal particles with a diameter of about 12 nm and a height of 7 nm. TOM complex particles were found with one, two and three pores with a diameter of about 2.1 nm, which we propose represent protein-conducting channels.

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4E-9
CHOLESTEROL IMPORTANCE FOR THE ACTIVITY OF MULTIDRUG TRANSPORTER P-GLYCOPROTEIN

Alexia Garrigues, Stéphane Orlowski

The multidrug transporter P-glycoprotein (Pgp) is responsible for the efflux of various amphiphilic molecules and seems to be particularly sensitive to its membrane environment. Cholesterol is a physiological component of the lipid phase which modulates the membrane fluidity. We investigated the cholesterol effects on the basal (i.e. without any apparent substrate) and on the drug-stimulated Pgp ATPase activities. Measurements of these enzymatic activities are performed on Pgp-rich native membrane vesicles prepared from multidrug resistant cells. Increasing cholesterol concentrations have no significant effect on basal and drug-stimulated Pgp ATPase activity. The depletion of membrane cholesterol is mediated by methyl-β-cyclodextrin, a water-soluble cyclic oligosaccharide with a hydrophobic cavity capable of dissolving hydrophobic molecules. No difference in the enzyme turn-over is observed on the drug-stimulated ATPase activity of cholesterol-depleted vesicles and control vesicles. In contrast, the basal activity disappears when the vesicles are depleted in cholesterol. This cannot be attributed to membrane perturbations because the other membrane ATPases are not affected. Furthermore, the reloading of depleted vesicles with cholesterol restores the basal activity which is thus cholesterol-dependent. In conclusion, the basal and drug-stimulated Pgp ATPase activities can be enzymatically dissociated. This suggests a specific interaction between cholesterol and Pgp. Thus cholesterol might be a transport substrate coupled with the basal ATPase.

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4E-10
SUBSTRATE AND VOLTAGE DEPENDENCE OF THE INVERSE MODE OF THE RABBIT Na^+/GLUCOSE COTRANSPORTER SGLT1

Gudrun A. Sauer, Georg Nagel, Hermann Koepsell, Ernst Bamberg, Klaus Hartung

The aim of this study was to investigate the properties of the cytoplasmic binding sites of rabbit Na^+/glucose cotransporter SGLT1, expressed in *Xenopus* oocytes using the giant excised patch clamp technique. Substrate and voltage dependence of the outwardly directed currents were studied by varying cytoplasmic sugar and sodium concentrations at different holding potentials. The experiments show that the application of a glucose derivative α-Methyl-D-glucopyranoside (αMDG) generates outwardly directed currents due to SGLT1. The αMDG dependence can be described by Michaelis-Menten kinetics. The apparent affinity for αMDG depends on sodium concentration and voltage. $K_M^{\alpha MDG}$ increases with decreasing sodium concentration ($K_M^{\alpha MDG}$ = 7 mM at 110 mM Na^+ and 31 mM at 10 mM Na^+ at V = 0 mV). The currents and the affinity increase at positive potentials. At 250 mM αMDG and V = 0 mV, the Na^+ dependence of the currents can be described with $K_M{}^{Na}$ = 38 mM and n = 1.5. The $K_M{}^{Na}$ for Na^+ increases slightly with positive potentials. The results show that SGLT1 can also transport sugar out of the cell. However, under physiological conditions, the low cytoplasmic affinity for sugar makes an outward transport highly improbable. The results are compatible with weak voltage dependence of Na^+ binding on the cytoplasmic side and negative charge translocation by the empty carrier. Furthermore there is evidence that cytoplasmic substrate binding is ordered, i. e. Na^+ binding precedes sugar binding.

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4E-11
PROTON AND SODIUM DEPENDENCE OF THE EXCITATORY AMINO ACID CARRIER EAAC1

Natalie Watzke, Thomas Rauen, Ernst Bamberg, Christof Grewer

The activity of glutamate transporters is essential for the temporal and spatial regulation of the neurotransmitter concentration in the synaptic cleft, and thus, is crucial for proper excitatory signaling. The driving forces for glutamate translocation are the transmembrane gradients for Na^+, K^+ and H^+, as the transport of one glutamate anion is coupled to the cotransport of three Na^+ and one H^+ and to the countertransport of one K^+. Since the binding order of these ions at the extracellular side with respect to glutamate is still under debate, it was the topic of this work. EAAC1 was heterologously expressed in HEK293 cells and the transporter function was monitored with the patch clamp technique. We found that at saturating concentrations the glutamate-induced maximum currents (I_{max}) show only a sodium dependence, but not a pH dependence. In contrast, the K_M for glutamate depend on both, sodium and proton concentration. For the order of substrate binding, these findings indicate that the proton binds before glutamate and at least one sodium binding step follows the glutamate binding process. In conclusion, the results are consistent with a cyclic transport model in which ordered, but not random cation and substrate binding steps precede glutamate translocation across the membrane.

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4E-12
ROLES OF THE BETA BARREL AND "PLUG" DOMAINS IN STABILITY OF FHUA, AN *E. COLI* OUTER MEMBRANE TRANSPORTER

Mélanie Bonhivers, Pascale Boulanger, Michel Desmadril, Greg Moeck, Lucienne Letellier

FhuA (MM 78,9 kDa) is an *Escherichia coli* outer membrane protein that transports iron coupled to ferrichrome and is the receptor for a number of bacteriophages and protein antibiotics. Its three-dimensional structure consists of a 22 stranded transmembrane beta barrel, of extracellular hydrophilic loops loops, and of a globular domain, the "plug" located within the beta barrel and occluding it. This unexpected structure raises questions about the connectivity of the different domains and of their respective roles in the different functions of the protein. To address these questions we have compared the functional and structural properties of the wild type receptor to those of a mutated FhuA from which a large part of the plug had been removed. Differential scanning calorimetry experiments on wild type FhuA indicated that the plug and the beta barrel behave as autonomous domains. Ferrichrome although binding to a loop had a strong stabilizing effect on the plug. Removal of the plug destabilized the beta barrel even in the presence of ferrichrome, increased the sensitivity of the protein towards proteolysis and denaturant agents and impaired phage T5 binding. Taken together these studies indicate that a change of conformation upon ligand binding on the external loops of FhuA can be sensed by the plug. Such results are expected to apply for proteins of the same family (like FepA) which share similar structures.

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5A-1
DIRECT OBSERVATION OF DNA ROTATION DURING TRANSCRIPTION BY ESCHERICHIA COLI RNA POLYMERASE

Yoshie Harada[1,2,3], Nobuo Shimamoto[4], Kazuhiko Kinosita Jr[2,3]

RNA polymerase plays a key role in the transcription of gene expression by synthesizing RNA transcripts from DNA templates. To transcribe DNA, RNA polymerase moves along the DNA helix. Whether RNA polymerase precisely follows DNA helix is an unanswered question bearing directly on the mechanism.Direct and real-time measurement is desirable, however, particularly because assessing the fidelity in helical tracking is important for elucidating the transcription mechanism. Here, we show by optical microscopy that a single RNA polymerase molecule attached to a glass surface rotated DNA for > 100 revolutions, in the direction of a right-handed screw as anticipated. The rotation rate was consistent with precise helical tracking, and the rotary torque was > 4 pN nm. The real-time observation of rotation opens the possibility of resolving individual transcription steps allowable.

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5A-2
ACTIVELY CONTRACTING BUNDLES OF POLAR FILAMENTS

Karsten Kruse, Frank Julicher

We introduce a phenomenological model to study the properties of bundles of polar filaments which interact via active elements. The stability of the homogeneous state, the attractors of the dynamics in the unstable regime and the tensile stress generated in the bundle are discussed. We find that the interaction of parallel filaments can induce unstable behavior and is responsible for active contraction and tension in the bundle. Interaction between antiparallel filaments leads to filament sorting. Our model could apply to simple contractile structures in cells such as stress fibers.

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5A-3
STOCHASTIC INTEFERENCE IN ENZYME COUPLED REACTIONS

Ken Mogi

In enzyme coupled reactions, the coupling ratio is not necessarily one. A striking example is the multiple powerstrokes observed in muscle contraction (originally reported in Yanagida et al. *Nature* 316, 366–369. 1985). The stochastic intereference effect (Mogi, K. *Proc. Roy. Soc. Lond.* A, 445, 529-541. 1994) was proposed to account for the energetics involved in enzyme coupled reactions, in particular where the coupling ratio is not one. In this model, it is assumed that the enzyme puts a constraint on the relative ratio of the reaction rates of the coupled degrees of freedom. This assumption leads to an interference effect between the coupled degrees of freedom, in a way compatible with the basic principles of chemical reaction energetics. One of the predictions is that the free energy is evenly distributed among the multiple events (e.g powerstrokes) driven by the enzyme coupled reaction. Here, I develop a detailed model of this mechanism by taking muscle contraction as an example, taking into account the recent evidences from structural and imaging studies. I elaborate on how we could experimentally verify this model as opposed to other models that tries to explain the multiple powerstrokes, such as the thermal ratchet model. The essential role played by thermal fluctuations is examined in terms of the structural change the enzyme undergoes in the course of the coupled reaction.

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5A-4
ENERGETICS OF ISOTHERMAL RATCHETS AND APPLICATIONS TO MOLECULAR MOTORS

Andrea Parmeggiani, F. Jülicher, A. Ajdari, J. Prost

We study the energetics of isothermal ratchets which are driven by achemical reaction between two states and operate in contact with asingle heat bath of constant temperature. We discuss generic aspectsof energy transduction in the linear response regime as well as theefficiency and dissipation close to and far from equilibrium. Studyingthe properties for specific examples of energy landscapes and transitions, we observe in the linear response regime that theefficiency can have a maximum as a function of temperature. Far fromequilibrium, in the fully irreversible regime (the typical regime for motor proteins), we find for some system the maximum of the efficiency. We show that the efficiency of isothermal ratchets can be of the order of 50% and higher. Finally we discuss some application of energetics of these simplified models to different biological motor proteins.

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5A-5
NUCLEOTIDE DEPENDENT (DIS)ORDER IN THE KINESIN-TUBULIN INTERACTION

Erwin J.G. Peterman, Hernando Sosa, Larry Goldstein, W.E. Moerner

We have studied the orientation and flexibility of the kinesin-tubulin binding with fluorescence polarization microscopy of individual and many kinesin motors bound to axonemes. For these experiments we prepared a monomeric, cystein-light kinesin construct to which we attached a bisfunctional rhodamine derivative. We measured the orientation of individual and many motors on axonemes using wide-field epi-fluorescence microscopy, utilizing alternating polarization of the excitation laser light. We have observed that kinesin in the nucleotide free, ATP-, and ADP-Pi-bound states attaches to tubuline in an ordered way. In the ADP-bound state however, the binding is disordered and flexible. The transition between this flexible state and a rigid state after ADP release could contribute to force generation and translocation of kinesin along microtubules

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5A-6
MOTILITY BY SINGLE MOLECULES OF THE KINESIN-RELATED NCD MOTOR

Christoph F. Schmidt, Michael J. Decastro, Regis M. Fondecave, Leigh A. Clarke, Russell J. Stewart

The ncd protein is a dimeric ATP-powered motor belonging to the kinesin family of microtubule motor proteins. We used optical tweezers-based instrumentation and a three-bead, suspended-microtubule assay to resolve single mechanochemical cycles of recombinant dimeric full-length ncd. Under conditions of limiting ATP, isolated and transient microtubule binding events were detected with exponentially distributed and ATP-concentration dependent lifetimes. We observed no consecutive steps along the microtubule, quantitatively confirming that ncd is non-processive. At low load a single motor produced ATP-triggered working strokes of about 9 nm, which occured at the end of the binding events.

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5A-7
COMPLIANCE OF SEMIFLEXIBLE FILAMENTS IN THREE-BEAD MOTILITY ASSAYS

Joost V. Mameren, Jeroen V. Zon, Wolfgang Möhler, Frederick Gittes, Christoph F. Schmidt

A typical single-molecule assay for non-processive motor proteins is the three-bead assay, where an actin filament or a microtubule is suspended with the help of two optical tweezers and "handle beads" and then brought into contact with a motor protein attached to a substrate-immobilized bead. In most cases the handle beads are laterally attached to the protein filament. This introduces an additional series compliance in the assay that hampers the detection of conformational changes of the motor protein. We present a simple mechanical model for this non-linear compliance and compare the model to experimental results. Counterintuitively, at relatively low tension in the filament, the effective compliance with microtubules is very similar to that achieved with actin filaments although the bending stiffness of actin is about 100× lower than that of microtubules.

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5A-8
DECORATION OF MICROTUBULES WITH KINESIN: A THEORETICAL STUDY

Andrej Vilfan, Erwin Frey, Franz Schwabl

We study the binding of two-headed proteins like kinesin on microtubules. We assume that each kinesin molecule can either bind with one head on one bindng site or with both heads on two adjacent sites on the same protofilament. Taking into account the crowding on the lattice, we derive an analytical expression for the binding stoichiometry. Depending on kinesin concentration, it can lie between 1 and 2 heads per tubulin subunit.
We further introduce an attractive interaction between attached kinesin molecules and show that it can provide an explanation for the observed 16nm longitudinal periodicity (1) and cooperative decoration (2).

1) M. Thormählen et al., J. Mol. Biol. 275, 795–809 (1998)
2) A. Vilfan, E. Frey, F. Schwabl, M. Thormählen, Y.-H. Song, E. Mandelkow, submitted (2000)

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5B-1
MYOSIN-INDUCED ACTIVATION OF STRIATED MUSCLE THIN FILAMENT: A COOPERATIVE EFFECT OVER 260 ACTIN MONOMERS

Juliette Van Dijk[1], Alex Knight[2], Justin Molloy[2], Patrick Chaussepied[1]

Myosin motor domain (S1) is known to participate in the Ca^{2+}-tropomyosin-troponin (Tm-Tn) linked regulation of striated muscle contraction. In order to understand its activating effect, we measured the ATPase activity and the sliding velocity of thin filaments containing S1 (at a 1:50 S1:actin molar ratio) chemically cross-linked to either one or two actin monomers. The actin-S1 complex (with one cross-linked actin) showed optimum Tm-Tn-linked regulation of its ATPase with an inhibition rate higher than 80% in the absence of Ca^{2+}. In contrast, the $actin_2$-S1 complex lost its regulation but also increased the (EGTA)ATPase activity of adjacent S1 molecules up to 70% of Vmax with a very strong cooperative effect saturating at one $actin_2$-S1 for 260 monomers. On the other hand, the Ca^{2+}-linked regulation of the sliding velocity of the thin filaments along myosin in *in vitro* motility assay was not affected by up to one $actin_2$-S1 for 20 monomers. The data support the three (blocked, closed, open) states regulatory model with S1 being able to inhibit specifically the blocked to closed transition over 260 actin. The results also show that in contrast to the ATPase activity, the sliding velocity of striated muscle actomyosin does not need the blocked state to be fully regulated by the Ca^{2+}-tropomyosin-troponin system.

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5B-2
OLIGOMERIZATION OF SMOOTH MUSCLE MYOSIN LIGHT CHAIN KINASE AS A POSSIBLE MECHANISM OF ITS ACTIVATION

Valentina M. Danilova, Anatoly M. Filenko, Eduard B. Babiychuk, Basil S. Omelyanuk, Apolinary Sobieszek

Myosin light chain kinase (MLCK), enzyme playing key role in the regulation of smooth muscle contraction, is activated by Ca^{2+}-calmodulin (CaM). At the study of the kinase activation we found its oligomeric species with different affinity to CaM (Sobieszek et al., Biochem. J., 1993, 295, 405; Filenko et al., Biophys. J., 1997, 73, 1593). In this work we show that MLCK may be regulated through its oligomerization. Using zero-length covalent cross-linker 1-ethyl-3(dimethylaminopropyl) carbodiimide, together with laser correlation spectroscopy and light scattering we showed that MLCK exists in solution as dynamic equilibrium of monomer, dimer and oligomer species. Oligomeric state of kinase was shown to depend on melittin, an effective antagonist of CaM, which can be a useful instrument at the study of subtle mechanisms of MLCK activity modulation. The studies showed that kinase dimer formation is the result of interactions between autoinhibitory domain of one kinase molecule and C-end titin-like domain II of another one. Kinase aggregates are formed due to the interactions between C-end domains of individual molecules. But additional interactions between C-end domain of one molecule and N-end of another one are needed to form soluble oligomers. We propose enlarged model of the kinase activity regulation considering its oligomeric properties.

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5B-3
THE RAPID BURST OF ATP HYDROLYSIS AT THE BEGINNING OF CONTRACTION IN PERMEABILIZED SKELETAL MUSCLE FIBRES OF THE RABBIT

Zhen-He He, Michael A. Ferenczi

The ATPase rate is higher at the beginning of a contraction than in the steady state (J. Physiol. 501: 125–148, 1997). Is this transient phase of P_i release caused by shortening of the fibre against its compliant ends, is it a feature of the ATPase mechanism itself, or is it a combination of both? The ATPase rate was measured by following fluorescence enhancement resulting from P_i binding to modified phosphate binding protein MDCC-PBP (J. Physiol. 517: 839-854, 1999). Contractions were initiated from the rigor state by photolytic release of ATP from caged-ATP. Cross-linking a fraction of myosin cross-bridges to actin with the cross-linker EDC prevents sarcomere shortening whilst maintaining the contractile characteristics of muscle fibres (Bershitsky & Tsaturyan, 1995 Biophys. J. 69: 1022-1021). With ~10% cross-linking, sarcomere shortening was mostly eliminated and the ATPase was found to proceed in two distinct phases. The initial phase had a rate of $5.8 \pm 0.4\ s^{-1}$ ($n = 13$) at 12 °C and an amplitude corresponding to $64 \pm 4\ \mu M\ P_i$, 47% of non-cross-linked cross-bridge sites. The second phase proceeded linearly until near exhaustion of P_i-free MDCC-PBP, with a rate constant of $3.0 \pm 0.3\ s^{-1}$. About half of the initial high ATPase rate observed previously results from the enhanced ATPase rate which accompanies shortening, and the rapid phase of P_i release which remains once shortening is eliminated is caused by accumulation of the A.M.ADP species which predominates during the plateau of isometric contraction.

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5B-4
EXPERIMENTAL EVIDENCE FOR HYSTERESIS OF THE MEMBRANE POTENTIAL IN SKELETAL MUSCLE

R.J. Geukes Foppen, H. van Mil, J. Siegenbeek van Heukelom

Previously a computer model was constructed to simulate the behaviour of the membrane potential (V_m) of skeletal muscle fibers in low potassium media. The model reproduced the experimental results, which showed, that Vm depolarizes when extracellular potassium (K_o) was reduced beyond a certain critical potassium value (K_c). These results deviate from the hyperpolarization predicted by the Goldman-Hodgkin-Katz equation. The model consists of three passive fluxes for Na, K and Cl, including an empirical equation for the inward potassium rectifier channel, and a kinetic equation for active transport through the Na/K pump. Subsequently, a mathematical analysis of this model was performed, and it suggested the existence of hysteresis for V_m around K_c.
We used intracellular microelectrodes (3 M KCl: 20–80 MW) to monitor V_m in fibers of the mouse lumbrical skeletal muscle. In this preparation and at an osmolality of 290 mOsm, K_c is found between 1–3 mM K_o. We reduced K_o to K_c in small steps. This hyperpolarized V_m. Then K_o was stepped beyond K_c. This depolarized V_m, after which K_c was returned to the K_o value above K_c. This did not repolarize V_m to the previous value. These results comply perfectly with the mathematical analysis suggesting hysteresis. Na/K/2Cl cotranporter activity and β2-adrenergic pathways can modulate the hysteresis loop.

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5B-5
MYOFIBRILLAR CONTRACTION ON-LINE

Corinne Lionne, Robert Stehle*, Franck Travers, Tom Barman

Muscle contraction results from the conversion of the chemical energy of ATP hydrolysis by the myosin heads into mechanical energy used for producing force or movement. The key problem is to solve the temporal and spatial relations between these different events. With skeletal muscle, this coupling is regulated by calcium. Classically, ATPase activity has been studied on isolated molecules in solution (myosin sub-fragment 1 and actin) whereas the mechanical properties have been obtained with muscle fibres. Moreover, structural studies have used truncated and modified proteins, often with nucleotide analogues. However, despite this bulk of interesting data, the key problem has not yet been resolved.
Our work is based on 3 approaches :
experimental model as simple as possible, yet organised and fully regulated : the myofibril;
acto-myosin interactions (intrinsic tryptophan fluorescence measurements), ATPase (Pi measurements) and shortening (video microscopy) studied in parallel;
these processes, which are very rapid and interconnected, slowed down by cryoenzymology.
We show that the coupling between the chemical and mechanical cycles is not straightforward and the results obtained are not explained easily by classical models of muscle contraction.
Corinne Lionne is grateful to AFM and the Société de Secours des Amis des Sciences for financial support.

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5B-6
ELECTROMYOGRAPHICAL MAPPING OF THE FROG EXTRAOCULAR MUSCLES ACTIVITY

M.S. Talaat, Ibrahim I.H., Abdelsattar M. Sallam

Electromyographic (EMG) mapping of the frog extraocular muscles have been carried out using surface wick Ag-AgCl electrodes. Eight muscles were investigated. The characteristics of potentials obtained by the EMG mapping of the frog extraocular muscles showed significant differences. They ranged from 20–800 μV in amplitude. They were diphasic or triphasic in form,with occasional polyphasic potentials encountered as in case of the medial rectus muscle and the levator palpebrae superioris muscle. The duration of the unit discharge of EMG records ranged from 4.5–665 msec. The rate of firing of the whole waveform was in the range of 0.4–2/sec, where the highest firing frequency was recorded from the lateral and medial rectal muscles.

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5B-7
STUDY OF GLOBAL AND LOCAL CONFORMATIONS IN MYOSIN BY DSC AND EPR

Dénes Lorinczy*, Nóra Hartvig, Nelli Farkas, Joseph Belagyi

EPR and ST EPR in combination with DSC were used to examine the effect of nucleotides on myosin in muscle fibres. A new procedure was applied to obtain ST EPR spectra with precise phase setting. The main goal was whether the different order of spin labels come from the same or different global conformations of myosin heads.
Muscle fibres were labelled with isothiocyanate probe molecules at Cys-707 of myosin. In comparison to rigor significant differences in the orientational order of spin labels were detected upon addition of ADP or AMP.PNP to myosin evidencing changes in the domain orientations. In the presence of AMP.PNP about half of myosin heads were in strongly binding ADP-state, whereas the other half of heads were in weakly binding state.
In rigor four transitions were identified (52 °C, 59 °C, 63 °C, 67 °C); the two higher were affected by nucleotide binding. The contribution of the third transition to the total melting enthalpy reduced to its half, whereas the enthalpy of the last transition markedly increased after binding of AMP.PNP.
The results suggest that in the presence of ADP and AMP.PNP the attached heads have the same global orientation as in rigor, but the internal structure undergoes a conformational change.
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5B-8
A CORRELATIVE ANALYSIS OF MUSCLE MYOSIN FILAMENT ASSEMBLY AND STRUCTURE

N.S. Miroshnichenko, V.I. Ursulenko, A.O. Halyleev, A.P. Slabchenko, I.V. Balanuk

Myosin is present in all eukaryotic cells, being especially abundant and ordered in muscles. It is an actin-based molecular motor providing muscle contraction, cytokinesis, organelle movement and other intracellular motilities. In addition, myosin can self-associate to form myosin filaments or thick filaments. The backbone of the myosin filament is an aggregate of α-helical coiled coil myosin rods. Currently there is no adequate model to describe the organization of the myosin filament. We have suggested a model of the thick filament organization based on molecular modeling, principles of aggregation, and analysis of X-ray and electron microscopic data. It is proposed here that, in cross-section the LMM of 18 myosin molecules form an outer tube, with nine S2 forming the interior core. At the surface of the thick filament heads of two myosin molecules are organized at an angle of 120 degrees to each other on the same level, while the third is shifted 7.2 nm along the filament axis. Iteration of this structure forms a three-stranded helix and gives a striation pattern of 7.2 and 14,3 nm by electron microscopy. The packing of individual molecules within the myosin filament is based on a regular pattern of charge on the 28 amino-acid repeat in the rod domain. Computer modeling and calculation of electrostatic interaction between coiled coil rods of myosin molecules forming the backbone of filament confirm the validity of proposed myosin filament assembly.

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5B-9
EFFECTS OF FREE RADICALS ON MUSCLE PROTEINS

Márta Kiss, Nóra Hartvig, Dénes Lorinczy*, Joseph Belágyi

Using EPR and DSC techniques the reaction of -SH-containing proteins with Ce(IV)-ions in the presence of spin trap PBN were studied. The EPR spectrum is characteristic of a strongly immobilized spin adduct that is rigidly attached to the protein.
In G-actin the most reactive -SH groups are Cys-235 and Cys-374, whereas in F-form the most accesible thiol group is Cys-374. The free radical concentrations after Ce(IV)- treatment were 0.42 mole of PBN and 0.37 mole of PBN per mole of actins. The hyperfine splittting of the two forms of actin were about the same (G-actin: $2A_{zz}$ = 63.71 G, F-actin: $2A_{zz}$ = 63.54 G) showing that mild oxidation by Ce(IV)-NTA can modify the protein structure in the neighbourhood of the thiol sites. DSC measurements of F-actin resulted in a narrow thermal transition at 70.4 °C showing the large cooperativity between the actin subunits. After Ce(IV)-NTA treatment the transition temperature shifted to 65.5 °C.
The mild oxidation of myosin in muscle fibres in the presence of PBN generated strongly immobilized nitroxide free radicals. Saturation transfer EPR measurements revealed that the rotational correlation time was 170 μs evidencing the rigid attachment of the PBN to the oxidised thiol sites. The ATPase activity of the myosin supports that the free radicals are located partly on the essential -SH groups of myosin.
(Grants: OTKA T 030248, CO-123, CO-272 and FKFP 0387/2000).

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5C-1
ENERGY TRANSFER IN F_1-ATPASE STUDIED BY MOLECULAR DYNAMICS SIMULATIONS

Rainer Böckmann, Helmut Grubmüller

ATP synthase [1] is believed to be a rotatory engine driven by proton- or sodium-motive force and is the smallest molecular motor known [2,3]. The proton flow through the membrane portion F_O drives the synthesis of ATP in the three active sites of the headpiece, F_1.The energy transfer from the F_O portion to the active sites in the F_1 portion is assumed to be mediated by the γ-subunit, an asymmetric, coiled-coil shaft. This subunit acts like a crankshaft within the trigonal $(ab)_3$ subunit of the F_1 headpiece.
The goal of the present project is to study the mechanical properties of the crankshaft. In particular we studied (a) if the γ-subunit is stable enough to transmit the necessary torque and (b) if the shaft can act as a transient energy buffer by elastic deformation. Our results indicate that the γ-subunit as a part of the ATPase can not withstand the necessary applied torques. Accordingly, the γ-subunit may unfold/refold during rotation.
Future work will concentrate on conformational changes in the headpiece of the ATPase associated with the rotation of the γ-subunit and with the binding and release of ADP and ATP.
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[2] D. Sabbert, S. Engelbrecht and W. Junge, Nature 381(1996), 623
[3] H. Noji, R. Yasuda, M. Yoshida and K. Kinosita, Nature 386 (1997), 299 302

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5C-2
2D-CRYSTALLIZATION AND STRUCTURAL CHARACTERIZATION OF THE CHLOROPLAST H^+-ATP SYNTHASE

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ATP synthases are the smallest rotary motors in biology. The flow of protons propels the rotation of a transmembrane entity composed of identical protein subunits III. The number of subunits in this proton turbine determines the H^+/ATP stoichiometry and therefore the efficiency of energy conversion. In all current models of the structure-function relationship of ATP synthases, this rotating oligomer consists of 12 subunits. In chloroplast ATP synthase, however, by atomic force microscopy we visualize 14 subunits III arranged in a cylindrical ring, surrounding subunit IV. The central position of subunit IV requires redesign of the proposed functional models of F_oF_1-ATP synthases.
The narrow and the wider orifice of the subunit III oligomer exhibit outer diameters of 5.9 ± 0.3 nm and 7.4 ± 0.3 nm, respectively. Both orifices have inner diameters of 3.5 ± 0.3 nm. The 7.3 ± 0.3 nm long oligomer traverses the 4.1 ± 0.2 nm thick lipid bilayer. In most cases 14 subunits per oligomer can directly be counted from the images recorded. From angular power spectra calculated from 320 individual images of well preserved particles, the 14-fold symmetry of the proton turbine is confirmed.
Additional information is gained from the projected structure of subcomplexes obtained by electron crystallography of 2D crystals.

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5C-3
SUBUNIT ARRANGEMENT IN THE MEMBRANE PART OF CHLOROPLAST ATP-SYNTHASE INVESTIGATED BY ELECTRON MICROSCOPY

Jochen Eisfeld, Peter Gräber

ATP-Synthases consist of a hydrophilic F1-part containing the nucleotide binding sites and a hydrophobic membrane integrated F0-part which is involved in proton translocation. We isolated the F0-part from *Spinacia oleracea* chloroplasts using a new protocol. Starting with the holoenzyme, the F1-part was removed by treatment with Na-thiocyanate, then the F0-part was purified by a sucrose density gradient centrifugation followed by native preparative electrophoresis.
Due to the mild purification conditions all four F0 subunits I, II, III & IV were found in the resulting complex. Since the protein was obtained in dodecylmaltoside-lipid-micelles, 2D crystallisation was initiated by dialysis in presence of phospholipase A.
Imaging in transmission electron microscopy after negative staining yielded pictures of large flat sheets of aggregated F0, not yet crystalline but amenable to single particle image processing.
Data obtained so far show an asymmetrically segmented outer ring of protein surrounding an annular central mass. This suggests an F0 assembly of a ring of subunits III, in which subunits I & II are embedded, arranged around central subunit IV.
Atomic force microscopy investigations on the sheets are currently being conducted in order to obtain a detailed surface map.

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5C-4
STRUCTURAL STUDIES OF SUBUNIT C OF THE ATP SYNTHASE

Ulrich Matthey, Georg Kaim, Daniel Braun, Kurt Wüthrich, Peter Dimroth

ATP synthases catalyze the formation of ATP driven by an electrochemical gradient. The enzymes consist of two subcomplexes: the membrane embedded F_0 part and the associated F_1 moiety. On expense of an electrochemical gradient ions are translocated through the F_0 part coupled to ATP synthesis in F_1. A central γ subunit in an $\alpha\beta$ hexamer activates the three catalytic centers of the F_1 moiety by rotation. Therefore, the ion translocation has to be converted into the generation of torque. Biochemical investigations led us propose the following "one channel model", which comprises a revolving subunit c ring as part of the rotor and the subunit a as part of the stator: A subunit a channel connects the periplasm with an ion binding site on subunit c. Directed rotation of the subunit c ring *versus* subunit a is mediated by the attraction of charges and the membrane potential. After leaving the rotor/stator interface the subunit c-bound ion diffuses into the cytoplasm.
We investigated in structural analysis of subunit c from *Propionigenium modestum* by NMR. In SDS micelles, the *P. modestum* subunit c folds into four clearly defined α-helices connected by short linker peptides with non-regular structure. The Na^+ binding residues are located in the I • II, III • IV helix connectivities, probably near the membrane surface on the cytoplasmic side. These results favour the one channel model. Further investigations of subunit c monomer and the subunit c ring are in progress.

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5D-1
ENHANCED DIFFUSION OF AN INTRACELLULAR PROBE

Avi Caspi, Rony Granek, Michael Elbaum

We study the motion of a probe driven by cytoskeleton-associated motors within a living eukaryotic cell. The measured mean square displacement, $< x^2 >$ of an engulfed micro-sphere shows enhanced diffusion scaling as $t^{3/2}$ at short times, with a clear crossover to ordinary or even sub-diffusive scaling at long times, i.e. t^{γ}, with γ less or equal 1. Interpretation of the broken integer power law is based on two diverse topics widely studied on purified cytoskeleton filaments: 1) the microtubules and f-actin associate with ATP-hydrolyzing motor proteins to generate directed forces, resulting in production of mechanical work and dynamic transport processes. 2) thermal bending modes of these semi-flexible polymers lead to anomalous diffusion phenomena of particles embedded in purified gel networks or attached to single filaments, with $< x^2 > \sim t^{3/4}$. The motion of the intracellular probe results from random forces generated by motor protein interactions rather than thermal collisions for the classical Brownian particles. The average duration of such interactions is of order seconds rather than of pico-seconds for thermal collision, which renders the ballistic time regime observable. The $t^{3/2}$ scaling is a result of the time-dependent friction imposed by the non-Newtonian medium in which the driven motion takes place, following a generalized Einstein relation. This sub-ballistic driven motion is analogous to the sub-diffusive motion observed in passive networks of semi-flexible biopolymers.

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5D-2
LOCAL MEASUREMENTS OF VISCOELASTICITY AND INTRACELLULAR FORCES IN DICTYOSTELIA CELLS BY MAGNETIC BEAD MICRO RHEOLOGY

Wolfgang Feneberg, Günther Gerisch, Erich Sackmann

We applied a micro rheology technique based on magnetic tweezers to measure local viscoelastic moduli and active forces on colloidal probes in cells of dictyostelium discoideum. The nearly random motion of colloidal beads was analyzed by a particle tracking algorithm which allowed to measure the length of nearly straight steps of movement and the corresponding velocities. The motion consists of a superposition of nearly straight long range steps (length scale in micrometer range) and local random walks of step-widths of the order of 100 nm. The velocities for the former range from 1 to 3 μm/s and they decrease with increasing bead size. They are attributed to transportation along microtubuli. The short range local motions exhibit velocities of less than 1μm/s and can be determined by the wiggling of the microtubuli.
The viscoelastic behavior was studied by application of external forces of 50 to 400 pN. For high forces the beads are deflected in the magnetic field direction enabling measurements of the cytoplasmic viscosity η_D. For low forces the beads exhibit similar quasi-random walks as in the absence of external forces while the velocities along long range trajectories are modified in a force dependent way. This is attributed to the superposition of the active force and the magnetic force enabling absolute measurements of the active forces. The forces lie in the range of 50 pN and correspond to the cooperative action of up to 6 Kinesin motors.

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5D-3
STUDY OF THE NUCLEAR IMPORT OF PROTEINS BY FLUORESCENT CORRELATION SPECTROSCOPY

Cécile Fradin, Micheal Elbaum

In cells, proteins containing certain peptides called nuclear localization signals (NLS) are directed from the cytoplasm to the nucleus, through the nuclear pore complexes (NPC). It is known that this transport involves binding to soluble proteins found in the cytoplasm, namely transportin α and transoprtin β, which mediate the passage through the NPC. We study the location and coordination of this binding, by measuring (by fluorescent correlation spectroscopy) the diffusion coefficient of fluorescent protein cargo labeled with NLS, and comparing it to the diffusion coefficient of the similar cargo labeled with a modified peptide that does not promote nuclear import. Preliminary results in cell extract show that the NLS binds to other proteins, whereas the mutant NLS does not, hinting that the specific binding of proteins labeled with NLSs occurs already in the cytoplasm, and not only at the NPCs entrance.

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5D-4
VISCOELASTIC MICROSCOPY OF HETEROGENEOUS *IN-VITRO* ACTIN NETWORKS

Manfred Keller, Erich Sackmann

The viscoelastic properties of heterogeneous networks of the semiflexible polymer F-actin and the motor-proteins myosin and its active subfragment HMM are investigated. The method used for this purpose is mainly the local measurment of the viscoelastic parameters by means of micro-rheometry with the Magnetic-Tweezers method.
An aim is to establish an *in-vitro*-model of the actin-myosin-cortex of cells that plays a central role for many chemo-mechanical processes in the cell. Another aim is the investigation of the microstructure of a heterogeneous gel of semiflexible polymers and the special viscoelastic properties of these biologically important micro-gels. The actin-myosin-system is an ideal model for the investigation of micro-gels. The heterogeneity of the networks can be observed and characterized by electron-microscopy and by measuring the spatial distribution of the local viscoelastic parameters by the Magnetic-Tweezers method. This latter method can thus be used as a viscoelastic microscope for measuring the spatial distribution of actin filament density.
Another aim is to further investigate the transport mechanism of vesicles or phagosoms along filamentous actin tracks. Beads coated with a membrane that contains myosin molecules are embedded in a F-actin-solution. Their trajectory is then tracked and searched for so-called Levy-flights, a kind of anomalous diffusion. The force on these phagosoms is to be measured.

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5D-5
OBSERVATION OF ANOMALOUS DIFFUSION OF SINGLE P4K MOLECULES IN THE CELL NUCLEUS

Ulrich Kubitscheck, Thorsten Kues, Reiner Peters

How molecules travel inside the nucleus and between genetic loci and nuclear periphery is still poorly understood. We have studied this topic at the level of single protein molecules. As a probe of macromolecular mobility the recombinant protein P4K was employed, a tetramer of 125 kD subunits containing a stretch of 30 amino acid residues of the SV40 T antigen and the complete E. coli β-galactosidase moiety. P4K was labelled Alexa 488, and introduced into the nuclei of digitonin-permeabilized 3T3 cells by signal-mediated transport through the nuclear pore complex. By combining a high-numerical objective lens with laser illumination of a small observation field and image acquisition by a cooled, highly sensitive slow-scan CCD camera operated in a high speed framing mode it was possible to visualize single P4K molecules in the nuclear interior. The trajectories of single P4K molecules could be followed at a time resolution of 10 ms and a spatial resolution of • 35 nm in the focal plane. The analysis of molecular trajectories disclosed different modes of molecular motion. A substantial fraction of P4K molecules was immobile, a further fraction was confined to small regions or appeared to be tethered to larger structures with restricted moblitity, and a third fraction was not confined but also displayed anomalous time-dependent diffusion. We interpret the observed constraints onto the free mobility of P4K molecules as indications of multiple association-dissociation events.

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5D-6
SIGNAL INDUCED MOTILITY IN A COMPLETE TWO DIMENSIONAL MODEL FOR AMOEBOID MOTION BASED ON THE POLYMERIZATION OF ACTIN

Ralf Sambeth, Arthur Baumgaertner

Starting from the treadmilling phenomenon of actin filaments acomplete two dimensional model for the locomotion of amoeboid cells like keratocytes and fibroblasts ist presented. The head to tail polymerization of actin monomers inside a two dimensional model vesicle can rectify a random motion if it is accompanied by a traction force generating process. This salient feature is the coupling of the filament network to an underlying substratum. The strength of the coupling takes influence on various parameters of the motion such as speed and direction.
Starting from a mesoscopic description of a lamellipodium on the level of actin monomers we perform brownian dynamics simulations and show how the concerted action of polymerization and substrate coupling leads to a cyclic relaxation process. This has a ratchet like nature similar to the flashing ratchet process. Further we show that the strength of the coupling to the substratum is sufficient to give guidance cues to the migrating cell. Thus the presented system is capable of performing motion steered by haptotacic signals.

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5D-7
SELF-ORGANISATION OF MOTORS AND MICROTUBULES

Thomas Surrey, François Nédélec, Stanislas Leibler, Eric Karsenti

Motor proteins and microtubules are of crucial importance for self-organization of the intracellular architecture. Here, we study the morphogenetic properties of mixtures of oligomeric motor proteins and dynamic microtubules. We observe their collective behavior by light microscopy in *in vitro* systems consisting of purified proteins. We use numerical computer simulations to reproduce these observations and to study the influence of individual kinetic parameters on pattern formation. We find that oligomeric motors are capable of organizing microtubules into a variety of large-scale structures like asters, vortices or interconnected microtubule networks. The polarity of the self-assembled structures depends on the directionality of the organizing motors. Competition of two motors with opposite directionalities leads to separation of the motors into distinct domains connected by microtubule bundles of antiparallel orientation. These assemblies can have spindle-like topologies. We find kinetic principles governing cytoskeletal mechanics mediated by motor protein activities. This will lead to a more quantitative understanding of the collective aspect of motor protein and microtubule interaction resulting in self-organization of spatial supramolecular order.

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5D-8
MECHANISM OF CELL DIVISION IN THE ABSENCE OF MYOSIN II

Günther Gerisch, Jan Faix, Ralph Neujahr, Igor Weber

The motor protein myosin II is dispensable for mitotic division in *Dictyostelium* if the cells are attached to a substratum, but is required when the cells are growing in suspension. Cortexillins are actin-bundling proteins that translocate to the midzone of mitotic cells and are important for the formation of the cleavage furrow, even in attached cells.
We show how cortexillins and myosin II cooperate to determine the position and shape of the cleavage furrow. Absence of myosin II leads to an asymmetric cleavage and, in extreme cases, to the abortion of cytokinesis. Absence of cortexillins leads to abnormal accumulation of the filamentous actin in the cleavage furrow and to obstruction of cytokinesis.
Actin polymerization in a direction perpendicular to the plasma membrane at the cell poles leads to ruffling activity. The polymerized actin is engaged in a flow directed towards the midzone, where the actin depolymerizes in a cortexillin-dependent process. A model for cytokinesis in the absence of myosin II is proposed.

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5D-9
MODELS FOR OSCILLATORY MICROTUBULI POLYMERISATION AND SPATIAL PATTERNS

Walter Zimmermann, Markus Breidenich, Klaus Weber

A minimal model for microtubuli polymerisation is analysed which takes into account the growth and shrink kinetics of microtubuli, their coupling to the density of tubulin and the regeneration of GDP loaded tubulin dimers to GTP loaded ones. Under steady conditions this polymerisation leads to a length distribution of microtubuli. It will be shown analytically that the polymerisation kinetics may become also oscillatory under certain conditions, leading to traveling waves in the length distribution of microtubuli. The conditions for either a continuous or discontinuous bifurcation to the oscillatory state are determined.
It is well known that mixtures of rods of different length, such as the long cylindrical microtubuli, may separate with increasing density into ranges with high (low) and low (high) concentration of long (short) rods close to an orientational phase transition (isotropic-nematic) of the rods. If the length of rods is fixed as for usual lyotropic liquid crystalline systems the kinetics of the phase separation is known. If the length is not fixed as during the continuous, nucleation, growth and decay during microtubuli polymerisation the competition between an entropically driven length separation and the kinetically driven changes of the length of the rods leads to spatially periodic patterns.

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5D-10
MECHANISM OF PURINERGICALLY INDUCED CILIARY BEAT FREQUENCY ENHANCEMENT

Natalya Uzlaner, Zvi Priel

The primary function of the cilia of airway epithelium is to transport mucus over the cell, thereby contributing to mucociliary clearance. This important task is performed by the beating of cilia and the consequent movement of mucus from the lungs to the upper airways. Several hormones and neurotransmitters stimulate ciliary motility. One of the most potent stimulators is extracellular ATP, which acts by releasing calcium ions from internal stores and by activating calcium influx. In this study we investigated the signal transduction pathway underlying the strong and sustained enhancement in ciliary activity induced by purinergic stimulation, based on simultaneous monitoring of the changes in CBF and in $[Ca^{2+}]_i$ from the same ciliary cell. Airway ciliated cells express two kinds of purinergic receptor: a G-protein-coupled (P2Y) receptor, sensitive to both ATP and UTP, and a novel ATP-gated cation-selective channel ($P2X_{cilia}$). Activation of P2Y receptor stimulates a massive release of Ca^{2+} from internal stores. Ca^{2+}-calmodulin complex activates NO-pathway which leads to activation of PKG. Both of them: activated PKG and elevated $[Ca^{2+}]_i$ induce a rapid and robust enhancement in CBF. Activation of $P2X_{cilia}$ contributes to Ca^{2+} influx. Extracellular sodium ions competitively inhibit an ATP-gated channel and thereby attenuate purinergically induced ciliary motility.

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5D-11
ADENOSINE ENHANCES CILIARY BEAT FREQUENCY IN CULTURED CILIARY CELLS

Irena Gertsberg, Zvi Priel

Cilia are densely packed cellular protrusions whose main task is to transport a blanket of mucus over the tissue. To perform this mission the cilia beat in a synchronous and periodic pattern called methachronal wave and are commonly stimulated from outside. Ciliary cells can respond to a variety of stimuli (mechanical, electrical, chemical and hormonal) by altering the pattern of the ciliary beating. This system is unique in providing the opportunity to follow the endpoint of the signal transduction cascade. Monitoring of ciliary beat frequency (CBF) in an intact cell provides a physiological non-invasive sensor of cell stimulation. Extracellular adenosine regulates many physiological processes in mammalian systems as a result of its release from cells that are metabolically active, stressed or damaged. Many cellular effects of adenosine have been attributed to its interaction with cell-surface purinoceptors (P_1) classified into three types of receptors (A_1, A_2 and A_3).
In the present work the dual photoelectric method was applied to investigate the adenosine influence on CBF in ciliary cells cultured from frog esophagus. Adenosine enhances the CBF in dose-dependent manner with the maximum at 10 μM. Three selective agonists for adenosine receptors (each at relatively low concentration – 1 nM) produce a prolonged CBF enhancement indicating the presence of all three classes of adenosine receptors in the cells. The signal transduction cascade, induced by the activation of adenosine receptors will be presented and discussed.

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5D-12
PHENOMENON OF MECHANICAL SCREENING IN EXPERIMENTS ON BIOMEMBRANES: ADHESION, MAGNETIC TWEEZERS AND AFM

A. Boulbitch

Animal cells possess membranes consisting of a lipid bilayer and associated integral proteins attaching it to a cytoskeleton. The latter represents a membrane-associated actin network (so-called, actin cortex) coupled to the bulk cellular cytoskeleton. Mechanical properties of cells and cellular organelles (as, for example, vesicles) are difficult to measure because of their small size and low rigidity. Their study demands a possibility to apply extremely small forces and of registration of small displacements. Present experimental technique is unable to measure global deformations of cells. This represents difficulties, since action typically causes deformation of the whole membrane. However, experimental technique makes it possible to register membrane displacements locally.

We show that application of local load onto a biomembrane causes a displacement that is localized within a small domain. This phenomenon is referred to as the "mechanical screening". It enables us to study mechanical properties of membranes by their local deformations by various localized loads. In the present contribution we report on the application of the mechanical screening to study adhesion, to experiments on magnetic tweezers on the cell surface and to AFM measurements on bacteria.

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6A-1
FAST AND SLOW GATING RELAXATIONS IN THE MUSCLE CHLORIDE CHANNEL CLC-1

Alessio Accardi, Michael Pusch

At negative voltages the macroscopic gating of the muscle chloride channel, CLC-1, is characterized by a double-exponential deactivation, whereas at positive voltages no relaxation is visible. The single channel behaviour of CLC-1 suggests that, like the *Torpedo* channel CLC-0, it also has a "double-barreled" structure with two gates, a fast single protopore gate and a slow common pore gate. We studied the kinetics and steady-state voltage dependence over a wide voltage range (−160 to 200 mV) of CLC-1 using "envelope protocols". Applying prepulses of varying duration to different potentials and monitoring the activation by measuring the peak current after repolarization to −140 mV we found that fast and slow gating at +200 mV are respectively characterized by a time constant $\tau_f \sim 16$ μs and $\tau_s \sim 1$ ms. Both τ_f and τ_s have an exponential voltage dependence for $V \geq -50$ mV where they reach a plateau. The kinetic difference of τ_f and τ_s at 200 mV allowed us to separate the two processes yielding a precise measure of P_o^s and an approximate measure of P_o^f.
We characterized the pH_{int}-, and $[Cl]_{ext}$-dependence of the two gates, and the effect of the dominant myotonia inducing mutation, I290M. These manipulations affect in a parallel manner both processes suggesting that they cannot be considered independent. Our results are inconsistent with a previously published non Markovian model for CLC-1 (Fahlke et al., 1996) and show that, instead, gating behaviuor of CLC-1 is qualitatively similar to that of CLC-0.

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6A-2
INDEPENDENT REGULATION OF HUMAN-*ERG* K^+ CHANNEL ACTIVATION AND DEACTIVATION GATING BY TWO DIFFERENT DOMAINS IN THE AMINO TERMINUS

Francisco Barros, Cristina G. Viloria, David Gómez-Varela, Teresa Giráldez, Pilar de la Peña

Potassium channels of the human-*ether-a-go-go* related gene (HERG)-type play a fundamental role in repolarization of cardiac action potential, but also participate in control of resting potential and electrical activity of neuronal, neurosecretory and tumour cells. One exclusive structural feature of HERG is a long domain in the amino terminus (named here "proximal" domain), that separates the initial conserved *eag* or PAS domain from the channel core. Using different deleted and mutated HERG variants we demonstrate that proximal domain removal causes a huge acceleration of channel opening associated to a strong shift in activation voltage dependence to hyperpolarized values. However, deactivation time constant from fully activated states and channel inactivation remain almost unaltered following deletion of the proximal domain. Additional elimination of the *eag* domain significantly reverses the effects of proximal domain deletion on activation, and accelerates channel closing. It is concluded that whereas HERG activation is dominated by structural rearrangements in the proximal domain, closing rates are mainly dependent of *eag* domain dissociation from the channel core. Thus, the particular combination of HERG proximal and *eag* domains determines the slow activation and deactivation gating, essential for neuronal spike-frequency adaptation and maintenance of cardiac action potential plateau.

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6A-3
BROWNIAN DYNAMICS SIMULATION AND CONTINUUM APPROACHES OF ION PERMEATION THROUGH A SIMPLIFIED CHANNEL; A COMPARISON

Sebastian Bergling, Eberhard von Kitzing

Currently, a public dispute is going on between different schools of thoughts concerning the question on which forces dominate ion permeation through open biological channels (see the extensive perspective section on this topic in the June and October issue of the Journal of General Physiology (1999)). Two opposing views concerning the dominant contributions to ion conductance in biological ion channels become evident: a) interactions of the single permeating ion with particular atoms in the protein structure or b) ion-ion interactions in general are the determining factors for the electrophysiological properties of open single channels.
We carry out 3-d Brownian Dynamics (BD) simulations of ionic flux through a simplified membrane channel in order to test some aspects of continuum electrodiffusion approaches such as the Poisson-Nernst-Planck and Continuity equations. Our simplified BD – channel/semipermeable membrane allows us to obtain reasonable statistics of the channel conductivity. As expected we find that the actual local electric field is different from the mean field used in continuum approaches.
Many theories used the mean field instead of the correct local field to describe ion permeation. Therefore, we investigate the influence of one or the other field on current voltage curves.
This research is supported by the DFG.

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6A-4
INTRINSIC ATPASE ACTIVITY: A NOVEL PROPERTY OF CARDIAC K-ATP CHANNELS

Martin Bienengraeber, Alexey E. Alekseev, M. Roselle Abraham, Antonio J. Carrasco, Christophe Moreau, Michel Vivaudou, Petras P. Dzeja, Andre Terzic

ATP-sensitive K^+ (K_{ATP}) channels are unique metabolic sensors, formed by association of Kir6.2, an inwardly-rectifying K^+ channel, and the sulfonylurea receptor SUR, an ATP-binding cassette (ABC) protein. We identified an ATPase activity in purified fusion proteins containing nucleotide binding domains, NBD1 and NBD2, of the cardiac SUR2A isoform. NBD2 hydrolyzed ATP with a higher rate compared to NBD1. The ATPase required Mg^{2+}, and was sensitive to ADP and Vanadate. K1348A and D1469N mutations in NBD2 reduced ATPase activity, and produced channels with increased sensitivity to ATP. K_{ATP} channel openers, which bind to SUR, promoted ATPase activity in purified sarcolemma. K1348A and D1469N mutations attenuated the effect of openers on K_{ATP} channel activity. Opener-induced channel activation was also inhibited by the creatine kinase/creatine phosphate system that removes ADP from the channel complex. Thus, the K_{ATP} channel complex functions not only as a K^+ conductance, but also as an enzyme regulating nucleotide-dependent channel gating through an intrinsic ATPase activity of the SUR subunit. Modulation of the channel ATPase activity and/or scavenging the product of the ATPase reaction provide novel means to regulate cellular functions associated with K_{ATP} channel opening.

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6A-5
EFFECTS OF ION CHANNEL REDISTRIBUTION ON CURRENT RESPONSE

Volker Binding, Reiner Kree

Inhomogeneous distributions of ion channels are ubiquitous in biological membranes and important for many biological functions. Therefore many theoretical models have been set up, which try to explain the emergence of observed patterns. On the other hand little is known about the quantitative response of an inhomogeneous distribution of ion channels.
We have studied the modifications of the current response to voltage pulses, which can be induced by the shape of an inhomogeneous distribution of channels in membranes close to electrically insulating surfaces (like other membranes or glass walls). We find that rather large deviations from the response of a homogeneous ensemble are possible. Even qualitative changes in the kinetics (e.g. an apparent inactivation behaviour of standart potassium channels) can be observed. If the channels are mobile and carry electrophoretic charge, dissipative patterns of channel proteins may occur (Fromherz 1995). We have studied the effects of repititive stimulation by voltage pulses on such a system and find significant changes in the current response on a time scale of a few seconds. This indicates, that a complete analysis of experimentally obtained current responses requires both a model of the channel kinetics and a model of the distribution of ion channels over the membrane.

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6A-6
THE INTERACTION OF κ-CONOTOXIN PVIIA WITH *SHAKER* K^+-CHANNELS: DEPENDENCE ON MONOVALENT CATIONS

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κ-PVIIA is the first conotoxin known to interact with voltage-gated potassium channels by inhibiting *Shaker* mediated currents. We investigated the mechanism of block of *Shaker* Δ6-46 channels, lacking N-type inactivation, by using the *Xenopus* oocytes expression system. We show that PVIIA blocks differently the open and the closed channel. The time scale of the block allows the evaluation of the kinetic parameters of the block. In 2.5 mM-$[K]_o$ and 115 mM-$[K]_i$, PVIIA block of the open channels decreases with the voltage, with a slope of 40 mV, due to an increase of the toxin dissociation rate. The block of the closed state is voltage independent and about 4 times more effective than that of the open channel at 0 mV. In analogy with CTX-block, the binding of PVIIA to open channels appears to be destabilized by the occupancy by K ions of an outer site in the permeation pathway. Raising $[K]_o$ to 115 mM does not affect the open channel block, but decreases the affinity for the closed state by a factor of 8. We replaced $[K]_i$ with different permeable and impermeable cations. No effect is observed for the interaction of PVIIA with the closed channel. Replacement of $[K]_i$ with impermeant large cations (NMG and TRIS) roughly halves the slope of the voltage dependence of the open channel block. These results characterize PVIIA as a valuable tool for probing indirectly the intimate properties of the permeation pathway of the potassium channel.

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6A-7
MURINE EMBRYONIC HEARTS BEAT IN THE ABSENCE OF THE L-TYPE CALCIUM CHANNEL $\alpha_1$1.2 -GENE TILL DAY 14 P.C.

A. Welling, C. Seisenberger, V. Specht, N. Klugbauer, F. Hofmann

Regulation of L-type calcium channels varies in different types of cells depending on membrane potential and subunit composition. The main subunit of the heterooligomeric protein complex is the α_1 subunit which is encoded by several genes that give rise to a number of splice variants. To inactivate the $\alpha_1$1.2 subunit of the L-type channel in different tissues we established two mouse lines with different disrupted exons. Mice homozygous for the disrupted $\alpha_1$1.2-gene (−/−) die in utero before day 14.5 p.c.. Till day 14 p.c. $\alpha_1$1.2 −/− embryos are normal with a beating heart later than day 9.5 p.c.. Electrophysiological experiments revealed a dihydropyridine sensitive Ba^{2+} inward current in +/+, +/− and −/− cardiomyocytes. β-adrenergic stimulation increases the Ba^{2+} inward current even at day 12.5 p.c. in (−/−) and control cells.
At a membrane potential of −80 mV, the dihydropyridine nisoldipine blocked the current of +/+ (and +/−) cells with a high affinity constant of 0.1 (0.084) and a low constant of 3.9 (2.1) μM. Inhibibtion of −/− cardiomyocytes could be fitted with only the low affinity constant. This low affinity constant was absent in $\alpha_1$1.3 −/− cardiomyocytes. RT-PCR confirmed the existence of transcripts for $\alpha_1$1.2 and $\alpha_1$1.3 in embryonic heart cells.
These results indicate that the murine embryonic heart beats in the absence of $\alpha_1$1.2-gene before day 14 p.c.. The $\alpha_1$1.2-gene is necessary for the development after day 14 p.c..

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6A-8
GRAVITATIONAL INFLUENCE ON NATIVE ION CHANNELS

Markus Goldermann, Wolfgang Hanke

We investigated the effect of gravity on native ion channels isolated from the outer membrane of the bacterium Escherichia coli. The channels were purified from cultured strain MRE 600 and reconstituted into planar lipid bilayers. The aim of this study was to find out whether and how gravity influences the highly simplified system membrane-ion channel. An effect would provide an explanation for the perception of gravity by single cells having no perceptive organelle.
The bilayer technique was adapted to the requirements of gravity research. Microgravity experiments were performed in the german drop tower in Bremen and in a parabolic flight campaign in Bordeaux, France, whereas moderate hypergravity was reached by centrifugation in a home build, low-speed centrifuge.
We could prove that the open probability of bacterial porines from outer membrane depends on gravity. The dependency is sigmoid with the steepest region between 1 and 1.5 g. Derived from former experiments with the artificial pore forming peptide Alamethicin we propose a model for graviperception on the molecular level with the bilayer plane as the sensor for gravity. The mechanism might be similar to that of mechanosensation by ion channels.

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6A-9
STOCHASTIC RESONANCE AND INFORMATION TRANSFER IN VOLTAGE DEPENDENT ION CHANNELS

Igor Goychuk, Peter Hänggi

We identify a unifying measure for stochastic resonance (SR) in voltage-dependent ion channels, which comprises periodic (conventional), aperiodic and nonstationary SR. Within a simplest setting, the gating dynamics is governed by two-state conductance fluctuations, which switch at random time points between two values. The corresponding continuous time point process can be characterized by the voltage-dependent opening and closing switching rates and is analyzed by virtue of information theory. In pursuing this goal we evaluate for our dynamics the t-information, the mutual information and the rate of information gain. As a main result we find an analytical formula for the rate of information gain that solely involves the probability of the two channel states and their noise averaged rates. For small voltage signals it simplifies to a handy expression. Our findings are applied to study SR in a potassiumselective Shaker channel. We find that SR occurs only when the closed state is predominantly dwelled. Upon increasing the probability for the open channel state the application of an extra dose of noise monotonically deteriorates the rate of information gain, i.e., no SR behavior occurs. We connect this type of behavior with the steep, thresholdlike dependence of the channel's opening rate on the applied voltage.
[I. Goychuk and P. Hänggi, Phys. Rev. E 61, 4272 (2000)].

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6A-10
VARIABLE RATIO OF ION PERMEABILITY TO GATING CHARGE OBSERVED IN RAT BRAIN IIA SODIUM CHANNELS IN *XENOPUS* OOCYTES

Nikolaus G. Greeff, Frank J.P. Kühn

Recordings of gating currents (I_g) using a fast TEVC at Xenopus oocytes with high expression of rat brain IIA voltage-gated sodium channels show I_g of unexpectedly large size compared to ionic currents (I_{Na}) (Greeff et al., Biophys. J., 74:A402). I_g displayed normal properties and was already used to characterize the inactivation process from mutant channels (Kühn and Greeff, J. Gen. Physiol. 114:167). We now report further studies to understand the small ratio of I_{Na}/Q_g (Q_g = integrated I_g). Asymmetry artifacts were discriminated from I_g. We observed a continuous decrease of the sodium equilibrium potential E_{Na} during the experiments suggesting an influx of Na from the bath into the cell. $[Na]_i$ was calculated from the Nernst equation. The influx due to leak at holding potential (assumed to be Na) and I_{Na} from pulses into the cytoplasmic volume would account for the elevated $[Na]_i$. This suggests strongly that at high channel expression the oocyte is permanently forced to pump Na ions out. A systematic study in several oocyte batches demonstrated that the ratio of P_{Na}/Q_g (permeability P_{Na} was used instead of I_{Na} to account for different Na concentrations) strongly correlates with $[Na]_i$. This could be further validated by partially changing choline vs. Na in the bath. The Na load appears to represent an energetic stress for the cell which modulates the opening probability by an as yet unknown mechanism, whereas the gating machinery appears undisturbed.

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6A-11
COMPUTER SIMULATION STUDY OF THE BINDING EFFECT OF VARIOUS IONS IN CYCLIC NUCLEOTIDE-GATED CHANNEL

Jean-Fang Gwan[1], Arthur Baumgaertner[1], Reinhard Seifert[2], U. Benjamin Kaupp[2]

The binding effect in Cyclic nucleotide-gated (CNG) channel of different ions is of the main interest of this study.
Cyclic nucleotide-gated (CNG) channels are cation permeated channels, which function on providing the calcium ion signal in the rod and cone photorecepters and olfactory neurons. When conducted with the monovalent cation, like sodium ions or potassium ions, the calcium ions effect as blocker of the current in CNG channels, which means the existence of binding sites to calcium ions inside the pore. As shown in the experiment done by mutation methods, the four intrapore glutamate are responsible for binding effect in the channels.
We study the binding effect of different ions in a mutated CNG channel by molecular dynamics simulation method using AMBER. Based on the homology of the sequences in the pore region, we obtained the structure of the mutated CNG channel by mutating the essential part from the KcsA channel of Streptomyces Lividans. The dynamics simulation data shows local structure change of the mutated CNG channel which is responsible for the binding effect. The different binding effect of various ions agree with the experiments.

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6A-12
SIMILAR EFFECTS ON INACTIVATION GATING OF RBIIA NA^+ CHANNELS BY THE β_1-SUBUNIT, CULTURING IN PRESENCE OF TTX, AND A PORE MUTATION

Frank J.P. Kühn, Nikolaus G. Greeff

Voltage-dependent Na^+ channels of brain and skeletal muscle display an unusually high proportion of slow current decay if expressed in *Xenopus* oocytes in absence of the β_1-subunit (e.g. Moorman et al., 1990; Zhou et al., 1991). In this study we compare the inactivation properties of wild-type (WT) and mutant (D384N) rat brain IIA Na^+ channels expressed in *Xenopus* oocytes with and without co-expression of the β_1-subunit. Mutation D384N located in the selectivity filter domain mediates insensitivity to guanidinium toxins and strongly reduces single channel conductance (Pusch et al., 1991; Terlau et al., 1991). We used a high expression system and an optimized two-electrode voltage clamp for the recording of well resolved ionic and gating currents (Greeff and Kühn, 2000). Furthermore, we analyzed the effect of culturing the injected oocytes in presence of 2 μM TTX. The data show that co-expression of the β_1-subunit accelerates the onset and recovery from fast inactivation of both WT and D384N. Interestingly, the TTX-incubation of WT-injected oocytes causes similar effects on the inactivation kinetics of the Na^+ channel α-subunit as the co-expression of the β_1-subunit. On the other hand, the pore mutation D384N selectively hastens the recovery of the immobilized gating charge. The data suggest that the β_1-subunit, the culturing of oocytes in presence of TTX, and the mutation of the TTX-binding site influence the gating mode of the Na^+ channel α-subunit.

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6A-13
A1 AND A2 PURINERG RECEPTOR AGONISTS INVERSELY MODULATE POTASSIUM PERMEABILITY AND TRANSMEMBRANE POTENTIAL OF DDT1 MF2 SMOOTH MUSCLE CELL

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The effect of A1 and A2 adenosine ligands on the transmembrane potential -measured with flow cytometer using oxonol fluorescence dye- and potassium conductance was investigated in the hamster vas deferens smooth muscle cell line DDT1 MF2. A1 adenosine receptor agonist CPA elicited a rapid and maintained increase in potassium conductance and hyperpolarised the membrane. A1 antagonist DPCPX as well as 4AP a potassium channel inhibitor eliminated the CPA effect.
In the presence of A2A adenosine receptor agonist CGS 21680 the potassium conductance of the cells decreased, while the transmembrane potential was slightly shifted to positive values. These effects could be eliminated by the A2A antagonist CSC. These data suggest that adenosine A1 receptor activation in DDT1 MF2 cells stimulates the potassium channel activity and results in a hyperpolarization, while A2A adenosine receptor activation has opposing effect on the potassium channel activity and membrane potential.

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6A-14
LOCAL ANAESTHETICS: BINDING SITES OF BUPIVACAINE IN K^+ CHANNELS

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Local anaesthetics block voltage-gated channels in a complex state-dependent way. In a previous study we showed that bupivacaine mainly blocks the Kv1.1, 1.2, 1.5, 3.1 and 3.2 channels in open state. In contrast, the effect on the Kv2.1 channel was mainly state-independent. The results suggest the primary binding site of the Kv1 and 3 channels to be located in the inner pore mouth region, while that of the Kv2.1 channel is suggested to be located in the external side of the membrane. The internal site most likely is associated with the S6 segment, which forms the wall of the inner mouth. The external site seems probably to be associated with the S5-S6 linker. We have studied the effect of introducing the S6 segment and S5-S6 linker (or parts thereof) of the Kv1.2 channel into the Kv2.1 channel on the bupivacaine action. The results show that it is possible to introduce a low-affinity external site and a high-affinity internal site from the Kv1.2 channel in the Kv2.1 channel. However, although the two binding types are associated with specific locations, it was not possible to associate them with specific residues, suggesting that the bupivacaine binding depend on more general structural properties of the channel.

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6A-15
EFFECT OF CA^{2+} IONS ON THE INACTIVATION OF L-TYPE AND T-TYPE CALCIUM CHANNELS

Lubica Lacinova, Franz Hofmann

A prominent feature of L-type calcium channels is Ca^{2+}-dependent inactivation. It is not known if Ca^{2+} also regulates T-type calcium channels. We expressed $\alpha_{1C} + \beta_{2a} + \alpha_2\delta$-1 as a representative of L-type calcium channels and α_{1G} as a representative of T-type calcium channels in HEK 293 cells. Whole cell current was analysed with variable intracellular environments: 10 mM EGTA-buffer; 0 EGTA; 0 EGTA + 200 μM Ca^{2+}. Non-buffered L-type channels had five-fold smaller current amplitude while the amplitude of T-channels was only 30% smaller. When intracellular Ca^{2+} was buffered, voltage dependence of the speed of L-type calcium current inactivation was U-shaped and amplitude inactivation was relieved by high depolarisations. Both effects were suppressed when intracellular Ca^{2+} was not buffered. In contrast, intracellular Ca^{2+} had no effect on the steady state inactivation of this T-type calcium channel. Kinetics of T-current inactivation was accelerated in the presence of Ca^{2+} in the pipette solution. Recovery from the inactivation has fast and slow components in both channels. In the L-channel, the fast component of recovery is suppressed when free Ca^{2+} is present in the cell. In the T-channel, the intracellular solution had no effect on channel recovery. In conclusion, when Ca^{2+} ions are present in the cell expressing this L-type calcium channel, part of the channels is probably permanently in the Ca^{2+}-dependent inactivated state. T-type calcium channel is regulated by intracellular Ca^{2+} only to a minor degree.

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6A-16
SPECIFIC INHIBITION OF VOLTAGE-SENSITIVE CA^{2+} CHANNELS BY PROTEIN KINASE C ε ISOFORM IN BOVINE ADRENAL CHROMAFFIN CELLS

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Protein kinase C (PKC) has been implicated in the modulation of ion channels in several cell types. We have investigated the involvement of specific PKC isoforms in the modulation of voltage-sensitive Ca^{2+} channels in bovine chromaffin cells. Exposure to the PKC activators PMA and PDBu inhibited the Ca^{2+} currents elicited by stepping membrane potential to 0 mV from an holding potential of -80 mV. PDBu inhibition of Ca^{2+} currents was occluded by the PKC-specific inhibitor Ro 31-8220 but remained unaffected by G 6976, a selective inhibitor of classical PKC isoforms. PDBu-induced translocation of the different PKC isoforms from cytosol to membrane was investigated by western blotting. This not only confirmed the presence of PKC-α and -ε but allowed the identification of yet another atypical PKC isoform (PKC-ι) besides PKC-ζ. PKC-α and -ε reached maximal translocation in approximately 10 min after PDBu treatment. PKC-ι and -ζ showed no signs of translocation. It is concluded that VSCCs are specifically inhibited by the activation of the PKC-ε isoform in chromaffin cells, suggesting that either activated PKC is targeted to discrete sites in the plasma membrane in an isoform-specific fashion or that classical and novel PKC isoforms display some degree of selectivity towards the Ca^{2+} channel phosphorylation sites.

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6A-17
ELECTRICAL ACTIVITY IN GAP-JUNCTION CONNECTED CELLS OF TRANSGENIC MICE OVEREXPRESSING CONNEXIN CX32

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We use pancreatic islets of Langerhans as a model tissue for studying electrical behavior of gap-junction connected cell networks. Membrane potential of beta cells in intact islets of control and transgenic mice overexpressing connexin Cx32 was recorded intracellularly. For an intermediate glucose concentration (11.1 mM), both cellular types show the typical bursting pattern. Nevertheless, both duration of active phase and total period are significantly bigger in transgenic beta cells. These differences do not affect neither EC50 nor the slope of the dose-response curves to glucose in control and transgenic cells, indicating that despite the differences in total duration of the oscillation, the degree of activity (active phase duration / total period) is similar, and therefore transgenic beta cells keep their capability to answer properly to glucose changes. A detailed analysis of burst characteristics reveals further differences: action potentials are homogeneous in amplitude and duration in the active phase in transgenic beta cells, having a high firing rate. There is only a slight decrease in spikes frequency towards the end of the active phase. This is rather different in control beta cells, where different spike populations can be observed and where high frequency firing is only seen during the first seconds of active phase.

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6A-18
CHARACTERIZATION OF THE nAcChR SEGREGATED LIPID DOMAIN BY FLUORESCENCE STUDIES

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The nicotinic Acetylcholine Receptor (nAcChR) from *Torpedo* is a large transmembrane glycoprotein composed of four different polypeptide subunits (α, β, γ and δ) in a 2:1:1:1 stoichiometry. Binding of cholinergic agonists to the α subunits causes the formation of a transient cation channel within the protein, responsible for the initiation of postsynaptic membrane depolarization.
Studies of reconstitution on purified nAcChR protein into multicomponent artificial liposomes have shown that presence of certain lipids, namely cholesterol and phosphatidic acid, is required to preserve nAcChR function.
Recent experiments using FT-IR and DSC on protein reconstituted in eggPC:DMPA:CHO vesicles show that presence of nAcChR directs the formation of specific lipid domains, composed mainly by DMPA, that become segregated from the bulk lipid matrix.
Fluorescence studies, including anisotropy and RET, have been done in order to analyse this lipid domain. For this purpose, we have used protein intrinsic fluorescence and that from selected membrane probes (*t*-PnA, DPH, PA-DPH and a fluorescent analogue of DMPA). All this probes are suitable to make RET experiments with protein tryptophan as donor and membrane probes as acceptors, so we can obtain distribution and distances between them. Once analysed these results, we propose a model of the lipid-nAcChR interaction, detailing domain composition, stoichiometry and distribution of its components.

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6A-19
REGULATION OF K_{ATP} CHANNELS BY INTRACELLULAR AP_4A IN MOUSE PANCREATIC β-CELLS

Juan M. Rovira, Bernat Soria, Cristina Ripoll

K_{ATP} channels maintain the resting potential in β-cells and close in response to glucose, coupling metabolism with secretory function in this cell type. ATP/ADP ratio has been considered to be the main regulator of K_{ATP} channel activity. However, the estimated ratio in the cytosol is too high to enable an effective K_{ATP} channel regulation. Furthermore, ratio changes induced by glucose stimulation are relatively small. Diadenosinpolyphosphates, namely Ap_4A, have been proposed as mediators in stimulus-secretion coupling. In contrast to ATP/ADP, Ap_4A basal levels in the cytosol are too low to block K_{ATP} channel activity. But when β-cells are stimulated, Ap_4A cytosolic concentration substantially increases, allowing a high degree of channel closure. In the present study we show that Ap_4A induces a decrease in K_{ATP} channel current. This inhibition is achieved by a 2-fold increase in long closures that separate bursts of openings, as measured by dwell time analysis. Ap_4A did not alter K_{ATP} channel unitary current. Linear regression of unitary current-voltage relationship gave a conductance of 21.4 $\pm$ 1.8 and 22.3 $\pm$ 1.6 pS in the absence and in the presence of 40 μM Ap_4A, respectively. This data can be explained by an allosteric model of the K_{ATP} channel, which includes a closed state that depends on the binding of a single molecule of Ap_4A. This model qualitatively and quantitatively describes Ap_4A K_{ATP} channel inhibition.

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6A-20
MODULATION OF THE GATING PROPERTIES OF THE MAXIK CHANNEL BY HYDROPHOBIC COMPOUNDS

Olaf Scheel, Rikard Blunck, Klaus Brandenburg, Ulrich Seydel

In macrophages, a MaxiK channel is supposed to act as an early transmembrane signal transducer for endotoxin-induced activation of immune cells subsequently leading to the induction of Gram-negative inflammation. Channel and cell activation are strongly correlated to the physicochemical properties of the hydrophobic part of the amphiphilic endotoxin molecules. Since these are known to intercalate into the macrophage membrane, we investigated the influence of hydrophobic and non-hydrophobic compounds on channel gating. Patch-clamp recordings in the outside-out configuration of membrane patches were performed to determine the influence of different channel blockers on channel gating. FTIR measurements were done to investigate a possible interaction between the lipid bilayer matrix of the macrophage membrane and the blockers. Blocking mechanisms were determined by calculating the transition matrix for the model C=O=G, in which O is the open and C and G are different closed states. While charybdotoxin stabilized the inactivated channel, chlorpromazine, quinine and tetraethylammonium blocked the open channel in a flickering manner. Unlike tetraethylammonium, chlorpromazine and quinine showed an interaction with the membrane lipids and caused a decrease of channel inactivation. These data suggest that the MaxiK channel underlies modulation by the surrounding hydrophobic compounds. This modulation may also explain channel activation by endotoxin.

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6A-21
ION SELECTIVITY AND FLEXIBILITY OF THE FILTER: A MOLECULAR DYNAMICS SIMULATION APPROACH

Indira H. Shrivastava, M.S.P. Sansom

Molecular dynamics simulations of a bacterial channel potassium channel (KcsA), embedded in a fully solvated POPC bilayer have been used to explore the physical basis of selectivity of this channel for K^+ over Na^+. A number of simulations have been run, each of the order of 2ns but differing in their initial configurations of ions and water molecules in the selectivity filter. Comparison of simulation with K^+ ions and Na^+ ions reveal significant differences in the ion-protein interactions. The K^+ ions and the water in the selectivity filter, undergo single file motions and are seen to hop from one binding site to another, over a 2ns time scale. In contrast, the Na^+ ions seem to remain bound at their sites and do not show significant motion on a nanosecond timescale. In addition, the entry of K^+ ions into the selectivity filter from the extracellular mouth has been observed, whereas this is not seen in Na^+. Furthermore, the K+ ions sits within a cage of eight carbonyl oxygen atoms (mean K-O distance is 0.29 nm), while the Na^+ ions prefers to sit in a centre of a ring of four oxygen atoms (mean Na-O distance is 0.24 nm). These results correlate with the known selectivity of KcsA for K^+ and with the blocking of KcsA by internal Na^+ ions.

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6A-22
AMPHOTERICIN- AND AMPHOTERICIN ALKYL DERIVATIVES-INDUCED CONDUCTANCE IN FROG MUSCLE FIBRE MEMBRANE

Nora Shvinka

The cation conductance and effluxes induced by polyene antibiotics amphotericin B, amphotericin B methyl ester (metamphocin) and ethyl ester (etamphocin) have been investigated, using isolated frog skeletal muscle fibres and whole sartorius muscles. The conductance was measured under current clamp conditions, using a double sucrose-gap technique. The cation effluxes were studied by flame emission photometry.
At the application from the external side of the membrane, polyenes formed cation-selective channels. The cation conductance and effluxes induced by amphotericin B alkyl derivatives were much lower than those induced by amphotericin B. By the magnitude of the effect on the K^+ efflux the polyenes were in the series: amphotericin B > metamphocin > etamphocin, and on the Na^+ efflux – amphotericin B ≥ etamphocin > metamphocin. The power dependence of amphotericin- and metamphocin-induced cation conductance (1.8- and 1.5–2.5-fold, respectively) was shown to be lower than on artifical membranes. A very fast decline in the equilibrium conductance caused by rapid removal of the polyenes from solution was observed. The metamphocin-induced conductance decreased in polyene-free medium with a half-time of about 0.57 min. The sensitivity of *Candida albicans* was higher for methyl ester than for amphotericin B. By the minimal fungistatic concentration (μg/ml) following series of the sensitivity was obtained: metamphocin (0.39) > etamphocin (0.78) > amphotericin B (0.79).

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6A-23
ACCUMULATION OF RECOMBINANT POTASSIUM CHANNELS IN CELL ADHESION PROBED BY TRANSISTOR

B. Straub, P. Fromherz

Electrophysiology of ion channels in adhesion regions can be studied with transistors in the substrate. The functionality of channels there is a key information to understand extracellular recordings of neuronal activity using neuron-transistor couplings. To get a defined model system we expressed the voltage dependent human slowpoke (hSlo) potassium channel in HEK-293 cells. The transfected cells were subcultivated on a collagen coated silicon chip with fieldeffect transistors. Transfected cells on a gate of a transistor were contacted by a patch pipette in whole cell configuration. By applying voltage pulses the gating curve of the ion channels is obtained from the current through the cell membrane. Simultaneously a transistor records a signal that is caused by the local potassium current in the cleft between cell and gate. This signal has two components: an instantaneous voltage drop along the ohmic resistance of the junction and a slow change of the surface potential at the gate due to a change of local potassium ion concentration.
To get the specific conductance of the ion channels in the attached membrane, the fast signal component is analysed. It turned out that the functionality of the hSlo potassium channel was not affected by the adhesion region. Position and slope of the gating curve were not changed. But the specific potassium conductance in the adhesion region was distinctly higher than in the average membrane. This is interpreted as an accumulation of ion channels in the adhesion region.

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6A-24
CONTROLLING ION CHANNELS ON SILICON CHIPS BY CAPACITIVE TRANSIENTS

Max Ulbrich, Peter Fromherz

Action potentials in individual neurons are elicited by capacitive stimulation from oxidized silicon chips as shown with leech neurons [1]. The mechanism of this process in unclear. The primary signal of a voltage step applied to a chip is an exponential transient in the attached and in the free cell membrane with a time constant in the microsecond range as shown with voltage-sensitive dyes [2]. We investigated the problem, whether these short transients are able to affect voltage-gated ion channels in the attached and free membrane. In particular we studied the cumulative effect of a train of rectangular stimulation pulses applied to the chip. In the computations we considered potassium channels of the delayed rectifier type. We used the parametrization of Hodgkin-Huxley and a similar model for the Kv1.3 channel. Numerical simulations showed that the channels in the attached and free membrane can be openend almost completely by pulse trains choosing a suitable voltage, frequency and duty cycle. The capacitive control of the potassium channels takes advantage of the specific features of the voltage-dependent rate constants of opening and closing. Relevant for the stimulation of neurons may be a similar control of sodium channels.

[1] P. Fromherz, A.Stett, Phys. Rev. Lett. 75 (1995) 1670–1673.
[2] D. Braun, P. Fromherz, in preparation

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6A-25
TRANSISTOR ARRAY PROBES ION CHANNELS ALONG A XENOPUS OOCYTE-SILICON JUNCTION.

Volker Kiessling, Stefano Vassanelli

We consider a Xenopus oocyte with expressed ion channels and attached to a poly-lysine coated silicon surface as a simple model of cell adhesion. Here the adherent membrane and the silicon surface form an artificial junction where known ion channels interact with known adhesion molecules. A "sandwich-cable" electrical model describes the junction whose channels were represented by a resistance-inductance circuit.
We expressed in the oocyte membrane a mutated non inactivating Shaker potassium channel. We applied intracellular voltage pulses of different amplitude and superimposed ac voltages in a range from 5 to 1000 Hz. A linear array of field effect transistors integrated in the semiconductor substrate measured the voltage along the cleft between membrane and silicon.We analyzed the ac voltage transfer from the intracellular compartment into the junction when the fraction of open channels was in a steady-state. The electrical parameters of the junction were estimated: in most cases they were compatible with an adhesion area of circular shape, a diameter of 300–600 μm and a distance between membrane and silicon of 10nm to 100 nm. By comparing the impedance of adherent and free membrane we found a difference between the two regions in the time constant of channels activation and in the fraction of open channels. The effect could be caused by the voltage developing in the adhesion cleft during changes in the intracellular potential. Similar processes could take place in a tissue playing a role in modulating channels gating.

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6A-26
THE PORE OF PLANT K^+ CHANNELS IS INVOLVED IN VOLTAGE- AND PH-SENSING: DOMAIN-SWAPPING BETWEEN DIFFERENT K^+ CHANNEL A-SUBUNITS

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Plant K+ uptake channels differ with respect to their voltage- and pH-dependence. Here, we constructed recombinant chimeric channels between KST1, a member of the inward rectifying, acid-activated KAT1-family, and AKT3, a member of the weakly voltage-dependent, proton-blocked AKT3-family.
The AKT3-pore (amino acids 216–287) was substituted by the homologous region of KST1 (amino acids 217–289). In contrast to AKT3, these chimeric channels, AKT3/(p)KST1, revealed a strong inward rectification reminiscent to that of KST1. Besides the voltage dependence, the interaction between the chimera and extracellular H^+ and Ca^{2+} resembled the properties of the inserted KST1- rather than the AKT3-pore such as acid activation and Ca^{2+}-insensitivity. The fact that the chimeric channels were inhibited by cytoplasmic protons alike AKT3, however, indicated that the intracellular pH-sensor is located outside the P-domain.
We thus conclude that essential elements for external pH- and Ca^{2+}-regulation, and for the rectification of voltage-dependent K^+ uptake channels are located within the channel pore.

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6B-1
COMMON FORMS OF NEURONAL PLASTICITY IN DROSOPHYLA MELANOGASTER REQUIRE BIDIRECTIONAL MODULATION OF SYNAPTIC ION CHANNELS: COMPARISON BETWEEN MODELS AND EXPERIMENTS

A. Bazzani[1], G.C. Castellani[1,2], A. Megighian[3], M. Zordan[4]

The fly giant fiber pathway is a specific and accessible neuronal circuit that allows a direct functional analysis of habituation in presence of biochemical changes induced by mutations. The Caki protein belongs to a subfamily of guanylate kinases (*M*embrane-*A*ssociated *G*uanylate *K*inase homologs), which are involved in cell junction organisation and signalling. These new MAGUKs may regulate transmembrane molecules that bind calcium, calmodulin and regulate synaptic and ion channel activity. To study the latency responses, we model the circuit of the giant fiber pathway as a three layers neural network: the sensitive neurons, the intermediate inhibitory neurons and the motoneurons. Moreover we state a specific hypothesis for the Caki role by a more microscopic model focused on the synapse between the sensitive neuron and the motoneuron. In particular we consider a single ligand gated ion channel that is reversibly bound to the neurotransmitter with a fast equilibrium kinetic. The transition constant is modelled as a reaction variable with a double well internal dynamics. This bistable behaviour is used for the description of the transition between a habituate and a dishabituate state: the role of Caki is supposed to be similar to other kinases. The explicit calculation of these transitions is obtained by Kramers reaction rate theory.

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6B-2
INHIBITORS OF VESICLE FILLING SELECTIVELY AFFECT HIGH-FREQUENCY TRANSMISSION AT STRIATAL GABAERGIC SYNAPSES *IN VITRO*

Jan C. Behrends, Eva Rumpel

Synaptic vesicles are loaded with transmitter by specific transport proteins of the vesicular membrane which use a H^+-electrochemical gradient (established by a vacuolar H^+-ATPase) to pump transmitter molecules from the cytosol into the vesicular lumen. At present, little is known about the importance of this mechanism for the regulation of synaptic strength at central synapses. In our preparation, the protonophore FCCP (5 μM) selectively suppressed synaptic transmission on the 2nd to 4th impulse of a 33 Hz train of 5 presynaptic impulses, changing the depression index from 0.32+/−0.02 in control to 0.23+/−0.01 in the first 3 trains after drug application ($n=6$, $p<0.005$). Preincubation with the Ca^{2+}-chelator BAPTA-AM (50 μM) and lowering extracellular Ca^{2+} to 0.5 or 0.8 mM had a protective action against FCCP-induced depression, while EGTA-AM or loading the presynaptic cell with 20 mM ATP were without effect. Furthermore, FCCP (5 μM)-application significantly and progressively reduced the mean amplitude of mIPSCs (−28.7+/−4.3% in the first 1-2 min of application, $n=6$, $p<0.0001$, Kolmogoroff-Smirnoff test) and decreased their time constant of decay (−18.98+/−5.26%, $p<0.01$, paired t-test). Similar results were obtained with a blocker of the vacuolar H^+-ATPase, Bafilomycin A1. We hypothezise that slower refilling of synaptic vesicles may selectively affect rapidly cycling synaptic vesicles at high frequency of transmission.
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6B-3
THE EFFECT OF PURINES ON NON-QUANTAL ACETYLECHOLINE RELEASE IN MICE DIAPHRAGM

A.V. Galkin, S.N. Grishin, R.A. Giniatullin

The effects of purinergic compounds ATP and adenosine on non-quantal release of acetylecholine (ACh) from motor nerve ending were investigated at mice hemidiaphragm preparation. Mice of male sex were killed under ether anaesthesia by decapitation (in accordance with EEC). Membrane potentials in synaptic region were recorded using high-resistance (10 MOm) glass microelectrodes. Experiments were started 30 minutes after irreversible inhibition of acetylcholinesterase (AChE) by armine (1 microM). Non-quantal ACh release was evaluated from hyperpolarization (H-effect) produced by D-tubocurarine (10 microM) in muscle treated by antiAChE agent (Katz, Miledi, 1977). H-effect was 6.98 +/- 0.26 % (n=4) in control. Application of 10 microM ATP abolished H-effect (1.04 +/- 0.86 %; n=4; $p < 0.001$), whereas adenosine was virtually devoid of such effect on non-quantal release. After 10 microM adenosine application H-effect approached 7.05 +/- 1.51 % (no significant difference vs control). But when we applied ATP on the background of staurosporin depressive effect of ATP on NQR disappeared and resulted in 8.05+/-1.62%. Thus, ATP but not its derivative adenosine decreases non-quantal release, suggesting different mechanisms of their presynaptic action.

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6B-4
PRESYNAPTIC ZINC TRANSIENTS INDUCED BY REPETITIVE STIMULATION OF THE HIPPOCAMPAL MOSSY FIBER PATHWAY

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The mossy fiber terminals in hippocampal CA3 area contain large amounts of vesicular zinc which is co-released with glutamate following high-frequency stimulation. We studied, with high temporal resolution, properties of zinc release evoked by different trains of stimuli. Experiments were carried out in the mossy fiber synapses of rat hippocampal slices (400 μm), loaded with the permeant zinc indicator N-(6-methoxy-8-quinolyl)-para-toluenesulfonamide (TSQ). Repetitive synaptic activity was induced by trains of 2–100 current pulses applied at 20-100 Hz to the mossy fiber pathway. Responses were monitored by recording extracellular field potentials and fluorescent TSQ signals (λ_{exc} = 400 nm). The latter signals, which were transient and downward, were blocked by 20 μM of the heavy metal chelator N,N,N',N'-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) but unaltered by CNQX (10 μM) and D-APV (50 μM). These results suggest that the optical transients were presynaptic and due to the dissociation of TSQ/zinc complexes. Both their amplitude and the rate of decline increased with the number and frequency of the stimuli. For the lower frequencies each pulse induced a clear fluorescence change which added to the previous one, suggesting that zinc is released following individual stimuli.

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6B-5
COMPUTER MODELING OF ACH AND ATP TIME-COURSE IN SYNAPTIC CLEFT

Najl Valeyev, Andrei Skorinkin, Rashid Giniatullin

ATP is cotransmitter to release together with ACh in synaptic cleft during neuro-muscular transmission. But the functional role of ATP remains still obscure. We have developed the mathematical model of one quantum action containing ACh and ATP. The model has been created basing on Monter-Carlo method in analogy with model previously developed by Bartol et. al. 1991 for ACh. Our model allows to predict time course of ACh and ATP concentration in the synaptic cleft during different patterns of nerve stimulation.We show, that when AChE is inactive and 90 % of receptor are blocked, theoretical curve is similar with the bungarotoxin action. When AchE is inactive the ACh concentration have been examined and established really presence of the second wave of the concentration.

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6C-1
OPTICAL RECORDING: ACTIVITY-DEPENDENT BACKPROPAGATION IN DENDRITES OF CULTURED HIPPOCAMPAL NEURONS

Bernd Kuhn, Peter Fromherz

We examined the backpropagation of action potentials from the soma into the dendrite of cultured hippocampal neurons of the rat. It is known from hippocampal slices that backpropagation during a train of action potentials is attenuated.[1] We used a voltage-sensitive dye for our measurements.
Dissociated neurons (ED 17) were cultured at very low density for 8 to 20 days. The cells were stained with the novel voltage-sensitive dye ANNINE 5 [2] with a new 'HCl-method'. Measurements were done with a 90 × 60 pixel CCD-camera with a spatial resolution of 4.5 μm × 4.9 μm and a time resolution of 0.75ms. In contrast to earlier measurements of backpropagation with photodiodes [3], an area of 400 μm × 300 μm is observed without piecing together and without averaging.
We stimulated neurons at the soma for 800 ms, causing a train of about 15 action potentials. We observed the backpropagation of the first and the last action potential and compared both. There is only a weak difference.
So backpropagation in dendrites of hippocampal primery cultures is much less activity-dependent than in hippocampal slices.
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6C-2
EFFECTS OF COUPLING REDUCTION ON PASSIVE ELECTRICAL PROPERTIES OF RETINE CELLS

E. Andreu, E. Fernández, E. Louis, G. Ortega, J.V. Sánchez-Andrés

Dopamine seems to reduce the extent of the receptive field (length constant), in horizontal cells of turtle retina Most authors ascribe this behavior to changes in the resistance of the gap junctions between the cells that form the tissues. Here we point out that this may not always be the case. We compare the theoretical results obtained with different models for the membrane k_m and coupling k_c conductances as functions of the input voltage V_0 and the length constant .While in the two cells model the membrane conductance is proportional to k_0, in periodic arrangements or Bethe lattices with coordination greater than 2, the contact conductance results proportional to the input conductance. This result may be the cause of frequent confusion in the available literature.
We discuss the effects of dopamine on the length constant of horizontal cells of turtle retina. Dopamine decreased the length constant, hypothetically through changes in the contact conductance. Our analysis leads to a different conclusion, namely that the contact conductance is not affected by dopamine additions, and that changes in the length constant should be ascribed to changes in the membrane conductance.

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6C-3
A RANDOM WALK MODEL ANALYSIS OF SPINAL DORSALHORN NEURON DISCHARGES

S. Blesic, M. Ljubisavljevic, S. Radovanovic, M. Björklund, S. Milosevic, H. Johansson

Methods of statistical physics have been recently successfully applied to the study of spatial and temporal randomness in various biological systems, like the analysis of the DNA nucleotide sequences and the heartbeat time series. We study the interspike intervals (ISI) time series of the spinal dorsal horn nociceptive-responsive neurons (DHN) activity in decerebrate cats, applying the detrended fluctuation analysis (DFA) that is a modification of the standard random walk model analysis. Specifically, we focus on ISI variability as an important quantity to help elucidate sensory coding and signal processing performed by DHN. DFA has been applied for it permits quantification of correlation properties of a nonstationary time series of neuronal discharge.
Changes in DHN activity were extracellularly recorded with high impedance glass microelectrodes from superficial laminae of the dorsal horn, during different experimentally simulated conditions. We have analyzed DHN discharge patterns during spontaneous activity, as well as in the presence of different noxious and non-noxious mechanical stimuli. Application of DFA method showed significant changes in dynamics of neural discharge when the external stimulus is applied. These findings demonstrate the relevance of the application of methods of statistical physics to identify the changes in afferent inflow, and to characterize temporal patterns of activity of neurons under study.

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6C-4
MODELLING ION CHANNEL DISTRIBUTION AND DENDRITIC MORPHOLOGY WITH ANALOGUE INTEGRATED CIRCUITS

Catherine Breslin

Modelling neurobiological phenomena with analogue integrated circuits and other forms of electronic hardware is known as neuromorphic engineering. Described here is an analogue integrated circuit design technique that facilitates modelling of the morphological and ionic properties of neurons. Rather than matching mathematical descriptions of neurobiological phenomena with the transfer functions of "off-the-shelf" circuits used in traditional integrated circuit design, this alternative design technique aims to exploit more fully the intrinsic, physical properties of the transistors and the materials from which they are composed. The use of analogue integrated circuits in neurobiological modelling originated with the concept of a physical equivalence between field-effect transistors and populations of ion channels in neuronal membranes. This work represents a return to focussing on the physical equivalences between neurobiological phenomena and electronic hardware, rather than concentrating on the algorithmic ones. The possibility of a conjunction of the physics of silicon with the physics of biological membranes is held as a long-term goal. The physical design technique has been used to construct an array of integrated circuits, representing different morphologies and ion channel distributions, to test the effects of altering these variables upon output. Results from both simulated and fabricated ICs will be presented.

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6C-5
INVESTIGATION OF THE OPTICAL PROPERTIES OF R-NEURON AT NEURO-GLIA INTERACTION

T.A. Brindikova, S.M. Novikov, G.V. Maksimov, T.V. Vyshenskaja, V.P. Tychinsky

It is known that neuro-glial interaction depend on extracellular K^+ and acetylcholine concentrations. Using the new method of DPM (Dynamic Phase Microscopy), the phase image of R-neurons of Herudo medicinalis segmental ganglia in native conditions was investigated. It was found that the object's phase image changes has the temporal periodical components. DPM time-dependencies processed with a Fourier transform revealed that spontaneous rhythmic activity (SRA) contains 8-10 characteristic frequencies in a range of 0.5–10 Hz with a maximal intensity at 1 and 2 Hz. The intensity of these periodic components changes within a period of 0.5–1 min. The increase of intracellular K^+ concentration [50 mM] produced the SRA blocking and decrease the intensity of DMP-spectrum components within interval 2–6 Hz. The addition of acetylcholine [10^{-7} mM] to R-neuron decreased the intensity of components within interval 2–6 Hz and stimulates the neuron's oscillations at 15 Hz. Periodic components were localized mainly on cell's membrane. The cellular mechanisms of DMP frequency spectrum changes at neuro-glia interaction was discussed.

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6C-6
STEADY STATES OF HETEROGENEOUS POPULATIONS OF ACTIVE ION CHANNELS IN SIMULATED DENDRITES

Natalia S. Korogod, Serge M. Korogod, Valery I. Kukushka

A rarely assessed issue of biophysics of dendrites is the relation between spatial maps of the steady transmembrane potential E and the effective equilibrium potential E_q of the total transmembrane current. We studied these on a multicompartment model of CA3 hippocampal pyramidal neuron with uniform cylindrical apical and basilar dendrites over which mixed populations of ion channels were heterogeneously distributed. Each dendritic compartment contained a passive leak conductance and up to five active conductances (fast inactivating Na, high-threshold Ca, delayed rectifier K, long duration afterhyperpolarization Ca-dependent K, and short duration voltage- and Ca-dependent K), which were present in different proportion as described in the literature. Intracellular Ca binding and diffusion mechanisms were included. E_q is the weighted sum of the partial equilibrium potentials E_{Na}, E_{Ca}, and E_K with the weighting factors equal to proportions of the corresponding ion conductivities in the total membrane conductivity. Taken in isolation, each dendritic compartment had certain E_q and the local steady current-voltage relation with one or more stable steady states depending on the cocktail of the conductances. In the whole cell, spatially heterogeneous E_q caused intercompartment currents, which produced non-uniform E and, depending on the cell geometry, changed the number of electrical steady states of the coupled compartments as compared to those in the isolated ones.

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6C-7
IMPACT OF GEOMETRY AND MEMBRANE PROPERTIES ON CURRENT TRANSFER EFFECTIVENESS IN TONICALLY ACTIVATED DENDRITES

Serge M. Korogod, Iryna B. Kulagina

Tonic synaptic excitation, a common input signal, unveils the rules, by which geometry and membrane properties define the effectiveness domain of an individual neuron dendritic field. We used the elementary core current increment related to the total membrane current per unit path length as an estimate of the current transfer effectiveness. This was mapped on the dendrites with distinct geometry and/or cocktails of the ion conductances. The local relation between the steady current density and voltage (I-V) was either monotonic with positive slope, linear (1) or nonlinear (2), or N-shaped with a negative slope in a certain voltage range (3). Uniform excitation of the whole dendrite with a given uniform I-V produced steady depolarization, which always monotonically increased with path distance from the soma indicating the somatopetal core current flow. This was due to common electrical asymmetry determined by the major structural asymmetry: large leaky soma at the proximal end vs. thin sealed tips of all dendrites. As determined by I-V, more depolarized distal sites produced inward current of smaller density in cases (1) and (2). In case (3), smaller or greater density in the distal sites depended on whether the dendritic depolarization was out of or within the range the negative slope of the N-shaped I-V, that was an important feature of the bistable dendrites. Products of the current density and the perimeter at each site define the effectiveness map.

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6C-8
SPATIAL EFFECTS OF DISTRIBUTED TONIC INPUT TO ACTIVE DENDRITES (DETERMINISTIC VS STOCHASTIC MODELS)

Serge M. Korogod, Olexiy O. Kotchubey, Iryna B. Kulagina, Leonid P. Savtchenko

Stochastic behavior of the ion conductances influences neuronal firing patterns. Spatial aspects of that behavior remain unclear. We studied path profiles of the steady membrane voltages, conductances and currents during uniform tonic synaptic excitation in 800 μm long and 3 μm thick dendrites homo- and heterogeneously populated with Na and K channels. The heterogeneity was due to equal partition of these channels between equidistant 2 μm long hot zones (clusters) separated by passive cold zones. We varied the cold zone length from 2 to 200 μm and correspondingly changed the number and the population of individual clusters to conserve the total number of the channels. Voltage-dependent behavior of populations of Na and K channels was described by either deterministic or stochastic version of Hodgkin-Huxley equations. In the deterministic case, the excitation caused steady depolarization monotonically increasing with path distance from the soma towards sealed distal end of the dendrite indicating somatopetal flow of current collected from the whole dendrite. In the stochastic case, the membrane potential fluctuated with larger fluctuations generated by more populated clusters. However, 100 ms time averaging revealed the path profiles of the mean membrane potential mostly the same as deterministic ones. The heterogeneity was manifested by small jumps of the voltage gradient in the hot zones separated by distances >80 μm.

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6C-9
CALCIUM-INDUCED CALCIUM RELEASE IN DIFFERENT TYPES OF LD THALAMIC NUCLEUS NEURONS

Illya A. Kruglikov, Leonid Shutov, Nana Voitenko

LD thalamic nuclei consists of 2 morphologically distinct types of cells -thalamocortical neurons and interneurons. We carried out our experiments on acutely isolated LD neurons of 12-day-old rats. The cytoplasmic free calcium concentration ($[Ca^{2+}]_i$) was measured using Fura2-AM based microfluorimetry technique. Initial application of caffeine (30 mM) produced no effect on $[Ca^{2+}]_i$ in 7 of 15 and 5 of 11 thalamocortical neurons and interneurons respectively. But after cell stimulation with KCl (50 mM) caffeine induced $[Ca^{2+}]_i$ elevation in all cells tested. Removal of Ca^{2+} from extracellular solutions did not affect amplitudes of caffeine-induced $[Ca^{2+}]_i$ transients. 2nd application of caffeine in Ca^{2+}-free solution did not produce $[Ca^{2+}]_i$ elevation that indicates depletion of ryanodine stores. To study the effect of Ca^{2+}-induced Ca^{2+} release (CICR) participation in depolarization-induced $[Ca^{2+}]_i$ transient generation we applied sub-threshold concentration of caffeine. This concentration was set at 0.5 mM. We found that application of KCl (50 mM) after treating cell with 0.5 mM caffeine produced $51 \pm 7\%$ ($n=16$) increase in $[Ca^{2+}]_i$ transient in thalamocortical neurons and has no effect ($n=11$) on interneurons. These data suggest that CICR can contribute to the depolarization-induced $[Ca^{2+}]_i$ transient only in thalamocortical neurons but not in interneurons. The observed difference between two types of LD thalamic neurons could be a basis for the difference in their physiological functions.

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6C-10
STRUCTURE ALIGNED MICRO CONTACT PRINTING CONTROLS NERVE CELL GROWTH AND NETWORK FORMATION

Lars Lauer, Chi-Kong Yeung, Martin Scholl, Andreas Offenhäusser

Neuronal cell growth in vitro can be controlled with micropatterned structures of extracellular matrix proteins (ECM) such as laminin produced by micro contact printing (μCP). This technique is useful in order to increase experimental reproducibility and to artificially design innovative experimental setups. When appropriate structures are chosen for the patterning, cells can be precisely directed to specific spots. We have studied the correlation between structural dimensions of the ECM pattern and the shape of the resulting cellular network. Aim was the systematic optimization of lead structures towards neuronal cell body positioning and induced directed cell differentiation. With a structure geometry of 4 μm line width, 20 μm node size and 10 μm gap size a nodal compliance of 86% (±10%) has been achieved. For signal recordings with biosensor devices precise location of the cell bodies is crucial since signal recordings are only possible from small sensitive spots on the device surface. We were able to successfully guide hippocampal neurons to the sensitive spots of multi-electrode and field-effect-transistor biosensors using embryonic cells (E18) from sprague dawley rats.

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6C-11
USE DYNAMIC PHASE MICROSCOPY FOR INVESTIGATION OF MYELINE NERVE AXOGLIAL RELATION DYNAMICS

E.S. Lazareva, S.L. Nikandrov, G.V. Maximov, V.P. Tychinsky

Dynamic Phase Microscopy method was used for investigation of myeline nerve axoglial changes. This new noninvasive method provides possibility to measure the changes of nerve volume or optical density of structure at rhythmic exitation and to obtain Fourier spectrum of fluctuations along the selected scan-line on the object phase image. In node of Ranvier the characteristic frequencies were 5.31 and 10.75 Hz and during rhythmic exitation (50 Hz) a new component of 5.58 Hz have appeared. The changes of intensity of local optic path difference fluctuations in paranodal region of myelinated nerve at stimulation were not observed.

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6C-12
INVESTIGATION OF THE ROLE OF ACETYLCHOLINE IN REGULATION OF AXON AND SCHWANN CELL INTERACTION DURING NERVE EXCITATION

S. Chatterjee, T.A. Brindikova, G.V. Maksimov, V.V. Revin

It was shown that during axon excitation the number of active acetylcholine receptors (AChR) of Schwann cell increased as a function of frequency rhythmic excitation (RE), extracellular concentration of K^+, Ca^{2+} and acetylcholine (ACh). At RE the activity of axon ACh esterase decrease that could increase the level of extracellular ACh. The nerve phosphoinositidespecific phospholipase C (PI-PLC) activity increase during extracellular ACh perfusion. It was proposed that during axon RE K^+- depolarization and ACh exocytosis the influx of Ca^{2+} activated through Ca-channels for PI-PLC dependent stimulation of Schwann cell AChR phosphorilation.

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6C-13
INTRACELLULAR CALCIUM STORES IN PRIMARY SENSORY NEURONS UNDER INFLAMMATION

Vyacheslav Shishkin, Platon Kostyuk, Nana Voitenko

Changes of calcium stores in primary sensory neurons at various types of neuropathy is still little studied. We carried out our experiments on acutely isolated dorsal root ganglion (DRG) neurons of adult control and carrageenan-injected mice. Cytoplasmic free Ca^{2+} concentration $[Ca^{2+}]_i$ was measured using indo-I based microfluorimetry. As it was previously shown endoplasmic reticulum take part in $[Ca^{2+}]_i$ homeostasis of large (proprioceptive) but not small (nociceptive) DRG neurons by releasing Ca^{2+} through ryanodine receptors and InsP3-receptors. $[Ca^{2+}]_i$ transients evoked by 20 mM caffeine and 100mM ATP were significantly smaller in animals with inflammation with respect to control mice. In control cells 10 μM CCCP applied before KCl-depolarization induced an increase of the amplitude of depolarization-induced $[Ca^{2+}]_i$, indicating the participation of mitochondria in fast uptake of Ca^{2+} from the cytosol during the peak of transient. In carrageenan-injected animals this increase became diminished. CCCP, applied after the transient has reached its peak, induced an additional rise of $[Ca^{2+}]_i$ that reflected massive release of Ca^{2+} previously accumulated by mitochondria. In small neurons from mice with inflammation such an elevation was dramatically smaller. We conclude that inflammation is associated with substantial decrease of mitochondrial Ca^{2+} uptake and subsequent release into the cytosol in all types of DRG neurons, and diminishing of Ca^{2+} accumulation ability of endoplasmic reticulum in large neurons.
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6C-14
STRUCTURE AND DYNAMICS OF MYELIN SHEATH IN THE PERIPHERAL NERVES OF DIABETIC RATS – AN ESR STUDY

M. Zuvic-Butorac, J. Kriz, A. Simonic, M. Schara

The peripheral neuropathy associated with diabetes has characteristical histopathological findings of axonal degeneration, secondary myelin breakdown and atrophy.In the present study we examine structural integrity of myelin sheath in the peripheral nerves from short-term streptozotocin treated diabetic rats, using ESR spectroscopy as a tool in determining the dynamic state and the structure of the myelin lipid phase.Experiments were performed on spin labeled sciatic and sural nerves from STZ-treated Hannover-Whitstar rats and age-matched controls.The spectrum analysis employed a numerical simulation model with the set of fitting parameters, that in the same time relate the ESR line shape and structure and dynamics of the probed environment.The simmulation considered three spectral components weighted and summed in the composite spectrum.The comparative analysis of results showed the fraction of the spectral component II to be significantly increased in the spectra of diabetic rats, indicating the significant increase in overall fluidity of the myelin structure.The origin of fluidity changes was further investigated using: experimental model for demyelinization (local injection of ethidium bromide *in vivo*), proteolitic action of tripsin *in vitro*, and osmotic myelin swelling *in vitro*. Analysis and comparison of the results suggested a conclusion in terms of pathologically changed biophysical properties of myelin lipid phase, that may represent the first step in development of diabetic neuropathy.

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6C-15
CAPACITIVE STIMULATION OF NEURONS ON SILICON MICROSTRUCTURES

Christian Figger, Peter Fromherz

Individual neurons of the leech can be stimulated by voltage steps and by trains of voltage pulses applied to microspots of oxidized silicon with respect to bath potential [1]. We optimized this capacitive stimulation and tried to get a better insight into its mechanism on the basis of numerical simulations. A silicon chip was fabricated with p-doped 570 μm x 570 μm squares covered with a thin layer of insulating silicon dioxide. These stimulation spots were bonded from the backside through micromachined wells. Retzius cells of the leech were attached to the spots and stimulated with trains of voltage pulses. We investigated the influence of amplitude, frequency and number of pulses on the depolarization of the neuron and on the delay of the first action potential. Instead of rectangular pulses we applied also asymmetric trapezoid pulses with a fast increase and slow decrease of voltage. The experiments were compared with simulations on the basis of the Hodgkin-Huxley equations using parameters of the neuron-silicon junction as obtained from neuron-transistors with A-type, B-type and C-type coupling [2, 3].

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6D-1
ORGANOTYPIC HIPPOCAMPAL SLICES CULTURED ON A SILICON CHIP

Brigitte Besl, Peter Fromherz

Until now field effect transistors have been used to record action potentials from single nerve cells of invertebrates and vertebrates attached to the gate. For the first time it was now possible to measure the evoked activity in cultured brain slices with transistors. A silicon chip was designed with 16 field effect transistors in a 4×4 array (100 μm spacing between the gates) with a gate size of 10 μm × 80 μm. We cultured hippocampal slices of 5–7 day old Wistar rats on the chip with the Gähwiler method. After 2 to 4 weeks in culture the slices were used for experiments.
By stimulating the mossy fibers or the Schaffer collaterals with an external tungsten electrode (pulses of 30 μA to 60 μA for 100 μs) we measured field potentials in the CA3 and CA1 region of the hippocampus in the range of 400 μV to 3 mV.
In order to compare these field potentials to signals detected with conventional electrodes we placed a glass electrode above the gate. The signals of both probes were identical in shape but the amplitudes recorded with the field effect transistors were always larger (about a factor of three). Even with stimulus strength so low that there was no signal detected by the glass electrode the transistor still recorded a field potential.
As the coupling of evoked signals of organotypic hippocampal slices to field effect transistors is possible, a chip can now be designed with a high density of transistors in a given area to investigate neuronal network behavior in cultured brain slices.
The project was supported by the BMBF.

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6D-2
ELECTRICAL TRANSFER FUNCTION FROM SILICON CHIP TO NEURON IMAGED BY VOLTAGE-SENSITIVE DYE

Dieter Braun, Peter Fromherz

Noninvasive interfacing of neurons relies on capacitive currents across the interface solid/electrolyte. We stimulated single cells through the interfacing capacitor of thin silicon dioxide on silicon [1] and imaged the membrane voltage (64×64 pixels; width ~1 μm) using the voltage-sensitive dye BNBIQ [2]. Either the transfer function from sinusoidal stimulation or the transient from rectangular stimulation were collected for each pixel. We could compare the electrical and the geometrical properties as the thickness of the sealing cleft was measured with FLIC [3]. We found the following features of the seal:
- The membrane voltage is high with electroporation above ~700 mV.
- The attached and free membrane voltage have inverted signs.
- Transients of the membrane voltage follow a single exponential.
- Time constants of the seal for isolated mammalian cells are 1–3 μs.
- Cell monolayers show a significantly enhanced time constant.
- The specific conductance in the cleft differs from the medium.

Depending on distance, cell type and coating it can be lowered and enhanced by an order of magnitude.
With a time constant of ~2 μs it is a challenge to stimulate action potentials in single mammalian neurons from an insulated chip.

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6D-3
CIRCUITRY OF RAT BARREL CORTEX INVESTIGATED BY INFRARED-GUIDED LASERSTIMULATION

A. Schierloh, M. Eder, W. Zieglgänsberger, H.-U. Dodt

Infrared-guided laserstimulation was used to examine the synaptic connectivity of neurons in rat barrel cortex. Borders of barrels in neocortical slices containing the posterior medial barrel subfield (PMBSF) were identified under the microscope at low magnification. Single neurons were visualized by infrared videomicroscopy at high magnification and the membrane potential of layer V pyramidal neurons was recorded with patch pipettes. Brain slices were superfused with medium containing 0.5 mM 'caged glutamate'. Presumptive presynaptic neurons were activated by uncaging glutamate with the light of a UV-Laser directed via the microscope objective under visual control onto these neurons. Synaptic connections were identified by postsynaptic potentials following laserstimulation.
By scanning cortical barrels with the laser, 115 neurons of layer II to VI projecting to neurons of layer V ($n = 24$) were identified. The highest frequency of synaptic input was evoked by stimulating other layer V pyramidal neurons. The probability of input from this layer declined monotonically if the lateral distance of recorded and stimulated neurons increased. In contrast, input from layer II/III showed a periodic organization. Synaptic connections originating from this lamina clearly reflected the barrel structure, with more input originating from the barrel septa, and less input from the barrel hollows. Thus a barrel-specific organization seems to be especially pronounced for synaptic input from layer II/III to neurons of layer V.

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6D-4
SYNCHRONIZATION AND OSCILLOTORY RESPONSE IN A NEURAL SYSTEM FOR OBJECT-BASED ATTENTION

Norbert Galm, Gustavo Deco

In the present work, we introduce a neurodynamical model for the coding and binding of visual information. The model consists of several layers composed of spiking neurons, which are modeled by the Spike-Response Model.
This neural network realizes a hierarchical coding of the visual stimuli. The neural activities in each layer give an internal feature representation of the neural activities in the previous layer.
The network is able to segregate different visual stimuli presented simultaneously. This is achieved by a binding mechanism based on the synchronization of the spike activity of the corresponding coding neurons. Oscillatory and synchronized spiking activity at a certain layer results from an inhibitory feedback from the internal representation in the next layer.
This binding mechanism is utilized for modeling object-based attention. We present simulations of psychophysical experiments showing this effect.

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6D-5
A MATHEMATICAL MODEL OF THE COLLECTIVE BEHAVIOUR OF EMBRYIONIC NEURONS CULTURED IN VITRO

Michele Giugliano, Marco Bove, Michela Chiappalone, Mariateresa Tedesco, Massimo Grattarola

The ability to generate synchronized oscillatory activity appears to be a distinctive feature of the mammalian nervous system. This kind of electrophysiological behaviour can be investigated thorough several recording techniques, in the case of in vivo as well as in vitro biological preparations. In the present work, the rhythmic electrical activity generated by assemblies of dissociated neurons cultured in vitro is experimentally approached by means of arrays of planar microelectrodes, coupled to such populations of neurons, allowing the researcher to record/stimulate for a long period of time signals from several units in a network. This represents a new framework for experiments expressly dealing with the collective behaviour displayed by networks of neurons, under various chemical-physical conditions. With the aim of quantitatively characterising such electrophysiological behaviours a mathematical model that describes networks of synaptically connected neurons was developed and computer simulated, including detailed mathematical descriptions of cellular mechanisms responsible for the generation of bursts, the spontaneous sub-threshold activity, the synaptic transmission, and the space/time co-ordinated electrical activity. The predictions of such a model can be directly compared with the results of ad hoc designed real experiments where patterns of coherent activity are expected to emerge and evolve in space and time.

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6D-6
CONTROL OF THE EXCITABILITY OF NEURONAL TISSUE AS PROBED BY THE RETINAL SPREADING DEPRESSION

Wolfgang Hanke, Meike Wiedemann

Neuronal tissue, and thus the CNS can be treated to be an excitable medium as it fulfils all the necessary prerequisites. According to this, oscillations, wave propagation and pattern formation can be observed in it. The spreading depression (SD), an excitation-depression wave in neuronal tissue, which can especially easy be observed in retinal tissue, is a very pronounced example of this behaviour. Additionally it is related to a variety of medical topics, in example migraine, stroke, epilepsy and transient global amnesia. It is of major interest to understand, how the excitability of neuronal tissue and thus also the properties of the retinal SD are controlled. The effects of drugs and ions, as well as of temperature, have been studied to some extent, however, about the effects of weak external forces on the retinal spreading depression, much less is known. In this study we present data concerning the effects of electromagnetic fields, currents and gravity on the retinal SD. It is shown that all these parameters influence the retinal SD, however, the effects are different in resting tissue, during a propagating wave, and in refractory tissue.

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6D-7
A SANDWICH TECHNIQUE FOR MULTICHANNEL RECORDING OF RETINAL NETWORK ACTIVITY AFTER APPLICATION OF MULTISITE ELECTRICAL STIMULATION

Thoralf Herrmann, Thomas Stieglitz, Alfred Stett

Questions concerning the development of a subretinal implant demand defined stimulation of separated points of the photoreceptor side of the retina while recording from the ganglion cell side. We have established a technique where explanted retinal segments (chicken, p1-p3) are sandwiched between a polyimide (PI) foil with integrated electrodes for stimulation at the distal side and a microelectrode array (MEA) for extracellular recordings at the proximal side.
Stimulation at the distal retina side was achieved by applying current pulses via selected electrodes of the PI foil. Ganglion cell activity was recorded simultaneously with 8 of the MEA electrodes. We evaluated 13 experiments. In 3 cases neurons lying beneath the foil continuously showed correlated response to the stimulus (threshold between 0.4 and 0.7 mC/cm^2). Specified spatial stimulus configurations evoked distinguishable spike activity patterns. An initial response which deteriorated more or less rapidly occurred 6 times whereas 4 of the preparations showed no measurable activity.
The experimental setup is suited to study the point discrimination capability of the retinal network after application of focal electrical stimuli. Stimulation of the outer retina at different sites results in associated retinal output. Loss of activity beneath the foil could indicate retinal damage caused by touching down the foil onto the retina or insufficient supply of the tissue with ringer solution.

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6D-8
INTERFACING OF A SILICON CHIP WITH SYNAPTICALLY CONNECTED, IMMOBILIZED NEURONS

Günther Zeck, Martin Jenkner, Peter Fromherz

To study small neural networks, we wish to stimulate and to record the electrical activity of each cell over long time periods. We built a silicon chip able to stimulate and to detect individual cells. In first steps it was shown that synaptic connections can be investigated [1,2]. Because neurons were mobile during cell culture we had to immobilize them mechanically. Hexagonal cages, made of polyimide, trapped the neurons on the stimulation spots and the detecting transistors. Neurons grew on this threedimensional chip and formed synapses. The activitity of synaptically connected pairs could be induced and recorded.

[1] A.A.Prinz, P.Fromherz, Biol.Cybern. 82, L1 (2000)
[2] M.Jenkner, P.Fromherz, submitted

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6D-9
CONTROL OF NEURITE OUTGROWTH BY TOPOGRAPHICAL GUIDANCE

Matthias Merz, Peter Fromherz

Small neuronal networks of defined topology would provide an ideal system for neurophysiological experiments. A key to such systems is the controlled growth of neurites with a defined location of synapses. We present a method for directing the growth by topographical guidance cues and compare it with a method based on photolithographic patterning of growth promoting proteins.
Neurons from the pond snail *Lymnaea stagnalis* were cultured on substrates uniformly coated with brain-derived conditioning factors promoting outgrowth. The substrates consisted of silicon chips covered with a polymer into which the topographical structures (grooves and pits) were processed. Grooves with steep walls provided sufficient mechanical guidance for the growth cones, if they were deeper than 10μm. Neurites growing in the structures reached lengths up to several hundred μm, similar to the length on unstructured substrates. T-shaped branchings of the grooves often resulted in bifurcations of the neurites, whereas no bifurcations and change of growth direction were observed at cross-shaped intersections.
With this technique we were able to build a pair of two neurons that was connected by an electrical synapse. In some cases, that junction was strong enough to trigger a postsynaptic action potential upon a presynaptic stimulus.

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6D-10
COMPARISON OF THE EFFICIENCY OF VOLTAGE AND CURRENT PULSES FOR RETINAL NETWORK STIMULATION

Alfred Stett, Andreas Mai

Subretinal prostheses aim at restoring vision to blind people by electrical stimulation of the retinal network. The applied stimuli should provide low stimulation threshold for low power consumption and an appropriate dynamic range for the modulation of the retinal output. We investigated evoked activity of ganglion cells in explanted retinas in dependence of various parameters of applied voltage and current pulses. Retinas from chicken were adhered with the receptor side to a microelectrode array for stimulation that was achieved either by applying voltage pulses (up to ± 3 V, duration ≤ 3 ms) or by biphasic current pulses (up to ± 120 μA, duration ≤ 0.5 ms). As a measure of the stimulation strength, we estimated the charge injected by single pulses. The evoked spike activity of ganglion cells could be modulated up to saturation with increasing stimulation strength. Anodic voltage pulses with duration of 0.5ms resulted in lowest thresholds (median 0.54 nC, 0.68 mC/cm^2, $n=11$), followed by so-called Zeta voltage pulses (ramp with negative slope interrupted by a positive voltage step). Current pulses had higher thresholds. The smallest dynamic range was found with anodic voltage pulses (0.4 nC), the widest with negative voltage pulses (2.1 nC). In further implants, anodic voltage pulses are favoured due to low thresholds and therefore low power consumption. The low dynamic range of about one log unit of charge (nC) requires implementation of adaptive mechanisms.

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6D-11

THE DYNAMICS OF A NETWORK OF SPIKING NEURONS WITH ACTIVE SYNAPSES

Jan Storck, Frank Jäkel, Gustavo Deco

We present a network of spiking neurons connected by dynamical synapses. Neurons are of the integrate-and-fire type and the synapses are given by a dynamical model based on the exact pre- and post-synaptic spike timing. Following the experimental evidence the model takes into account biophysical processes like vesicle and calcium resource adaptation resulting in facilitating and depressing behavior in the transmission of spike trains. The synaptic dynamics adapts in dependence of pre- and post-synaptic spike activity. Through this plasticity, the synapse is capable of implementing various forms of delay and efficacy of spike train transmission.
In the present work we demonstrate how spatio-temporal patterns in multidimensional signals can be learned and detected in this framework. Input neurons encode the signal into a temporal sequence of spikes. Hence, multiple inputs generate spatio-temporal spike patterns. These are transmitted to the output neurons via dynamical synapses. Each synapse generates an individual form of EPSP response resulting from the synaptic dynamics determined by the adaptable parameters. Each output neuron with its set of incoming synapses works as coincidence detector for a specific spatio-temporal pattern. Due to the learned delay and efficacy of the synapses complex patterns in the input can be captured.
The whole network functions as a temporal clustering mechanism with one output per input cluster. We present simulations demonstrating the network's dynamics and its computational capabilities.

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6D-12

THE HEMODYNAMIC RESPONSE OF THE AUDITORY CORTEX AND BRAINSTEM

Pim van Dijk[1], Walter H. Backes[2]

The hemodynamic response of the auditory cortex and the brainstem inferior colliculus was measured simultaneously in an event-related fMRI paradigm. Normal hearing subjects listened to brief (300 ms) wideband noise bursts. Single bursts were placed between each pair of successive MRI acquisitions. To minimize movement artefacts of the brain stem which correlated with heartbeat, MRI acquisitions were triggered by the subject's ECG. A single slice image was acquired every 10 heartbeats. For each examined subject the slice was placed in order to transect both the auditory cortex and the inferior colliculus. By randomly distributing the auditory stimuli in time, we were able to sample the time-course of the MRI signal and to obtain the hemodynamic response function.
The hemodynamic response reached a maximum at 3-4 sec after the onset of the auditory stimulus. Following the maximum, the MRI signal decayed to it's initial value in about 10 sec after onset of the stimulus. We did not observe differences between responses in the auditory cortex and brain stem. Auditory hemodynamic response are similar to those observed in other brain areas.

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7A-1
EFFECT OF ZINC ON THE THERMAL STABILITY AND CHROMOPHORE REGENERATION OF RHODOPSIN

Luis J. del Valle, Paulo Dias, Juan J. Pérez, Pere Garriga

Previous studies have reported direct Zn^{2+} binding to rhodopsin in rod outer segment membranes and in purified form in detergent. Zn^{2+} binding to rhodopsin has also been shown to increase rhodopsin phosphorylation. We have studied the effect of Zn^{2+} on the thermal stability of rhodopsin -obtained from bovine retinas and solubilized in dodecyl maltoside detergent- in the dark. We find a decrease in the thermal stability of rhodopsin in its ground state (dark state) with increasing Zn^{2+} concentrations (0-50 μM Zn^{2+}). The thermal bleaching process is accelerated in the presence of Zn^{2+} with k rate constants, at 55 °C, of 0.028 ± 0.002 min^{-1} (0 μM Zn^{2+}) and 0.056 ± 0.003 min^{-1} (50 μM Zn^{2+}), corresponding to $t_{1/2}$ values of 24.4 ± 1.6 min and 11.8 ± 0.1 min respectively. Thermodynamic parameters derived from Arrhenius plots -obtained from experiments at different temperatures- show a significant Ea increase at 50 μM Zn^{2+} for the process, with $\Delta G^{\ddagger}$ decrease and increase in $\Delta S^{\ddagger}$ and $\Delta H^{\ddagger}$. The extent of chromophore regeneration *in vitro* is also reduced to 60% in the presence of 200 μM Zn^{2+}. These structural effects and others (effect of Zn^{2+} on Meta-II stability and on bleaching efficiency) are discussed with regard to the suggested role of this metal ion in retinal degenerative processes.

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7A-2
THE EFFECT OF ILLUMINATION ON PHOSPHOLIPID EXTRACTION BY BSA FROM DISC VESICLES

Elke Hessel, Peter Müller, Andreas Herrmann, Klaus-Peter Hofmann

The transbilayer distribution of spin-labelled phospholipid analogues with choline, serine and ethanolamine head groups (SL-PC, -PS and -PE, respectively) was studied on osmotically intact disc vesicles of bovine rod outer segment membranes. In the dark, ESR measurements using the BSA back-exchange assay showed an asymmetrical distribution of PS, favouring the outer leaflet, while PC and PE were almost symmetrically distributed in the membrane leaflets (Eur. J. Biochem. 267 (2000) 1473). Illumination of discs (20 μM rhodopsin) with 520 nm light for 1 minute (pH 7.0; 20 °C), bleaching the rhodopsin to about 85%, caused reversibly an increased extraction of SL-PS from the outer leaflet of the membrane. SL-PE and SL-PC were less extractable from the disc membrane. The data suggest a different relationship between the change of phospholipid extraction from the membrane and rhodopsin's active intermediate metarhodopsin MII as well as the subsequent regeneration process.

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7A-3
A POSSIBLE ROLE OF IKCA IN ISOLATED SALAMANDER RODS

Andrea Moriondo, Bruna Pelucchi, Giorgio Rispoli

Larval salamander rods were mechanically isolated and recorded in room light, using whole-cell voltage-clamp technique. A large outward N-shaped response was consistently recorded, which was identified as a calcium-dependent potassium current (IKCa) on the basis of the following results: this current was selectively and completely removed by Ca2+ channel blockers (0.1 mM Cd2+ or 1 mM Co2+) and by specific IKCa antagonists as Charibdotoxin (100 nM) and Iberiotoxin (100 nM). The activation threshold of IKCa was −30 mV and peaked at +20 mV (average amplitude: 1390 ± 321 pA, n = 10). Furthermore, the IKCa current resulted highly selective for Ca2+, since it was not activated if external Ca2+ (1 mM in standard conditions) was substituted by Ba2+ or Sr2+. The physiological role of this current was investigated by means of simulated photoresponses: the light-adapted rods, held at −35 mV, were stimulated by an hyperpolarizing current step (−100 pA, 1 s), in order to simulate the response to a bright step of light. The voltage returned to −35 mV at the end of the current step with a damped oscillation when external Ca2+ was substituted by Sr2+ or in the presence of 0.1 mM Cd2+. When Ba2+ substitutes Ca2+ a strong depolarization also occurs. These preliminary data suggest that IKCa could act as a safety feedback device that controls the return to the dark resting potential, avoiding strong depolarizations or transient oscillations.

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7B-1
$INSP_4$ IS A POTENT ACTIVATOR OF THE $INSP_3$-GATED CHANNEL FROM FISH OLFACTORY CILIA PLASMA MEMBRANE

Hervé Cadiou, Gérard Molle

Beside cyclic nucleotide-activated pathways, there are also evidences for the presence of an inositide-activated pathway in olfactory receptor neurones (ORNs). Thus, the existence of an inositol 1,4,5-trisphosphate-gated channel ($InsP_3R$) in the plasma membrane has been proposed but this still remains to debate. In another hand, Inositol 1,3,4,5-tetrakisphosphate might be involved in calcium entry in several cell types. In this study, we present experiments on the activity of $InsP_4$ towards the $InsP_3R$ in vertebrate (carp) olfactory cilia. Ciliary membranes were isolated from carp ORNs by calcium shock then ultracentrifugated before their reconstitution into planar lipid bilayers. The "tip-dip" experiments were performed at several concentrations of $InsP_3$ and $InsP_4$ by using a 53 mM Ba^{2+} solution in the pipette and a 100 nM free Ca^{2+} solution in the bath. $InsP_3$ did not induce current fluctuations at sub-micromolar levels whereas $InsP_4$, at a concentration of 0.5 μM, exhibited a current of 1.5 pA (SEM = +/− 0.1, n = 3) at 0 mV. From the dose effect curves, the half-opening probability of channels (Po) was obtained for concentrations of 1 μM $InsP_4$ and 10 μM $InsP_3$. Moreover, the current induced by $InsP_4$ was voltage-dependent and was inhibited by 10 μg/ml of heparin, which also inhibits the ER InsP3R-gated channel. These results suggest that the inositide-gated channel from carp ORNs possess distinct properties from the ER $InsP_3$-gated channel and indicate that $InsP_4$ could play a role in olfactory transduction mechanism.

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7B-2
SELECTIVITY IMPROVEMENT IN OLFACTORY RECEPTOR NEURON

Alexander Vidybida

The primary perception of odor molecules in olfactory system is their binding with receptor proteins expressed at the receptor neuron. If two odors are applied at a definite concentration, the probability for a receptor protein to be bound with odor depends on the odor type. The selectivity of receptor protein with respect to two odors can be estimated as ratio of binding probabilities. The selectivity of receptor neuron can be estimated as ratio of firing probabilities. Our purpose is to compare the neuronal selectivity with that of its receptor proteins.
The neuron is modelled as population of identical receptor proteins. If some threshold number of receptor proteins are bound with odor, the neuron fires a spike. The binding-releasing statistics in the population of receptor proteins is analyzed based on the birth-death stochastic process. The neuronal firing rate is estimated based on the first passage time technique. It is established that the selectivity of the neuron can be much higher than that of its receptor proteins, provided the total numbers of receptor proteins per neuron is large. This effect is better displayed for low concentrations.
The same effect could explain improved selectivity of olfactory secondary cells as compared to receptor neurons, as well as analogous improvement at higher stages of sensory signal processing in other sensory systems. The mechanism considered here could be used for developing of artificial sensors having improved discriminating ability.

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7B-3
STOCHASTIC PROPERTIES OF THE ELEMENTARY ELECTRICAL EVENTS IN PHEROMONE-SENSITIVE OLFACTORY CELLS IN THE MOTH

A.V. Minor, K.-E. Kaissling, J. Thorson

Small transient receptor potentials (bumps) usually precede the spikes generated by the pheromone-sensitive olfactory cells in silkmoth antennae. Bursts of such bumps were recorded in response to low pheromone concentration in the bombykal-sensitive receptor cells in males of *Bombyx mori*. The probability density functions (PDFs) of bump and gap durations in a burst can be approximated by single exponentials with time constants of 6–10 ms for the bumps and 40–50 ms for the gaps.The number of bumps per burst followed a geometrical distribution (probability of one more bump = 0.7). These data were used to calculate the expected parameters of the burst PDF which agreed with those observed experimentally. These results can be explained by addition of a third (excited) state to the model of the olfactory receptor excitation (Kaissling, Chem. Senses 23:385–395,1998). We suggest that after a pheromone molecule is bound to a receptor each transition to the excited state initiates activation of several ion channels. To check it with a computer simulation the characteristics of the pheromone-sensitive ion channels in *Antheraea polyphemus* (Zufall & Hatt, PNAS 88:8520–8524,1991) were used. A bump was successfully simulated by overlapped activation of 6-10 of such channels. The PDFs parameters found experimentally were used to calculate the rate constants for transitions between the three states of the receptor. For $r_{31} \ll r_{21}$ we found $r_{21} = 7.9/s$, $r_{23} = 16.8/s$, and $r_{32} = 98/s$.

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7B-4
OLFACTIVE PERCEPTION BY FUNCTIONAL RECEPTORS EXPRESSED IN AN HETEROLOGOUS SYSTEM

G. Levasseur, M.-A Persuy, R. Salesse, E. Pajot-Augy

Perception of odorant molecules by transmembrane olfactory receptors is mainly mediated by a G_{olf} protein, and induces an intracellular calcium increase. Olfactory receptors were stably expressed in mammalian cell lines. Characterization of one of these lines expressing the rat I7 receptor showed membrane expression of this receptor, either by using an engineered receptor – GFP fusion protein, or by immunolabeling the receptor with a specific antipeptide antibody. Stimulation of the transfected cells with a range of concentrations of octanal (previously determined potentially preferential ligand) induced no measurable cAMP level, but intracellular calcium increase was dose-dependent. The cellular response consisted in a transitory calcium mobilization followed by a prolonged desensitization. Nonanal, a similar odorant ligand, generated a similar calcium response, but the dose-response curve was shifted towards higher concentrations. Also important was the absence of desensitization to nonanal contrary to octanal. Moreover, no calcium response was observed at any concentration with heptanal. Our system thus provides functional receptors with an important discrimination capacity towards closely related odorants. This may prove to be a valuable tool for screening olfactory receptor – odorant pairs. Furthermore, this system allows investigation of the influence of several drugs of the transduction pathway on calcium response and on desensitization to a specific stimulation. It is now being used to follow molecular mechanisms involved in transitory intracellular calcium increase and differential desensitization of transduction pathways.

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7C-1
AUDITORY SENSITIVITY PROVIDED BY SELF-ORGANIZED OSCILLATIONS OF HAIR CELLS

Sébastien Camalet, Thomas Duke, Frank Julicher, Jacques Prost

In the case of hearing, we argue that active amplification of faint sounds is provided by a dynamical system at the threshold of an oscillatory instability. A specific model of sound detection by the hair cells of the inner ear is discussed. We show that a collection of motor proteins within a hair bundle can generate oscillations at a frequency that depends on the elastic properties of the bundle. Simple variation of bundle geometry gives rise to hair cells with characteristic frequencies that span the range audibility. Tension-gated transduction channels, which primarly serve to detect the motion of a hair bundle, also tune each cell by admitting ions that regulate the motor protein activity. By controlling the bundle's propensity to oscillate, this feedback automatically maintains the system in the operating regime where it is most sensitive to sinusoidal stimuli.

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7D-1
DEVELOPMENT OF A SYSTEM FOR THE SIMULATION OF HUMAN COLOR PERCEPTION

Andreas Hub, Peter Fromherz

The simulation of human color perception is difficult, particularly in the case of natural scenes. Our aim was to develop a system which allows the categorization of object colors into linguistic color classes, even in natural scenes.
Images of natural scenes were captured with a color camera. Objects were segmented with the help of hue information using a threshold algorithm. Images with complex contents were reduced to simple stimuli (previous experiments have shown that the morphology of objects can be changed greatly in many cases without substantially changing the perceived object color). The color perception of subjects with normal color vision was implemented into the inference step of a fuzzy system. For segmented objects, degrees of membership to linguistic color terms were calculated. Terms with the largest membership values were given as results.
The results of this procedure agree in many cases with the perception of subjects with normal color vision. For blind and partially-sighted persons, the procedure was implemented in a prototype of a measuring instrument which provides acoustic information about object colors, object sizes and distances. This information can improve the orientation ability and the mobility of persons with impaired vision.

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7D-2
NEGATIVE ASSOCIATION BETWEEN THE RESPONSES TO VISIBLE LIGHT MEDIATED BY THE IMMUNE AND PHYSIOLOGICAL SYSTEMS

Arcady A. Putilov

Earlier we detected only weak positive interrelations between the beneficial effects of bright light on circadian, sleep-regulating, energy-regulating and sympatho-adrenal systems in winter depression. Each of these 4 physiological effects, however, correlated positively with reduction of depressive symptoms (Putilov, 1998). Since several studies investigating the number of immune cells in the peripheral blood of individuals with major depression have reported a decrease of the total number of lymphocytes, we suggested that similar abnormality could be a feature of winter depression. The enumeration of lymphocytes was made before and after 1-wk trial of non-drug therapy in 115 female subjects. A decreased absolute lymphocyte number was found in depressed patients compared to controls before, but not after light treatment. However, the observed changes in depression scores and total lymphocytes number correlated negatively. Thus, the results of the study provide evidence that lymphopenia may be a part of psychobiology of winter depression, and that the positive immune changes may be a component of therapeutic action of bright light. However, the clinical response of the immune function seems to be dependent upon neither antidepressant nor physiological responses. Several distinct ways for visible light transduction and several rather independent biological mechanisms for mediation of it's therapeutic effect might be suggested.

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9A-1
VISUALIZATION OF BIOLOGICAL TISSUES BY X-RAY MICROTOMOGRAPHY

Nora M. De Clerck, Alexander Sasov, Dirk Van Dyck

Recent developments in X-ray microtomography offer new perspectives for studying the internal microstructure of biological preparations, without destruction of the specimen. A full three-dimensional (3D) model can be reconstructed after scanning in a non-invasive manner. A desktop microtomograph (Skyscan 1072, Belgium) with X-ray source (spot size < 8 μm) and a cooled fibre optics CCD camera (1024 × 1024/12 BIT) was used. The preparation was mounted on a rotating stage, during scanning. In bony structures 3D architecture of trabecular bone together with relative bone volume and trabecular thickness could be measured. Soft tissues were scanned under nearly physiological conditions as the preparations were protected by oil from drying and shrinking. In a mouse embryo development of soft tissues and bony structures could be followed in different sections through the preparation. This technique is unique in that it allows consecutive serial measurements, without destroying the specimen. Moreover repetitive scanning and reconstruction as a function of time allow to follow biological developments of the same specimen at different periods in time.

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9A-2
FOURIER TRANSFORM INFRARED MICROSPECTROSCOPIC IMAGING OF STRATUM CORNEUM AND ITS COMPONENTS

P. Garidel, H C. Chen, D J. Moore, E. Paschalis, R. Mendelsohn

The stratum corneum (SC) is the outermost layer of the skin and functions to protect the body from its environment. This layer of tissue is only about 10-20 μm thick. A major role of SC is to maintain water homeostasis of the body. A number of skin diseases are related to the malfunction of this barrier e.g. psoriasis, atopic dermatitis or xerosis. The SC consists of cells and corneocytes, within a matrix of lamellar bilayer lipids. The corneocytes are filled with keratin. These annucleated cells are derived from terminally differentiated keratinocytes. In principle, three classes of lipids are found in the SC: namely, ceramides, free fatty acids and cholesterol.

We have analyzed SC and its components by Fourier transform infrared microscopic imaging. The SC was obtained from a hairless Yucatan pig. Images were generated from spectra collected on a BIO-Rad "Sting-Ray" instrument consisting of a step-scan interferometer coupled to a 64 × 64 mercury-cadmium-telluride focal plane array detector. This new technique allows us to map a sample area of 400 μm × 400 μm, generating 4096 spectra, one from each element of the array. A number of infrared images were then generated from these large data sets by measuring and plotting the integrated area of particular spectral components, such as the amide band of proteins, in each of the 4096 spectra. With this procedure we can image the distribution of each chemical species through the SC.

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9A-3
FT-IR MICROSPECTROSCOPIC IMAGING OF TISSUES

Dieter Naumann, Peter Lasch, Janina Kneipp

Transmissible spongiform encephalopathies (TSEs) such as scrapie in sheep, bovine spongiform encephalopathies (BSE) in cattle, or Creutzfeldt Jakob disease (CJD) in man are infectious neurodegenerative disorders of the central nervous system that are far from being completely understood. A new approach for the identification of pathological changes in scrapie-infected Syrian hamster brains based on FT-IR microscopic mapping and imaging of cryosectioned brain samples has been developed to obtain spatially resolved, multidimensional information on tissue composition and structure[1,2]. In order to account for the fingerprint-like nature of spectral information, we used pattern recognition techniques and artificial neural network analysis to reconstruct infrared images of the cerebellum, medulla oblongata and pons of infected and control hamster brains and to compare spectroscopic data from identical anatomical structures in scrapie-infected and control brains. Consistent alterations in membrane state-of-order, protein composition, carbohydrate, and nucleic acid constituents were detected in scrapie-infected tissues[2]. Cluster analysis performed on spectra of homogenized medulla oblongata and pons samples also reliably separated uninfected from infected specimens. This method provides a potentially useful tool for the development of rapid TSE diagnostic and screening techniques.

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2. Kneipp J, Lasch P, Baldauf E, Beekes M and Naumann D Biochim. Biophys. Acta in press

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9A-4
MAGNETIC RESONANCE IMAGING TO MONITOR THE BIOLOGY OF INFLAMMATION

J. Ruiz-Cabello, I. Rodriguez, P. Avilés, M. Graña, I. Echave, J. Regadera, D. Gargallo, M. Cortijo

Despite the acknowledged advance of MRI along these years, there is scant data about the type of infection, the kind of inflammatory response and the clinical phase of its evolution.

MRI studies were made at different stages of infection ranging from days 0-14 post- intramuscular inoculation with *Aspergillus fumigatus* on male mice. Images were analyzed for measuring size of affected regions and tissue classification. Pattern estimation was effected by a self-organizing map (SOM).

Signal intensity in a large area around a central locus of infection on T_2-weighted images was increased immediately after inoculation. The margins of the signal abnormality were poorly defined. In the intermediate stage of the inflammatory process (7 days), different types of lesions, distributed concentrically around the previously described oval-shaped centrally located necrotizing mass and with much better defined limits, were observed on MRI. In the well-established inflammatory stage evolutioning to chronicity, the lesion is well delimited because there is a clear interface between the centrally located lesion and normal muscle.

The MR patterns were described along the experimental period based on millimetric comparison with histopathological and imaging analysis for identification and quantitation of different tissues. Although our results varied depending on the degree of maturation and probably on the number and kind of muscles initially affected, they were totally coincident with histological evolution.

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9A-5
A CLUSTERING METHODOLOGY FOR DETECTING ACTIVATION SIGNALS IN FUNCTIONAL MR IMAGES

Carmen Santisteban, Manuel Cortijo

There is a considerable interest in the use of functional magnetic resonance imaging to study brain function. We have obtained our results in a 1.5T SIGNA MRI scanner by using previous published acquisitions techniques (Eur Biophys J (1996) 24:335–341), as well as other ones recently developed. They have been applied to several tasks, which produce activation in the visual, motor cortex or higher brain areas.
The main aim of the present work is to improve the final images by removing from our data all pixels with high noise levels and low information. We have applied several statistical approaches to this goal, by using parametric and non-parametric methods. The best results have been obtained applying a cluster analysis. We can calculate the Mahalanobis distances of the pixels filtering all those with these distances lower than a threshold value. For this purpose we usually need to define the critical size of the cluster to discriminate between active areas and group of pixels with high Mahalanobis distances by hazard. It is carried out determining the probability of clusters with several sizes when they are active by chance or by the signal.

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9A-6
PROGRAM FOR THE MEDICAL IMAGES ANALYSIS

Mircea Tîrziu

The paper presents an image processing program that implements new and powerfull analysis of the medical pictures. Using this software the user can transform his B & W tomograph in a color one (without buy a color option that is very expensive), he can convert his radiographies, his ECHO pictures or his scintigrams into color images. He can put all your medical images into the computer, with all the subsequent advantages. It may added that he gains all the freedom to define himself the colors that reveal the best what he seeks on the picture. He has a lot of very friendly tools that permit him to do this. But he can also use a big library with predefined colors – with a simple mouse click. The program has a lot of new operations that permits to the doctor to see new aspects on the images, that no one saw until now. Among the new features it may be remembered the intelligent selection of the interes areas, that permits to the user to extract what he wishes from a complex or deteriorated images, as the recorded on the paper EEG, EKG or EMG waves. It must be emphasized that the package works not only with the triplet "red-green-blue", but also with the triplet "hue-saturation-intensity". As an example, the user may convert the saturation of each point of the image to hue and, on this way, to obtain a picture that shows very clear the distribution of the saturation in the image. This is only one of the many new features introduced by this software, which may be used to reveal B & W and color pictures aspects hiden until today. The program can also made new and fine retouchings on the images, a very useful feature that permits to the user to give to his photos the best shape. So, he can have nice presentations and beautiful books.

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9A-7
TIME RESOLVED OPTICAL TOMOGRAPHY OF SCATTERING PHANTOMS

Chantal-Virginie Zint[1,2], Feng Gao[1], Patrick Poulet[1]

A near-infrared Optical Tomograph is being developed to obtain 3D images of scattering phantoms with optical properties similar to those of biological tissues. This experimental setup uses a femtosecond laser and a Synchroscan streak camera. The scanning is carried out by moving the phantom. The temporal point spread function (TPSF) of light transmitted through the phantom is acquired for various positions and then analysed. For the processing, we developed a light transport model based on the Finite Element Method (FEM) that solves the diffusion equation numerically, as well as an approach to the inverse problem. Based on the implicit computation of the Jacobi matrix of the Forward operator, the reconstruction algorithm use Algebraic Reconstruction Techniques (ART) as linear inversion scheme. It executes the mapping of absorption and scattering on the basis of TPSF characteristic data, such as the mean flight time of photon with or without the related variance.
The reconstruction algorithm has been validated for simulated data and is being validated for measured data. First images were obtained from measurements on liquid cylindrical phantoms consisted of Indian ink added to Intralipid-10%, with a scattering and/or absorbing inhomogeneity.

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9B-1
MAGNETIC DRUG TARGETING: AN INTERDISCIPLINARY METHOD IN TREATMENT OF CANCER

Christoph Alexiou, Wolfgang Arnold, Peter Hulin, Roswitha Klein, Ernst Huenges, Fritz G. Parak

Specific delivery of chemotherapeutic agents to desired target sites with a minimum of systemic side effects constitutes one of the ongoing challenges of chemotherapy. "Magnetic drug targeting" offers an novel opportunity to treat tumors locoregionally.
Magnetic particles were ionically bound to an anticancer agent (mitoxantrone). This compound (MP-CH) was intravascularly applied in tumor bearing rabbits and directed with an external magnetic field to the tumor. The magnetic field strength (1.7 Tesla) was produced by an electro magnet and focused on the tumor. Animals receiving intraarterially MP-CH in a concentration of 20% or 50% of the regular mitoxantrone dosage (10 mg/m^2 body surface) had complete tumor remission without any negative side effects. Animals receiving the same amount of magnetic particles without mitoxantrone did not show regression of tumor size and the rabbits developed metastases. The respective mitoxantrone dose alone did not affect the tumor size and intravenous application revealed mainly a stop of the tumor growth. The enrichment of magnetic particles could be visualized histologically and with magnetic resonance imaging technique.
The intraarterial application of ferrofluids bound to anticancer agents in conjunction with an external magnetic field focused on the tumor was very effective in treating squamous cell carcinomas and showed no negative side effects.

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9B-2
ACTIONS OF SEMAX ON CALCIUM RESPONSE IN HUMAN NEUTROPHILS

Julia Bespalova

Semax is the new Russian medical drug. It is synthetic heptapeptide of adrenocorticotrophic hormone ($ACTH_{4-10}$). This nonsteroidogenic fragment $ACTH_{4-10}$ is used for adapting in extreme situations. The study of mechanism action of semax on human neutrophils represents the considerable interest.
In whole blood the exogenous semax (5×10^{-5} M) led to respiratory brust. Semax caused an immediate concentration-dependent rise in $[Ca^{2+}]_i$. The maximal amplitude of the calcium response (110 nM) is observed at concentration of semax – 25 μM . Relationship between $[Ca^{2+}]_i$ and semax looks like a curve with saturation. However semax has no affects on undifferentiated promyelocytic HL-60cells, human lymphocytes and CTTL.
To determine whether semax increases inositol-1,4,5-trisphosphate (InsP3) level we compared its effect in Ca^{2+} free and Ca^{2+} mediums. In Ca^{2+} free medium semax (25 μM) did not cause the release of Ca^{2+} from intracellular Ca^{2+} stores. The subsequent addition of tapsigargin (400 nM, an inhibitor Ca^{2+}-ATPase of these stores) induced transient calcium response. It was concluded that the semax effects is not connected with InsP3 metabolism. FMet-Leu-Phe (FMLP, 10 μM) blocked the subsequent semax (25 μM) Ca^{2+} response. Semax (25 μM) decreased Ca^{2+} response to FMLP (10 μM). Thus, is established, that semax: Induces respiratory brust in whole blood; Increase $[Ca^{2+}]_i$ in neutrophils; Its action is not connected with FMLP receptors; FMLP down-regulates semax Ca^{2+} response; These results indicate that semax may be produced locally in extra-pituitary tissues.

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9B-3
PROTECTIVE ACTION OF POTASSIUM ASCORBATE AGAINST HAEMOGLOBIN OXIDATION IN RED CELLS

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The systemic action of oxidative substances like free radicals or other chemicals that are involved in many degenerative diseases is able to modify erythrocytes stability leading to peroxydation of the lipidic membrane, oxidation of the erythrocytes haemoglobin (Hb) and finally to the formation of endoerythrocyte inclusions called Heinz Bodies. Such a process can also be induced or enhanced by the action of strong oxidants like acetylphenylhydrazine (APH). In a previous work we have investigated the formation of APH induced Heinz bodies by Mössbauer spectroscopy. Since one of the mechanisms that are altered in many degenerative diseases is the K-Na ionic channel, potassium ascorbate seems able to re-establish the potassium concentration and at the same time have an antioxidant action. In the present work the properties of potassium ascorbate acting on red cells treated with APH are investigated. Venus blood collected from healthy people was used to prepare the samples, incubated with APH and potassium ascorbate. These samples were analysed by Mössbauer spectroscopy in order to determine the capacity of potassium ascorbate to neutralise APH action, both in the case they are acting at the same time or in sequence, i.e. ascorbate following APH. The dynamical transformation of the iron oxidation states in the oxidation products has been followed and a comparison of the Mossbauer parameters obtained with and without the protective action of potassium ascorbate is presented.

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9B-4
AFM STUDIES OF THE TOPOGRAPHY OF HYDROGEL BEAD SURFACES

Markus Hillgärtner, Heiko Zimmermann, Ines Westphal, Günter Fuhr, Ulrich Zimmermann

Hydrogel-based (and particularly alginate-based) microencapsulation is currently one of the most favoured approaches for the treatment of endocrine diseases (e.g. Diabetes mellitus). One of the urgent needs for advance in this area of encapsulated cell therapy is certainly the enlightenment of the relationship between surface topography of the microcapsules and induction of foreign body reactions.
Atomic force microscopy (AFM) provides the possibility for three-dimensional scanning of an alginate capsule surface in liquid environment. Application of AFM to alginate gels requires the use of dynamic modes, low forces and very slow scanning rates.
AFM studies have shown that there is apparently no significant difference in the micro-structure of the surface between empty alginate capsules stored in saline solution for several weeks and capsules after 4-week implantation in a rat. The surfaces are quite homogeneous, but reveal some imperfections, such as wrinkles. In contrast, AFM images of alginate capsules retrieved from rats after about 15 months of implantation show deposition of material (presumably collagen fibrils) on the surface. It is conceivable that the formation of fibril films was ultimately induced by the roughness of the gel surfaces and/or by crevices in the surface that may occur upon exposure of the capsules to mechanical shearing forces during implantation. In any case, the AFM results show that both the gelation process and the mechanical strength of the capsules should be improved.

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9B-5
BIOCOMPATIBLE MATERIAL FOR THE MICROENCAPSULATION-BASED CELL AND TISSUE THERAPY: ALGINATE

Anette Jork, Jörg Ludwig, Hubert Cramer, Frank Thürmer, Ulrich Zimmermann

Studies have revealed that highly purified alginate can provide for the immunoisolation of allogeneic and xenogeneic cells and tissues for the treatment of metabolic diseases (e.g. Diabetes mellitus) without any immunosuppression therapy. Alginate constitutes a family of unbranched anionic polysaccharides, mainly extracted from brown algae. The major problems in microencapsulation shown up in clinical trials were the long-term stability and the biocompatibility of the implants.
Here we present a novel method of purification of the raw material and demonstrate that this alginate does not evoke any immunological reaction due to its chemical and physical properties. For *in vitro* examinations a new system was established based on the mitogenic lymphocyte stimulation. The long-term stability was improved by consideration of the thermodynamic equilibrium between the bead and the surrounding solution. The biocompatibility and the long-term stability of the alginate was examined *in vivo* by implanting Ba^{2+} cross linked beads beneath the kidney capsule of BB rats and also by implantation of perfluorocarbon-loaded capsules in Wistar WU rats. The perfluorocarbon implants allowed the measurement of the local oxygen supply in different locations in the rats and could be observed over a period of two years by using non-invasiv ^{19}F-nuclear magnetic resonance imaging (MRI). The new alginates showed no inflammatorical reactions and are especially suited to medical applications.

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9B-6
THE EFFECT OF NEWLY SYNTHESIZED PHENOTHIAZINE DERIVATIVES ON LIPID MODEL AND ERYTHROCYTE MEMBRANE STUDIED BY FLUORESCENCE SPECTROSCOPY AND MICROCALORIMETRY

Krystyna Michalak, Olga Wesolowska, Andrzej B. Hendrich, Malgorzata Bobrowska-Hägerstrand, Noboru Motohashi

Multidrug resistance (MDR) of cancer cells to cytotoxic drugs is one of the major obstacles to successful chemotherapy. Phenothiazines have been shown to be among the drugs that modify MDR but mechanism responsible for their action is not clear. Interaction of MDR modifiers with lipid bilayer may be an important factor in MDR modulation and reversal. The development of pharmacologic agents that reverse MDR is a very promising way to overcome of the drug resistance. In this work interactions of the new phenothiazine derivatives with different substituents in tricyclic ring and with side chains of different length with model lipid membranes and erythrocyte membrane were studied. In fluorescence experiments liposomes were labeled with NPN. Studies revealed that alterations induced by phenothiazines in emission spectra of membrane-bound NPN were strongly dependent on the chemical modification of the drug, particularly on substitutions in position 2 of phenothiazine ring. The derivatives with CF_3 in this position were more efficient than others. Phenothiazines also affected lipid main phase transition - broadening of transition peak and T_m shift to lower temperatures were observed by microcalorimetry. In case of erythrocytes, apart of changes in fluidity of membrane, stomatocyte formation was detected in presence of tricyclic compounds.

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9B-7
BIOMEDICAL APPLICATION OF MÖSSBAUER SPECTROSCOPY

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Molecular diseases are caused or accompanied by the synthesis of anomalous biomolecules or any other protein biosynthesis disturbance. Some pathological states of the body are caused by environmental. Iron containing biomolecules play a vitally important role in biological systems. Therefore, relationship of the iron electronic and magnetic structure and protein molecular structure is of interest to study the molecular nature of various pathological processes. Mössbauer spectroscopy is the most sensitive technique to study the iron states in various materials. This review considers the main directions and results of the biomedical application of Mössbauer spectroscopy as follows: 1) Quantitative changes of iron containing proteins during hemoglobinopathies, erythremia, erythremia Friend and lung diseases related with the occupational groups; 2) Qualitative changes of the iron electronic structure in hemoglobin from patients with hemoglobinopathies, leukemia and erythremia; changes of the iron core in ferritin and hemosiderin from patients with the iron overload diseases; 3) Environmental factors: the effect of chemicals (hydrazine and its derivatives) and radiation (X–rays, γ–rays, electrons, microwaves) on hemoglobin; 4) Pharmaceutical compounds and blood substitutes: structural variations in iron–dextran and iron–polysaccharide complexes as well as changes in modified hemoglobins which used as blood substitutes.

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9B-8
MEMBRANE TOXICITY OF TRIPHENYLLEAD CHLORIDE

Stanislaw Przestalski, Halina Kleszczynska, Janusz Sarapuk, Anna Dziamska, Jerzy Hladyszowski, Jerzy Radecki

The aim of the studies on the effect of triphenyllead chloride on the lipid bilayer (BLM and liposomes) and biological membranes (erythrocytes) was: a) to determine toxicity of the compounds, which is of practical importance and b) to determine the interaction of a whole group of similar compounds (organic compounds of lead and tin) with the lipid and protein phases of membranes. Experiments were performed by using spectrometric, fluorimetric and electric methods. Influence of organometallic compounds on hemolysis of erythrocytes, stability of lipid planar membranes and fluidity of erythrocyte membranes was studied. Parameters measured were critical hemolytic concentrations (erythrocytes), critical mechanical concentrations (lipid membrane) and polarization or anisotropy coefficient (erythrocyte ghost membrane). Quantum chemical computations were performed to determine geometry (shape) and electronic properties of studied cation. The results obtained indicate at an essential role of electrostatic interactions between triphenyllead chloride and plasma membranes that suggest a marked effect of the lipid fraction on membrane disorganization. Potentially practical conclusion indicates that the change of the polarity of a membrane may limit availability of the membrane to ion studied. Cognitive results indicate which parts of the membrane are mostly influenced by external cationic species.

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9B-9
ACTION OF A VOLATILE GENERAL ANAESTHETIC ON A MODEL OF BIOLOGICAL MEMBRANE: PROTEOLIPOSOMES OF DPPC AND BACTERIORHODOPSINE

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The mechanism of action of anaesthetic molecules are still speculative. In particularly, the exact site of action of anaesthetics is still unknown : do the anaesthetics act directly on the protein or do they act via the lipidic phase. In order to test both hypothesis we have used DPPC liposomes containing a membrane protein, bacteriorhodopsin (bR). The interaction of anaesthetic with the lipids are followed by using a lipidic fluorescent probe, Laurdan, and the interaction of anaesthetic with the protein was followed by the absorption spectra of the retinal linked to the protein. Liposomes containing different quantities of proteins were formed and the general anaesthetic Enflurane was used in this study. At two steps action of Enflurane has been evidence. At low concentration, the anaesthetics drastically modify the absorption spectra of Br, whereas the transition temperature of the lipids was only slightly decreased. At higher anaesthetic concentration, the temperature of phase transition is shifted. Moreover, the action of anaesthetic on the lipids depends on temperature, whereas it does not on the protein. This experiments show that Enflurane modifies preferentially the protein, then the lipidic membrane indicating at least two classes of sites for the action of enflurane: one class in the proximity of the protein and an another class in the lipidic bulk.

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9B-10
MITOCHONDRIAL RESPIRATORY CHAIN DISORDERS IN GALACTOSAMINE INTOXICATION

Mykhailo Korda

The effect of galactosamine administration on rat liver mitochondrial respiratory chain and the protective effect of liposomes with incorporated antioxidant cresacin on galactosamine induced damage was studied. galactosamine treatment decreased significantly the rate of respiration, respiratory control ratio and po ratio a measure of phosphorylation efficiency upon oxidation of succinate. There was a significant decrease of the nadh dehydrogenase, succinate dehydrogenase and cytochrome oxidase activities of the mitochondrial respiratory chain as well as ATP level and membrane potential. But ATP-ASE activity was increased.
It is thus concluded that galactosamine exerts its effect on the mitochondrial respiration and oxidative phosphorylation through the impairment of the mitochondrial membrane and inhibition of the electron transfer activities of the respiratory chain.
The lipid peroxide level was increased in mitochondrial membranes of galactosamine treated rats. It is proposed that increased lipid peroxidation was responsible for the loss of membrane integrity and inhibition of both oxidation and phosphorylation in mitochondria of rats intoxicated by galactosamine.
Administration of liposomes with incorporated antioxidant cresacin prior to galactosamine treatment relieved the effects (induced by galactosamine) on all the parameters studied. This fact shows that liposomes with cresacin protects against galactosamine toxicity.

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9B-11
STUDIES OF THE INTERACTION BETWEEN NMDA AND t-PA BY A COMBINAISON OF EXPERIMENTAL AND THEORETICAL APPROCHES

Nathalie Colloc'h, Olivier Nicole, Fabian Docagne, Eric T. MacKenzie, Denis Vivien, Alain Buisson

During cerebral ischaemia, the acute interruption of blood flow leads to neuronal death distal to the occlusion which is concomitant with an increase of the extracellular concentrations of an excitatory neurotransmitter, glutamate. This increase induces an excessive stimulation of a class of post-synaptic glutamatergic receptors (NMDA receptor), provoking neuronal death, thus resulting in an extension of the damaged zone; this phenomenon is termed excitotoxicity. The neuroprotective activity of TGF-β1 (Transforming Growth Factor-β1) against NMDA-mediated neuronal death has been previously demonstrated. We have shown that an up-regulation of the serine protease inhibitor PAI-1 (Plasminogen Activator Inhibitor-1) in astrocytes mediates the neuroprotective activity of TGF-β. The Tissue-type Plasminogen Activator (t-PA) is a serine protease released following the onset of cerebral ischemia. We have shown, using double immunoprecipitation, that t-PA interacts with the NR1 subunit of the NMDA receptor. This interaction results in a cleavage of this subunit close to the N-terminal end. A putative binding and cleavage site on NR1 is proposed based on sequence analysis of t-PA partners. In the present study, we attempt to further understand, with a homology-based model of the N-terminal domain of NR1, how the truncated NR1 subunit of the NMDA receptor may affect the NMDA-induced Ca^{2+} influx and consequent neuronal death.

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9B-12
^{1}H-NMR STRUCTURAL AND THERMODYNAMICAL ANALYSIS OF ANTIBIOTIC MITAXANTRONE SELF-ASSOCIATION AND ITS COMPLEXATION WITH CAFFEINE

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The self-association of antibiotic mitaxantrone (novatrone) and its complexation with caffeine in aqueous salt solution has been studied by one- and two-dimensional 500 MHz ^{1}H-NMR spectroscopy. A statistical-thermodynamical model of self-association and hetero-association, in which molecules form indefinite aggregates has been used to analyse the NMR parameters of component molecules in solutions. Experimental concentration and temperature dependences of proton chemical shifts of aromatic molecules have been studied. The analysis leads to determination of the equilibrium reaction constants and the thermodynamical parameters (enthalpy and entropy) of the self-association and hetero-association reactions, the parameter of cooperativity and the limiting values of drug proton chemical shifts in the complexes. It is concluded that the self-association of novatrone is non-cooperative due to the existence of bulky side groups attached to the chromophore of the molecule. A relatively large magnitude of the self-association constant ($K = 12400 \pm 4000\ M^{-1}$ at $T = 318K$) of novatrone, structural and thermodynamical analysis of the self-association process and 2D-ROESY spectra indicate the formation of an intermolecular H-bond in the dimer complex of antibiotic. Quantitative analysis shows that hydrophobic interactions play a substantial role in the stabilization of hetero-complex between novatrone and caffeine.

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9C-1
PHOTODYNAMICALLY-INDUCED HAEMOLYSIS OF HUMAN ERYTHROCYTES – EFFECT OF TEMPERATURE AND ANTIOXIDATIVE AGENTS

Maria Bryszewska, Ilya Zavodnik, Emilia Krajewska

This study was undertaken to determine the effect of free radical scavengers and temperature on photoinduced haemolysis of human erythrocytes. Photosensitization was achieved by incubation of cells in the dark with Zn-phthalocyanine (ZnPc) dissolved in DMSO. First, the effect of DMSO on red blood cells was studied and a significant haemolysis was observed: 5% for 1% DMSO solution and 9.6% for 4% DMSO as compared to untreated controls (0.46% haemolysis). The presence of ZnPc in DMSO did not change haemolysis. Next, the photosensitized samples were irradiated with low-power laser red light (670 nm, 7 mW, 9 J) and the effect of antioxidants: butylated hydroxytoluene (BHT) and diphenylamine (DPA), the alkoxyl- and peroxyl radical scavengers, on post-irradiative haemolysis was studied. It was shown that DPA did not significantly alter this haemolysis, whereas BHT at a concentration of 0.5 mM increased the haemolysis over 10 times. It indicates that alkoxyl radical does not participate in photodynamically-induced haemolysis. Activation energy of erythrocyte lysis was calculated from Arrhenius plot after the cells were irradiated with red light in the presence of ZnPc and next kept at different temperatures up to 90 minutes. A value of 62.0 kJ/mol was obtained.

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9C-2
RETINAL INTRACELLULAR RECORDS FOR THE RABBITS AFTER TREATMENT WITH B-PARTICLES

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Purpose: This study aimed to investigate the effect of b-particles on the intracellular recording of local ERG at different depth for the New Zealand rabbits' retina at the doses 40-10 Gy.
Methods: This study included direct effects (after 24 h) and delayed effects (after 30 and 90 days) for the treated and scattered eyes.
Results: The obtained data showed dose dependent changes in ERG records in both eyes and the photoreceptors were the most radiosensitive cells in the retina. The significant changes in the retinal functions were observed above 60 Gy. The inner retina (ganglion cells) showed slight changes at the dose of 10 Gy only. These results are confirmed by histopathological examinations.
Conclusion: We conclude from this study that there is a need to protect the scattered eye and to use the radioprotectors.

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9C-3
SPECTROSCOPIC STUDY OF THE INTRACELLULAR BEHAVIOUR OF A NEW EFFICIENT CHLORIN-BASED PHOTOSENSITISER

A. Grichine, A. Feofanov, T. Karmakova, R. Yakubovskaya, A. Mironov, M. Egret-Charlier, P. Vigny

A new promising photosensitiser: 3-devinyl-3-formylchlorin p6 (FCp6) was investigated by optical spectroscopy in solution and in living cells to understand the mechanisms of its photodynamic action and their relationships with its intracellular interactions and localisation. The generation of singlet oxygen is a dominating process for FCp6. It efficiently occurs when FCp6 is free or bound to membranes, but sharply decreases when FCp6 is bound to proteins. A quantitative confocal spectral imaging (CSI) analysis revealed that the drug accumulates rapidly within cells and that its intracellular concentration reaches ca. 70% of the extracellular one after 6h incubation. A fast efflux of Chlp6 occurs during the first hour of cell exposure to the fresh culture medium. The main mechanism of FCp6 penetration into cells is endocytosis. The uptake increases when free FCp6 binds to the cell membrane, but decreases when FCp6 is bound to the components of serum. The CSI analysis with the selective labeling of cellular organelles in the presence of FCp6 proves preferential accumulation of the agent within mitochondria and plasmalemma. Mitochondria are the first target of FCp6-mediated photodamage.

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9C-4
USE OF OPTICAL WAVEGUIDE LIGHTMODE SPECTROSCOPY TO MEASURE UV DOSE

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In many indoor and outdoor measurements broad-band UVX meters are used to detect the UV dose-rate. To monitor the biological effect of environmental and artifitial UV radiation different methods have been developed. One of them is the polycrystalline uracil thin-layer dosimeter, which measures the decrease in optical density due to photodimerisation of uracil caused by UV irradiation. It is suitable for long term environmental monitoring and indoor measurements as well, giving a readout proportional to photocarcinogenesis effect. The exposure time is between two and six weeks for outdoor, while for indoor measurements it depends on the experimental set-up. Dimerisation of uracil monomers causes also a change in the refractive index of the thin-layer, which allows detection of an optical signal far from the absorption band. To measure these parameters the Optical Waveguide Lightmode Spectroscopy was applied. It is shown that the UV irradiation causes a decrease in the refractive index and an increase of the optical anisotropy. The kinetic parameters of the UV dosis–sensor response curve are similar to the method based on absorption measurements but the sensitivity of OWLS is much higher. These results prove that Optical Waveguide Lightmode Spectroscopy can be a powerful technique to detect and monitor UV irradiation.

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9C-5
PHOTODYNAMIC EFFECT ON ISOLATED CRAYFISH MECHANORECEPTOR NEURON AND GLIAL CELLS

A. Uzdensky, A. Zhavoronkova, M. Kolosov, D. Bragin

Isolated stretch receptor neuron together with surrounding glia were used as a model in the study of the complex tissue response to photodynamic effect (PDT). SRN preparations were stained with 10^{-7} or 10^{-5} M Photosens ($AlPcS_n$) and irradiated with He-Ne laser (0.3 W/cm^2) until irreversible firing cessation. Then, in 2; 4-6, or 10-20 h cells were fixed and stained with chromatin-specific fluorochrome Hoechst 33342. Two distinct irreversible firing cessation types were observed at high and low PDT intensities: (1) firing acceleration followed by abrupt depolarization block, or (2) gradual inhibition until irreversible firing cessation, respectively. Using different substrates or inhibitors it was shown that free radical processes, inhibition of ATP production, and increase in cytosolic Ca^{2+} enhanced the efficiency of PDT-induced cell killing. It was shown that firing acceleration phases in the cell responses were due to PDT-induced damage of neuronal membrane, whereas the inhibition phases were caused by the lesion of mitochondria and/or ER and Ca^{2+} release. Photodynamic or pharmacological impacts never caused apoptotic fragmentation of the sensor neuron nucleus whereas in glial cells it was often observed especially in 3-6 hours after functional neuron inactivation. Hence, glial cells were easier underwent to apoptosis and possibly protected neurons.
The work was supported by RFBR and Competition Center at SPbGU.

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9C-6
EFFECT OF PORPHYRIN-TYPE PHOTOSENSITIZERS ON LIPID MODEL SYSTEMS

Istvan Voszka, Gabriella Csik

Photodynamic therapy is a new method for tumor treatment. During this photosensitizers are given into the tumor cells. Illumination with light of proper wavelength results in photochemical reaction when reactive oxygen radicals are produced and finally the cell dies. The efficiency of photosensitizers depend on the selectivity and intracellular localisation of the compound. For the better absorption and selectivity new groups of photosensitizers were developed. One of these groups, tetraphenyl-porphyrins were examined. Since one important target of the photosensitizers is the cell membrane, the interaction with liposomes was examined. We used liposomes without net charge and with net negative charge. The interaction also strongly depends on the symmetry and hydrophobicity of the photosensitizers, so we used several compounds differing in their symmetry and side chains. The interaction was followed by measurement of fluorescence. The rate constants and binding constants were determined from the results. We used fluorescent probes to reveal the localisation of compounds within the membrane. The phase transition parameters of these systems were examined, too. According to our results the rate constant was different depending on the side chains. Both the rate and strength of binding was higher to liposomes without net charge for all porphyrin derivatives. The change of phase transition parameters were more expressed in case of asymmetrical derivatives. The intracellular localisation depends on the hydrophobicity.

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10A-1
SALT STRESS EFFECTS ON PHOTOSYNTHESIS IN DUNALIELLASALINA CELLS

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Eucaryotic unicellular green alga, Dunaliella salina, can adapt to very wide range of salt concentrations (0.5–5 M NaCl) and thus attend a convenient model system for investigating the molecular mechanisms of osmoregulation. In the present work we have studied the short time effects a salt shock on photochemical activity of photosystem (PS) I and PS II as well as on distribution of exitation energy between photosystems. A dramatic decrease in PS II activity, measured as oxygen evolution by whole cells in D. salina and a parallel increase in PS I activity occurred immediately with increasing the salt concentrations up to 4 M NaCl. The remarkable differences were observed between the low temperature fluorescence emission spectra of D. salina cells grown at low salt media (0.5 M NaCl) and then transferred to high salt media (4 M NaCl). The intensity of emission peak at 686 nm originated from PS II decreased with the increase of emission peak at 710 nm originated from PS I. These data also suggest the redistribution of exitation energy between two photosystems as well as the activation of PS I mediated electron flow under high salt conditions.

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10A-2
CHLORIDE MAY BE INVOLVED IN METABOLISM INHIBITION BY INFLUENCE ON CYTOSOLIC PH

Denis Andreyuk, Natalia Matveyeva, Olga Voytsekh, Igor Yermakov

Differentiation of plant pollen includes a transition from high to low metabolic activity just before dessication stage. While inhibitory role of low cytosolic pH is well established in different plant and animal cell types, chloride is not usually taken as possible participant of such regulation. Here we studied the role of pH and Cl^- in temporary inhibition of energy metabolism during tobacco pollen differentiation.
Respiration intensity was measured with a Clark-type oxygen electrode, ATP content – with a firefly luciferase assay, cellular $[Cl^-]_{in}$ – with ion-selective electrode, outer Cl^- content – with electron probe microanalysis on freeze-dried anther prints, cytoplasmic pH – with fluorescent microscopy.
Rapid inhibition of O_2 uptake and ATP content correlated well with increase of cellular Cl^- concentration and decrease of cytoplasmic pH. Simultaneously outer chloride content rose. High $[Cl^-]$ applied *in vitro* led to decrease of pH to the *in vivo* value. Standard culture medium caused much higher pH value. Only over a short differentiation period pollen grains were competent to change their pH when cultivated with high outer $[Cl^-]$. Ethacrynic acid known as chloride channel blocker caused rapid fall of cytoplasmic pH suggesting direct involvement of Cl^- in pH-regulation.

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10A-3
MODELING THE SPATIO-TEMPORAL METABOLIC DYNAMICS OF A PLANT BIOLOGICAL CLOCK

Andreas Bohn, Uwe Rascher, Marc-Th. Hütt, Friedemann Kaiser, Ulrich Lüttge

Crassulacean Acid Metabolism (CAM) is an ecophysiological adaptation of plants to drought stress and a model example for biological timekeeping: The temporal separation of CO_2 uptake during the night and its photosynthetic assimilation behind closed stomata during the day makes use of the reduced transpiration rate at nighttime, yielding an increased water use efficiency. When exposed to constant environmental conditions (e.g. light intensity, temperature), the CO_2 exchange of the CAM plant *Kalanchoë daigremontiana* shows endogeneous circadian oscillations, indicating the existence of an internal metabolic pacemaker. Recent experiments suggest that the macroscopic dynamics of this biological clock can only be understood as a collective phenomenon based on the microscopic interaction of cells in the leaf tissue. Experimental results of thermoperiodic perturbations of the rhythm are analyzed and compared with numerical simulations based on a recent theoretical model of CAM. It is shown that the dynamics of the observed gas exchange patterns corresponds to features well known from externally driven nonlinear oscillators. However, further experimental findings indicate some need for modification of the present macroscopic model in order to obtain a consistent theoretical description of CAM on the microscopic, i.e. cellular, level. This is discussed with respect to the simulation of the spatio-temporal dynamics of CAM-cell networks.

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10A-4
ION CHANNEL ACTIVITY IS MODULATED BY OXIDISING AGENTS IN VACUOLES FROM HIGHER PLANT CELLS

Armando Carpaneto, Franco Gambale

Plant must react to several external and internal stimula; there are many direct and indirect evidences that underline the key role of different ion channels in the transduction chain. In the cytoplasm, Active Oxygen Species (AOS) are involved both in processes of aerobic biochemistry (i.e. photosynthesis, oxidation of metabolic compounds, etc...) and also in defense mechanisms against the invasion of pathogens. The activity of AOS is controlled by the plant antioxidant system in which low molecular weight substances like glutathione and ascorbate are of relevant importance. We have demonstrated that reducing agents (like dithiothreitol) modulate the slow activating (SV) channels in vacuoles from higher plant cells. Here we investigate the effects of the oxidising agents chloramine-T on the vacuolar membrane from *Posidonia oceanica* and sugar beet. The presence in the cytoplasmic solution of 1 mM chloramine-T in vacuoles from the meristematic part of the *P. oceanica* leaf completely and irreversibly blocks the SV currents. In vacuoles from sugar beet taproots chloramine-T inhibits almost completely the activity of the SV channels and activates a voltage-independent potassium channel. Experiments are in progress to characterize this protein that could be involved in the defense mechanism pathways of the plant.
[1] Carpaneto, A., Cantù, A.M. and Gambale, F. (1999) FEBS Lett. 442, 129–132.

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10A-5
ENHANCEMENT OF ULTRAWEAK LUMINESCENCE AND ASCORBATE PEROXIDASE IN CHARACEAE INDUCED BY EXOGENOUS ASCORBIC ACID

Anna Jaskowska, Tamara Balakchnina*, Robert Borc, Andrzej Dudziak

Spontaneous ultraweak luminescence (UL) intensity from Characeae is at 10^4 photons s^{-1} cm^{-2}, several times higher than that emission from the cell medium within 350–700 nm. This emission is long-lived (a few days observable) and sensitized by chlorophyll.
We treated the cells with ascorbic acid (AsA), which enhanced UL intensity larger for higher concentration (at 10 mM 15 times greater) within 10–20 min. after the exposition of the samples (10 series with 30 cells in each) to AsA. After that time the emission level goes back to the initial intensity value. In about 10% of the population under study there were observed some oscillatory variations of UL intensity (with a 20-minute period). The exposition of these cells to AsA show a similar effect of emission enhancement, and retains its oscillatory character when the cells recover their physiological initial state.
It follows from the data of the absorbance kinetics of AsA, measured in the cell medium, that 50% of AsA diffusion into the cells takes place within first 7 min. A study of the enzymatic activities of ascorbate peroxidase and gluthatione reductase showed enhancement successively with the AsA concentration (at 10mM by about 140% and 60% respectively. The amount of chlorophyll *a* and *b* increased by about 36% at this AsA concentration.
These results correlated with UL spectral distribution, measurements showed the band intensity increase in the 680-700 nm range.

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10A-6
PHASE TRANSITIONS OF MEMBRANE LIPIDS AS MARKERS FOR HIGH-TEMPERATURE ADAPTIVE CAPACITY OF MARINE MACROPHYTES

Nina M. Sanina, Svetlana N. Goncharova, Maxim Gusarenko

Thermotropic behavior of anhydrous glyco- and phospholipids, isolated from 4 species of marine macrophytes, collected in Sea of Japanese in August, was studied by means of differential scanning calorimetry and polarizing microscopy. It was shown that on thermograms of both chloroplastic (monogalactosyldiacylglycerol and digalactosyldiacylglycerol), and non-chloroplastic (phosphatidylcholine and phoshatidylethanolamine) membrane lipids, alongside with the main heat absorbtion peaks (crystalline to liquid crystalline transitions), located at temperatures below zero, there were high-temperature zones with low enthalpy changes in the range of 30–40 °C. Earlier a similar thermotropic behavior we marked in phospholipids of marine invertebrates. Using polarizing microscopy, it was observed that these low-enthalpy transitions corresponded with either isotropic melt of lipids or mesophase transformations. The marked phase changes at temperatures above zero, probably, underlie the observed infringements of photosynthetic and mitochondrial activity of marine macrophytes at the seawater temperature increase up to 30–40 °C. Thus, the absence of effective compensatory mechanism of lipid viscosity may be the reason of poor adaptive capacity of poikilotherms to superoptimal temperatures of habitat in contrast to low- temperature adaptation.

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10A-7
STUDIES ON MECHANISM OF BIOLOGICAL ACTIVITY OF SOME NEW AMINOPHOSPHONATES

Janusz Sarapuk, Halina Kleszczynska, Anna Dziamska

Organophosphorous pesticides are widely applied. However, the mechanism of the interaction between them and biological cells is not quite clear. The most important of many factors that modify toxicity of aminophosphonates seems to be their lipophilicity. To relate lipophilicity with biological activity, we performed some studies using two model membranes; erythrocytes (RBC) and planar lipid membranes (BLM). The results obtained were compared with toxicity tests performed on *Spirodela oligorrhiza*.The aminophosphonates studied were structurally differentiated in the polar and hydrophobic parts attached to nitrogen, carbon and phosphorus atoms. Their influence on hemolysis of RBC and stability of BLM was studied. The concentration of the compound causing 50% hemolysis (C_{50}) or the destruction of BLM in 5 min (CC) was measured. The obtained values of C_{50} and CC were compared with effective concentration causing 50% inhibition of *Spirodela oligorrhiza* growth (EC_{50}).
Plant experiments showed that the most toxic compounds had butyl groups attached to P atom and long hydrocarbon chains attached to C and N atoms. Such results point at the lipid phase of the plant cell membrane as a place where the interaction between aminophosphonates and the cell membrane occurs. Also, both the C and CC values indicate that the more lipophilic a compound the more destructive its action on the models used.
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10A-8
THE MECHANISM OF WATER ASCENT IN PLANTS

Heike Schneider, Nicole Wistuba, Raphael Reich, Hans-Jürgen Wagner, Lars H. Wegner, Ulrich Zimmermann

Minimal- and noninvasive methods for the investigation of water and solute transport in higher plants, including the pressure probe techniques and NMR imaging, have proven to be superior to the established, more or less destructive methods to analyze plant water relations.
In the meantime, the novel techniques have become widely accepted and are applied for various questions in plant physiology. Extensive studies with the xylem pressure probe on herbaceous plants and tall trees have revealed that pressure gradients alone cannot account for the ascent of water under most environmental conditions. This result is at variance with the predictions of the cohesion-tension theory and highlights the significance of alternative forces like osmotic gradients across solute-reflecting barriers in the xylem, and electrical gradients. Evidence for the contribution of these forces to the transport of water and solutes in the xylem is presented here. These results support the multi-force hypothesis of long-distance water transport (Zimmermann et al. (1994), Plant, Cell and Environment 17, 1169–1181). By using NMR imaging, the dynamics of xylem flow can now be studied with a high spatial and time resolution in a noninvasive way. A combination of these methods leads to a complex picture of force-flow patterns and their response to environmental conditions.

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10A-9
SOLITONS AND DELAYED LUMINESCENCE IN BIOLOGICAL SYSTEMS

Agata Scordino, Antonio Triglia, Francesco Musumeci, Larissa Brizhik

Experimental results obtained studying the delayed luminescence (DL) in the seconds range from the unicellular alga *Acetabularia acetabulum* (*A.a.*) suggest the idea that DL is related to the integrity of the dynamic cytoskeleton organization and to the existence of a hierarchical order in the structure of the cytoplasm. The macromolecular polypeptides play important role in this structure and make it comparable to the band structure of a solid-state system. Analogies in DL from *A.a.* and Cadmium Sulphide samples strengthen this idea.
Such polypeptides favour the existence of solitons in form of localized nonlinear coherent electron states which are characterized by very high stability and large lifetime and can provide charge transport and energy storage and transfer on macroscopic distances necessary for the physiological processes. Soliton energy levels lie below the conductive band bottom, and give input into the optical properties of the system.
We apply the soliton model of charge and energy transport to explain the main properties of the DL of simple biological systems by assuming that DL from biological systems is connected with the formation and dissociation of solitons created by the pre-illumination of the sample. The system of coupled equations is derived which describes the time dependence of the intensity of luminescence. Its numerical solution is shown to give a good fit with the experimental data on the emission from unicellular alga *A.a.*

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10A-10
COUPLED EXCITABLE CENTRES AS DRIVING MECHANISM OF CIRCUMNUTATION

Jan Sielewiesiuk

Circumnutation is an universal phenomenon in growing vascular plants. The shoot twining implies that a wave of turgor and changes in rheological parameters of cell walls is propagated along the circumference of the shoot. Such a wave can be produced by a system of coupled excitable centres, but not by coupled oscillators. I investigated the loop of N coupled excitators represented by FitzHugh-Nagumo systems. The stable wave of excitation can be produced by the system provided that $N > (20–24)$ in a certain range of values of couplig (diffusion) coefficient D. For $N = 32$, the period is a decreasing function of D. The amplitude has a maximum at certain value of D. In experiment (*Helianthus annuus*), the period of circumnutations is bigger at bigger amplitude. Such a situation is reproduced by the model at high values of D. Dimensionless coefficient D can be scaled using experimental values of the period and the radius of the shoot. Its absolute value has been estimated as $(2–7) * 10^{-6}$ cm^2s^{-1} for *Phaseolus* and $(0.5–2) * 10^{-5}$ cm^2s^{-1} for *Helianthus annuus*. The numbers are quite acceptable as usual diffusion coefficients and are at least by six orders of magnitude smaller than corresponding coefficients for the action potential propagation.
Obtained results proove that the system of coupled excitators can be a driving mechanism of circumnutational movement. However, the excitation should be transfered from one centre to another by usual diffusion.

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10A-11
EFFECT OF CADMIUM STRESS AND DIFFERENT WATER AVAILABILITY ON IMPEDANCE PARAMETERS OF DEVELOPING PEA SEEDLINGS

Eszter Vozáry, Ildikó Jócsák, Gábor Horváth

The magnitude and the phase angle of the electrical impedance in a frequency range of 800 Hz – 1 MHz were determined in developing pea (*Pisum sativum*) at different water availabilities or Cd^{++} poising conditions (200, 400 μmol) by a HP 4284A precision RLC meter. Approaching by modified Hayden model, the impedance spectra, the apoplasmic (R_a) and the symplasmic (R_s) resistance in both roots and shoots were calculated. The transfer of seedlings from vermiculate to Hoagland hydroponic culture caused a decrease of either R_a or R_s values in roots of both 3 and 10 day old plants as well. The decrease of R_a and R_s values in the shoots, however was observed in 10 old day plants. This decrease can be explained by the increase of ion mobility as a consequence of increased water content.
In the presence of Cd^{++} the R_a and the R_s resistance were increased in the roots for both the 3 and 10 day old plants, but in shoots the R_s resistance was only increased in older plants. The extent of Cd^{++} effect was enhanced by higher concentration and longer incubation time of Cd^{++} treatment. The increase of R_s resistance can be caused either by formation of little veziculums in the cytoplasm, or by increased fitokelatin concentration in the presence of Cd^{++}.

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10A-12
THE XYLEM PRESSURE-POTENTIAL PROBE, A NEW TOOL IN PLANT ELECTROPHYSIOLOGY

Lars H. Wegner, Ulrich Zimmermann

By incorporating an Ag/AgCl electrode into the xylem pressure probe, pressure and the electrical potential in the xylem with respect to an external reference electrode can be recorded simultaneously. The xylem pressure-potential probe was used to measure the electrical potential difference between the root xylem and an external electrode, the "trans-root potential" (TRP), a parameter that is important for understanding salt transport across the root tissue into the xylem. In experiments performed on maize and wheat seedlings, the TRP was shown to respond to changes in light intensity, external osmotic pressure and nutrient supply. Oscillations of xylem pressure in wheat were accompanied by periodic changes of the TRP of various shapes. In maize seedlings grown in N-free media, changes of the cortical membrane potential induced by the addition of nitrate or ammonium to the bath were directly reflected by the TRP. The existence of electrical gradients in the xylem could be demonstrated on excised tobacco shoots. These gradients were likewise affected by changes of the light intensity.
For direct, minimal-invasive measurements of ion concentrations in the xylem sap, the pressure-potential probe can be combined with ion-selective electrodes by using multi-barreled glass tubing. Ion-selective barrels are filled with a pressure-tight matrix containing a "carrier" that translocates the ions of interest specifically (e.g. valinomycin for K^+ determination).

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11A-1
ENHANCEMENT OF PHOTOLUMINESCENCE PROPERTIES OF QUANTUM DOTS BY THIN ORGANIC FILMS

K. Adlkofer, M.Tanaka, E. Sackmann, E. F. Duijs, F. Findeis, A. Zrenner, M. Bichler, G. Abstreiter

Semiconductor nanostructures combined with biological systems are expected to play an important role for future applications as well as for basic research. One of the fundamental requirements is to fabricate biocompatible interfaces between devices and biomolecules.
In this study, InAs quantum dots (QDs) were first grown by molecular beam epitaxy (MBE), covered with a 5, 15, 25, and 50 nm $Al_{0.33}Ga_{0.67}As$ layer as a potential barrier, and completed with a 5 nm GaAs cap layer. As a first step of functionalization, self-assembled monolayers (SAMs) of octadecylthiol (ODT) were deposited onto the GaAs surface.
A significant increase of the photoluminescence (PL) signal after deposition of the SAM was observed, especially for the QDs close to the surface. For example, PL intensity was increased by a factor of 1.87 for the QDs with a 5 nm $Al_{0.33}Ga_{0.67}As$ layer. Such an increase in PL intensity can be explained by suppression of non-radiative recombination on the surface.
Slight modification of such molecular constructs has a large potential towards the design of smart biosensors using electrical and optical techniques.

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11A-2
MEASUREMENT OF CELLULAR RESPIRATION AND ACIDIFICATION WITH A SINGLE SILICON SENSOR

M. Lehmann, W. Baumann, I. Freund, M. Brischwein, R. Ehret, S. Drechsler, H. Palzer, B. Wolf

Over the past years there has been a lot of effort to develop non-invasive tools for cellular monitoring. The applications of this method extend for example from pharmascreening over chemosensitivity testing to environmental monitoring. In metabolism, both oxygen consumption and acidification showed to be important parameters. However it was shown that due to the complex cellular network is not sufficient to monitor only one of these parameters. For example, extracellular acidification and respiration may behave different. Only monitoring one parameter would then lead to the false conclusions. We therefore developed the idea of the Cell-Monitoring-System (CMS®) measuring different physiological parameters like cellular respiration and acidification online and in real-time.
For many applications it would be useful a) to have sensors without membranes, b) to work with small sensors (10 µm range), and c) to be able to detect cellular parameters at the same place.
Therefore we developed a sensor consisting of a pH-ISFET and a noble metal electrode which allows us to measure cellular acidification and respiration with the same sensor and incorporates the above mentioned features. The sensor chips were produced with silicon CMOS technology.
Several measurements with tumor cells showed the desired operation and promise the sensor to be a new tool for cellular analysis.

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11A-3
ELECTRIC READOUT BIOCHIPS FOR CELL BASED SCREENING

M. Brischwein, W. Baumann, S. Drechsler, R. Ehret, M. Lehmann, E. R. Motrescu, B. Wolf

The approach is directed to the development of biochips integrating multiple microelectric sensors for cell based screening. While functional assays of living cells relying on optical readout methods (using various kinds of specific fluorescent reporter dyes) are increasingly accepted, electric readout seems more adequate for long term monitoring of the cellular metabolic and morphologic status and for the kinetic assessment of cellular responses. Continous and sensitive long term observation, however, requires advanced measures for the precise control and maintenance of the cellular environment. The microsensor chips include sensor elements for pH, oxygen partial pressure and electric impedance. They are constructed on both silicon and glass substrate. Since non-specific parameters are detected, the chips are applicable to a wide range of living targets. The presented status of work includes issues of sensor construction and sensor function (e.g. sensitivity, long term behaviour), fluidic components, strategies for chip parallelisation and data evaluation.

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11A-4
MANIPULATION OF MOLECULES AND PARTICLES ON MICRO- AND NANO-ELECTRODE ARRAYS BY ELECTROPHORESIS AND DIELECTROPHORESIS

Manfried J. Dürr, Thomas Joos, Martin Stelzle, Dieter Stoll, Wilfried Nisch

Electrode arrays have been designed and evaluated to manipulate a variety of biomolecules and particles, including DNA oligomers, proteins and peptides by means of electrophoresis and dielectrophoresis. The ultimate goal of this technology is to enhance sensitivity and selectivity in bioanalytical applications while at the same time reducing the amount of analyte required.
Electrophoresis chips comprised focussing electrodes and a central micro-electrode array. A cover plate was glued on top to form a chamber. Theoretical considerations of current density and field distribution within the chamber resulted in a design with the accumulation electrodes arranged in a U-type fashion and the electrode to electrode distance monotonically decreasing towards the center of the chip. Readout was performed using a fluorescence imaging system. Time sequences of images of the fluorescence distribution over the chip were acquired and allowed for the calculation of molecular drift velocities and an assessment of the electrical field distribution. Accumulation factors as high as 1:200 were observed with DNA oligomers. Charge delivery by the electrodes and the transition from capacitive to faradaic charge transfer was investigated.
Nano-electrode arrays enable manipulation of sub-µm particles by means of dielectrophoresis. Several designs of arrays have been devised and experimentally evaluated.

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11A-5
NANOSTRUCTURED SUSPENDED APERTURE FOR PATCH CLAMP RECORDING AND SCANNING PROBE APPLICATIONS ON NATIVE CELL MEMBRANES

N. Fertig, C. Meyer, A. Tilke, R. Blick, J. Behrends

The recording bandwidth of patch clamp setups is commonly limited to below 100 kHz mainly because of stray capacitances and access resistance of the long tapered pipette. We present a new approach replacing the patch pipette by a nanostructured semiconductor/quartz chip in order to reduce the background noise. We define a nanoscale aperture in a suspended Si3N4-membrane on micromachined silicon/quartz substrate. The access resistance of this device used as a pipette is on the order of 100 kOhm. The geometry of the chip allows to reduce the stray capacitance by minimizing the distance between aperture and recording electrode. A method for the integration of an isolated cell membrane patch into the semiconductor/quartz device is established. This arrangement allows the change of solution on both sides of the cell membrane. The planar geometry of the chip enables the application of various scanning probe techniques such as atomic force microscopy or scanning near field optical microscopy in situ. Using standard planar processing techniques, this approach can be extended to an array of recording sites on a single chip, where a large number of cells or membrane patches can be studied simultaneously. Further reduction in background noise can be achieved with an on-chip pre-amplification by integrated FETs in the silicon chip.

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11A-6
COMPARISON OF ELECTRICAL MODELS TO THE 'CELL MEETS THE SILICON' PROBLEM

W. Baumann, I. Freund, M. Lehmann, M. Brischwein, R. Ehret, B. Wolf

The non invasive extracellular coupling of electrically active cells with appropriate transducers permits the e.g. long-term measurements of electrical signals from neural networks. Applications are, apart from the biological basic research, for example in the development of prosthesis (Retina Implant, Cochlear Implant,..) or new computer technologies. The quality of the contact between the electrically active cell and the transducer is thereby of crucial importance for practical application of such hybrids in biomedical research and sensor technology. As transducer, particularly electrodes with amplifiers or field-effect transistors (FETs) with direct cell/gate coupling are applied.
We developed FET transducers with different gate materials (sensitive gate areas from $6 \times 1\mu m^2$ up to $20 \times 2\ \mu m^2$) as well as various electrode designs integrated on one sensor chip. Measurements of different cell types showed however, that basic biophysical questions concerning the electrical coupling between cell and transducers are still open. Especially about the crucial parameters to be optimised for accomplishing the cell/transducer coupling.
In a first step the well-known models from Fromherz, Offenhäuser and Grattarola were implemented in SPICE. The results of the critical comparison of these models and the sensor chip with the diverse transducers will be presented.

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11A-7
INFLUENCE OF THE SEMICONDUCTOR SUBSTRATE ON ELECTRICAL PROPERTIES OF THE ADHERENT CELLS IN CELL-SEMICONDUCTOR HYBRIDS

Michael George, Wolfgang J. Parak, Michael Kudera, Hermann E. Gaub, Jan C. Behrends

For successful long-term coupling between neuronal assemblies and semiconductor devices neurones must be able to fully develop their electrogenic repertoire when growing on semiconductor substrates. While it is known that neurones may be cultured on silicon wafers insulated with SiO_2 and Si_3N_4, an electrophysiological characterization of their development under such conditions is lacking. The development of voltage dependent membrane currents, especially of the rapid sodium inward current, is of particular importance because the conductance change during action potentials determines the quality of cell-semiconductor coupling. Rat striatal neurones have been cultured on either glass coverslips or insulated silicon wafers using both serum-containing and serum-free media. We report evidence that not only serum-free culture media but also growth on semiconductor surfaces may negatively affect the development of voltage-dependent currents in neurones. Using surface-charge measurements with the atomic force microscope, we demonstrate a reduced negativity of the semiconductor surface compared to glass. This may affect cellular development through an effect on the binding and/or orientation of extracellular matrix proteins. Therefore, our findings suggest, that semiconductor substrates are not entirely equivalent to glass in terms of their effects on neuronal cell growth and differentiation.

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11A-8
A LOCAL BIOLOGICAL VOLTMETER: USING POLYMER SUPPORTED LIPID MEMBRANES FOR BIOSENSORIC APPLICATIONS

Heiko Hillebrandt, Motomu Tanaka, Erich Sackmann

The biofunctionalization of semiconductors with polymer supported lipid membranes seems to be a very promising approach towards realizing biosensors based on optoelectronic devices. We will present the design of lipid membranes on transparent Indium-Tin-Oxide (ITO) semiconductor electrodes coated with ultrathin polymer cushions out of cellulose derivatives. The cushion thickness can be controlled in the range of nm using successive Langmuir Blodgett (LB) transfer. Hydrophobic and insulating, as well as hydrophilic and hydrated polymer layers are possible. The stratified polymer supported lipid monolayer systems can be used to measure the change in membrane surface charge due to the complex formation of chelator lipids or due to the selective coupling of fusion proteins. The intermediated polymer layer serves as an insulator between the semiconductor space charge region and the membrane/electrolyte interface. Therefore, the whole system reacts on potential changes analogous to metal-oxide-semiconductor (MOS) devices. In addition, lipid bilayers on hydrated cellulose cushions can be applied as a platform for the incorporation of transmembrane proteins in active form.

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11A-9
CHARACTERIZATION OF THE NEURON-TRANSISTOR-COUPLING

Sven Ingebrandt, Michael Krause, Chi-Kong Yeung, Andreas Offenhäusser

We investigated the coupling mechanism of different types of neuronal cells with two types of extracellular biosensors: field-effect-transistors (FETs) with non-metallized gate structures and extended gate electrodes, where metal microelectrodes are connected to commercial low noise junction field effect transistors (JFET). In addition we designed a back-side-contacted FET array, where the active surface of the FET-chip is increased from 0.06 cm^2 to 0.36 cm^2. This will allow the use of this device for slice recordings and will minimize the interaction of the cells with the encapsulation.
We cultured neuronal cells from the hippocampus and from the cortex region of embryonic E15 and E18 rats. In addition we cultured thin brain stem slices from embryonic E15 rats. After a few days of culturing the brain stem neurons are spreading out from the slice over the whole sensor surface.
The neuron-transistor couplings were characterised by means of voltage clamp- as well as current clamp-methods. We will discuss the obtained signal shapes using the point-contact-model for the cell-sensor coupling. For both sensor types we observed similar coupling mechanisms.
Future work will be the controlled design of different neuronal networks on the sensor surfaces by means of micro contact printing and the readout of these networks with our sensor arrays.

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11A-10
CELLS AND MEMBRANES ON TRANSISTOR SI-CHIPS: PROBING THE 2D CORE-COAT CONDUCTOR MODEL

Volker Kiessling, Peter Fromherz

Understanding the electrical properties of the adhesion area beneath a cell or lipid bilayer is crucial for applications of neuron silicon junctions as well as for the development of biosensors based on semiconductor supported membranes [1-3].
Between the adhering membrane and the siliconoxide surface of the chip a thin sheet of electrolyte is in contact to the bath. This system forms a core-coat conductor or 2D cable [4]. Ohmic and capacitive currents through the membrane give rise to a voltage drop in the extracellular space. We proved the validity of the concept quantitatively with AC voltages, applied to different cell and membrane systems. Transistor arrays probed the voltage drop in the cleft. The AC stimulation was in the frequency range of 1 Hz to 100 kHz and was applied either to the surroundig bath, as with electrofused giant erythrocytes and lipid vesicles or to the cell interior, as with oocytes of Xenopus. The response of 36 transistors was detected simultaneously in magnitude and phase. The data were fitted to solutions of the 2D cable equation. We obtained the resistances of the adhering membrane and the sheet resistance of the cleft. The latter was in a range of 10 MΩ for oocytes up to 100 GΩ for lipid vesicles.
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11A-11
BIOCOMPATIBLE COATINGS FOR GALLIUM ARSENIDE BASED BIOSENSORS

Christian Kirchner, Michael George, Bernhard Stein, Wolfgang J. Parak, Amelle Vix, Tim Salditt, Hermann E. Gaub, Markus Seitz

In this work we present strategies to make GaAs-surfaces biocompatible. Biocompatibility here is defined by the growth and survival of NRK fibroblasts on the semiconductor substrate. Our most promising approach is based on the use of polyelectrolyte multilayers. This provides the possibility to introduce chemical functionalities by synthesis, which can act as arsenite-antidotes. Polyelectrolyte multilayers assembled onto glass slides by "dip-coating" from aqueous solution were found to be excellent substrates for the cultivation of NRK fibroblasts. Best results were obtained for poly(sodium 4-styrenesulfonate) as the polyanion, and polyethylenimine or poly(4-vinylpyridine) as the polycation. Thus, in a first step the buildup of polyelectrolyte multilayers on GaAs-wafers was verified by x-ray reflectivity. In a second step, sulfur passivation of the GaAs-surface using Na_2S as well as alkanethiols was characterized by atomic force microscopy. This not only allows for the improvement of the electronic properties of the semiconductor, but serves as a diffusion barrier for arsenite ions. A combination of such initial passiviation layers with functionalized polyelectrolyte coatings was employed and successfully tested for their use as biocompatible interfaces on gallium arsenide.

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11A-12
ELECTRICAL DETECTION OF MEMBRANE POTENTIAL TRANSIENTS OVER AN EXTENDED TIME SCALE

Winfried Leibl, Sonia Keller, Philippe Lebel

Vectorial transfer of electrons, protons, and ions across biological membranes are common features of energy transducing enzymes and the asymmetry of these transfer steps is essential for function. Photosynthetic systems are widely used as models for kinetic studies due to the possibility to synchronize the reaction sequences by flashes of light. The importance of electrogenic reactions makes direct electric measurements of potential changes a valuable tool and macroscopic model membrane systems created by fusion of lipid/protein vesicles to a solid support have been found to be well suited. We compared two different systems using chromatophores from purple bacteria as fusion material and either a lipid-coated thin Mylar film or a planar gold electrode modified by a self-assembled monolayer of octadecyl mercaptan as support. The result of the fusion process was independent of the support but the much higher capacitance of the gold electrode system offers advantages for some applications. This will be discussed together with the structural and electrical properties of both systems.
Furthermore a customizable data acquisition system will be described, designed for efficient single-shot recording of transients with optimized resolution of kinetic phases ranging from microseconds to seconds. It is based on a programmable logic circuit performing real time accumulation over a variable number of digitization steps during an acquisition cycle yielding a logarithmic time scale.

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11A-13
A CHIP-BASED BIOSENSOR FOR FUNCTIONAL ANALYSIS OF SINGLE ION CHANNELS

Michael Mayer, Christian Schmidt, Laurent Giovangrandi, Horst Vogel

Ion channels play essential roles in cellular signal transduction processes and are therefore directly or indirectly targeted by many clinically used medicines. The molecular details of these central cellular reactions are largely unresolved. Nearly unlimited numbers of mutant proteins provided by combinatorial genetics and huge libraries of potentially active compounds produced by combinatorial chemistry offer enormous possibilities for unraveling molecular details of channel function and for finding new medicines. Electrical recordings of single ion channels provide the most detailed insight into channel function, however presently available techniques are not ideally suited for efficient screening of the existing large numbers of compounds. Here we present a novel, silicon chip-based assay to probe the function of channel proteins. Membrane vesicles were electrophoretically positioned and fused across micrometer sized holes in the chip surface. Seal resistances higher than 200 GΩ, obtained after less than ten seconds positioning time, allowed the analysis of single ion channel currents in solvent-free membranes. Sample volumes in the microliter range strongly reduced diffusion times and the consumption of proteins and sample compounds, making the application of this technique in parallelized, highly sensitive biosensing devices for large-scale functional screening feasible.

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11A-14
BIOCHIP FOR WATER MONITORING

H. Palzer, W. Baumann, M. Brischwein, R. Ehret, B. Wolf

According to UN reports, lack of water prevails in 80 states on earth and affects approximately 40% of the world population. Five million humans die annually from contaminated water. So far, water monitoring is performed by analysing a set of abiotic parameters. Alternatively, various biotest assays can be used to assess physiological effects of the examined water sample. Existing biotests, however, are time- an cost intensive and are not suited for the implementation into remote control systems. This approach is directed to the use of multiparametric ISFET sensors (CMOS technology) for on-line monitoring of settled, benthic diatoms, (e.g. Cocconeis placentula (Ehrenberg)) in order to combine highly sensitive modern semiconductor sensor structures with the physiological response profile of selected water organisms (biohybrid sensor chips).

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11A-15
ELECTROCHEMICAL CHARACTERIZATION OF SUPPORTED LIPID MEMBRANES ON SILICON SURFACES BY IMPEDANCE SPECTROSCOPY

Oliver Purrucker, Motomu Tanaka, Erich Sackmann

The primary aim of this study is to fabricate protective and functional bridges between biomaterials and semiconductor devices based on silicon.
Lipid membranes were deposited either directly on solid surfaces or on soft polymer cushions by different preparation methods such as fusion of vesicles and exchange of solvents. The stratified films on silicon or silicon-on-insulator (SOI) substrates were characterized by electrochemical impedance spectroscopy in terms of electrical resistance and capacitance.
As a preliminary challenge to introduce biological functions, transmembrane ion channels were incorporated into these supported membranes to realize the selective ion transport.

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11A-16
MULTIVALENT RECEPTOR-LIGAND INTERACTIONS ANALYSED ON SINGLE CELLS WITH COMBINED LASER TWEEZERS AND DIELECTRIC FIELD CAGES

Christoph Reichle, Katrin Sparbier, Torsten Müller, Peter Walden, Günter Fuhr

Cell-cell interactions are among the most important communication principles in living organisms and involve multivalent interactions of different cell surface proteins. The initiation of signal transduction and the quantity and quality of the biological responses depend on the specificity of the receptor-ligand combination, the affinity of their interaction and the number of specific bonds. Usually, affinities are determined in systems where at least one of the binding partners is available in a soluble form. These affinities represent an equilibrium situation of monovalent interactions and do not reflect the multivalent interactions of intercellular contacts.
In the present study, the well defined receptor-ligand system biotin/streptavidin was used to study multivalent interactions between biotinylated life cells and streptavidin coated latex beads. A new technique based on the combination of optical and dielectrophoretical trapping was developed and employed to manipulate cells and beads contactless and with micrometer precision. The biotin density on the cell surface was varied to control the valency of the interactions. Binding force between cells and beads were measured that correlated with the densities of individual molecular bonds in the contact area, the size of this area and the time for adhesion.

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11A-17
ULTRASENSITIVE MULTIANALYTE IMMUNOASSAYS: THE SYNERGY BETWEEN PLANAR WAVEGUIDE- AND MICROARRAY TECHNOLOGY

E. Schick, M. Pawlak, E. Schürmann, M. Ehrat

A new powerful generation of biochips for fluorescence based multiplexed immunoassays will be presented. Multiple analyte information from a single sample, sensitivity in the range of attomoles, short assay time ("mix and measure") and the need of only minute sample volumes (< 50 µl) are the major benefits compared to traditional immunoanalytical techniques (e.g. ELISA). Capture antibodies against 3 different antigens (human interleukins 2, 4, 6) are immobilised on the biocompatible planar waveguide surface in microarrays consisting of spots with a typical size of 200 µm. Analyte solutions which were preincubated with a biotinylated tracer antibody mix against hIL-2,4,6 and fluorescence labelled streptavidin are added onto the chip, followed by a subsequent incubation. Since background fluorescence in the sample solution is eliminated by evanescent field excitation, antigen specific fluorescence signals can be directly imaged omitting any washing steps. The specific chip design allows the direct quantification of analytes measured in different microarrays leading to dose response curves with excellent sensitivities between 1–10 pg/ml and coefficients of variations in the range of 10-15% for all investigated interleukins.
The innovative combination of planar waveguide- and microarray technology offers extremely interesting possibilities for the simultaneous monitoring of specific biological marker sets, for example in the field of oncology, transplantation and inflammatory diseases.

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11A-18
TIME RESOLUTION AND POTENTIAL SENSITIVITY OF THE LIGHT-ADDRESSABLE-POTENTIOMETRIC-SENSOR (LAPS)

Michael George, Bernhard Stein, Wolfgang J. Parak, Stefan Kudera, Christian Schmidt, Stefan Kölblin, Hermann E. Gaub

The LAPS is a surface potential detector that can been applied in biosensing applications. In this study the relation between potential sensitivity and modulation frequency of the LAPS is investigated. A LAPS system with front-side illumination was developed and chips with various doping have been characterized in two different ways. Photocurrent-voltage (IV) characteristics have been obtained for different modulation frequencies of the light pointer. Additionally, capacitance-voltage (CV) curves have been recorded, when the chip was illuminated and under dark conditions. The difference in capacitance and the photocurrent showed a similar behavior. Therefore it can be concluded, that the IV characteristics can be derived from CV characteristics. This involves that theoretical and experimental investigations of CV might be utilized to find the optimal semiconductor substrate for a high-frequency LAPS. Both, the photocurrent and the capacitance measurements showed no loss in potential sensitivity for frequencies up to 30 kHz. This indicates, that a temporal resolution of 0.1 ms is achievable, without loosing potential resolution. The potential sensitivity was improved, when the resistance of the substrate was increased up to 10 Ωcm. No further improvement could be observed for higher resistances. These results may help to improve the LAPS towards the special needs for biosensing devices.

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12A-1
ELASTIC PROPERTIES OF SECONDARY STRUCTURE ELEMENTS: A MOLECULAR DYNAMICS STUDY

Rainer Böckmann, Berthold Heymann, Peer Geisendorf, Helmut Grubmüller

Unfolding and elastic properties of peptide secondary structure elements play an important role in many biological macromolecules. In the muscle protein myosin, e.g., an α -helix acts as a "lever" and induces the power stroke. Single molecule experiments allow to probe the underlying mechanisms by measuring elastic properties like the stretching force or the stiffness. In order to get insight in the involved interactions at the atomic level we performed enforced unfolding molecular dynamics simulations (a) of a $cysteine_3$–$lysine_{30}$–cysteine peptide which adopts an α-helical structure at high pH value [1], (b) of a coiled-coil leucine zipper and (c) of polyethylene-glycol polymers [2,3].
Our computed elastic properties and unfolding forces agree with the corresponding experimental data. The role of hydrogen bonds and water bridges is discussed.
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12A-2
DYNAMIC FORCE SPECTROSCOPY OF MOLECULAR ADHESION BONDS

Berthold Heymann, Helmut Grubmüller

Recent advances in atomic force microscopy, biomembrane force probe (BFP) experiments, and optical tweezers enable to measure the response of single molecules to mechanical stress [1-3]. Such experiments, due to limited resolution, typically access only one single force value in a continuous force profile or energy landscape that characterizes the molecular response, e.g., rupture, along a reaction coordinate. As a source of additional information, variation of loading rate has been proposed [3-5].
We present a theory [6] that relates single molecule dynamic force probe *spectra* (obtained from measurements at varying loading rates) to the underlying energy landscape. Our theory thus enables one to reconstruct energy landscapes and force profiles with high spatial resolution from measured force spectra. In particular, our transformation from the velocity domain into the space domain circumvents experimental spatial resolution limits.
We show that spectra obtained from measurements with soft and weak spring constants contain complementary information, and therefore, should be combined. As an example, the streptavidin/biotin unbinding energy landscape is reconstructed from recent BFP data [3].
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[2] E.-L. Florin, V.T. Moy, and H.E. Gaub, Science **264**, 415 (1994).
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[6] B. Heymann and H. Grubmüller, submitted.

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12A-3
MD SIMULATIONS OF THE UNBINDING OF THE AN02-ANTIBODY/DNP-HAPTEN COMPLEX

Berthold Heymann, Helmut Grubmüller

Molecular recognition is essential for many biochemical processes and is realized by highly specific binding of ligands to their receptors. We present molecular dynamics (MD) simulations of single molecule atomic force microscopy (AFM) experiments which enabled us to compute unbinding forces and to complement the experiments with microscopic interpretations [1]. Here we focus on the AN02/DNP-hapten complex. In the course of our MD simulations [2] the hapten molecule was pulled out of the AN02 binding pocket by means of a harmonic potential, which served as a model for the AFM cantilever.
The unbinding force of the complex was obtained as the maximum pulling force acting on the hapten molecule during the unbinding process. For the experiments we expect an unbinding force of 60 +/- 30 pN. Detailed inspection of the computed unbinding events revealed a large structural heterogeneity of the unbinding pathways. From principle component analysis of the unbinding trajectories we quantified the entropic contribution to the AN02/DNP-hapten bond to at least 4 kcal/mol. Unbinding simulations carried out with mutants of AN02 suggested a large flexibility of the AN02 binding pocket.
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[2] B. Heymann and H. Grubmüller. AN02/DNP-hapten unbinding forces studied by molecular dynamics atomic force microscopy simulations. Chem. Phys. Lett., 303:1–9, 1999.

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12A-4
VIBRATIONAL ANALYSIS OF GTP BOUND TO P21-RAS BY USING DENSITY FUNCTIONAL THEORY IN MOLECULAR DYNAMICS

Marco Klähn

p21-Ras is a decisive protein for signal-transduction leading to cell groth and cell differentiation and it's mutants are often responsible for cancer. During the simulation of the molecular dynamics of GTP bound to p21-Ras, it's vibrational frequencies and states are determined by using a hybrid method, combining quantum mechanical methods and classical force fields. Whereas GTP is modelled by density functional theory, the surrounding p21 is described by conventional electrostatics, where both parts are linked by a special interface. The aim is to produce vibrational spectra that can be compared to experimental ones. As a result it should be possible to explain the measured spectra and learning something about the GTPase mechanism in p21.

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12A-5
NOVEL SIMULATION OF CONFORMATIONAL TRANSITIONS: EFFECTIVE SEARCH FOR PATHWAYS, ACTIVATED AND STABLE INTERMEDIATES

Jürgen Schlitter, Benno Portmann

Conformational transitions are ubiquitous in the wealth of proteins. As they are usually rare events on the accessible nanosecond time scale of simulations, some type of bias must be applied to systematically induce and investigate such transitions. A previously developed technique, the Targeted Molecular Dynamics (TMD)1 has widely been used and shown to predict details of transitions which later could be confirmed experimentally2. It induces a transition by application of a constraint which enforces the approach to the target structure with constant velocity in terms of the rms distance.
The new technique, TMD2, is based on an adaptive strategy rather than a single constraint. At each time step the optimum direction is determined for the next step towards the target. This strategy of TMD2 avoids accumulation of potential energy and admits relaxation where possible.
The new technique was applied to detect pathways of conformational changes in a small peptide, and unfolding pathways of Apomyoglobin. A free energy estimate calculated by thermodynamic integration shows that TMD2 is superior to previous techniques in predicting realistic pathways and stable intermediates.
1 Schlitter, J., Engels, M., Krüger, P., Jacoby, E., and Wollmer, A. Targeted MD simulation of conformational change – application to the T-R transition in insulin. Mol.Simulation 10:291–308, 1993.
2 Schlitter, J. Molekulardynamik-Simulation von Makromolekülen. Nachr. Chemie, 48:301–5, 2000

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12A-6
ELASTIC PROPERTIES OF SUGAR POLYMERS STUDIED BY MOLECULAR DYNAMICS SIMULATIONS

Berthold Heymann, Gunnar Schröder, Helmut Grubmüller

Elastic properties of the three D-glucose polymers (1,6)-glucose (dextran), α(1,4)-glucose (amylose), and β(1,4)-glucose (cellulose) have been studied by molecular dynamics (MD) simulations [1]. In these simulations, the sugar polymers were stretched by an external force in close resemblence to recent AFM experiments [2,3].
From the simulations force extension curves were recorded. The elasticities derived from these curves agreed well with experimental data. Furthermore, the MD simulations provided interpretations for the measured elasticities in terms of structural changes induced by the applied mechanical stress. As a key result we found that small differences in the molecular structure of the polymers can lead to completely different elastic properties and to specific intramolecular structural transitions.
[1] B. Heymann and H. Grubmüller. 'Chair-Boat' Transitions and Side Groups Affect the Stiffness of Polysaccharides. Chem. Phys. Lett., 305:202–208,1999
[2] H. Li, M. Rief, F. Oesterhelt, H. E. Gaub, X. Zhang, and J. Shen. Single Molecule Force Spectroscopy on Polysaccharides by AFM. Chem. Phys. Lett., 305:197–201,1999
[3] M. Rief, F. Oesterhelt, B. Heymann, and H. E. Gaub. Single molecule force spectroscopy reveals conformational change in polysaccharides. Science, 275:1295–1297, 1997.

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12B-1
ANALYSIS OF LIGAND BINDING BY TWO-COLOR FLUORESCENCE CROSS-CORRELATION SPECTROSCOPY (FCCS)

Thomas Weidemann, Michael Tewes, Malte Wachsmuth, Karsten Rippe, Jörg Langowski

Fluorescence correlation spectroscopy (FCS) is a well established method for the analysis of freely diffusing, fluorescent particles in solution. When used with a two-color setup for simultaneous detection of two different dyes it allows the determination of both the auto correlation function of the single dyes and the cross-correlation function between the two dyes. Here we describe how a combination of the auto- and the cross-correlation function can be used for a quantitative analysis of ligand binding. Apart from hydrodynamic properties of the particles, a set of technical constraints, like the focal geometry, background signal and cross talk between the detection channels, as well as photophysical effects like quenching or differences in the number of bound fluorophores have to be considered. To account for these factors, a modified analytical correlation function for one-photon excitation in a confocal two-color setup is derived. Based on this anlytical framework a quantitative treatment for the general analysis of binding equilibria by FCS is presented.

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12B-2
SINGLE MOLECULE SPECTROSCOPY AND OBSERVATION OF SINGLE DYE LABELLED ANTIBODIES AND VIRUSES IN LIVING CELLS

Georg Seisenberger[1], Martin Ried[2], Michael Hallek[2], Christoph Bräuchle[1]

Gene transfer vectors based on adeno-associated viruses (AAV) show great promise for use in human gene therapy. While AAV can efficiently transfer genes to a number of different cell types it is apparent that a more detailed understanding of the interactions of the virus and the target cell is necessary for further improvements. So far little is known about the process of AAV infection, e.g. uptake and migration of theses viruses in the living cell. Recent developments in florescence microscopy of single molecules made this technique an important tool for the elucidation of such processes.
Here we present single molecule experiments to follow the migration of adeno-associated viruses in living cells. Only one fluorescent dye molecule is attached to a single virus in order not to influence the physiological behaviour of the virus on ist infectious entry pathway. Our investigations started with the observation of diffusion of single Cy5-labelled antibodies and Cy5-labelled adeno-associated viruses in aqueous solution. The next step involved the localisation of Cy5-labelled antibodies at an antigen which was expressed in the cell nucleus after prior exposure to the virus. Currently the migration of single labelled viruses in human HeLa cells is investigated.

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12B-3
A DEDICATED CONFOCAL/NSOM MICROSCOPE FOR SINGLE PROTEIN STUDIES AT THE CELL MEMBRANE

Bärbel I. de Bakker, María F. García-Parajó, Wouter H.J. Rensen, Niek F. van Hulst

Cell membrane is an import subject of study in molecular biology. The reason is obvious: the membrane encloses the cell interior and regulates the information transfer between the inside and the exterior of the cell. This communication is performed by 'sensor' proteins that ensure a proper reaction of the cell on its external environment.
We have developed a combined confocal/near field scanning optical microscope (NSOM) to study both individual and clusters of these biomolecules on the membrane of living cells. With this non-invasive setup we obtain a spatial resolution of 35 nm and single molecule sensitivity. This allows us to investigate the packing density of protein clusters, the distance between individual components in a given cluster and cluster spacing distances.
At present we are studying individual transmembrane receptor molecules fused to the Green Fluorescent Protein (GFP) and expressed on L-cells. Under these conditions the signal-to-background ratio is 4:1 for S56T-GFP. The fluorescence signal is discriminated with respect to the wavelength and/or polarisation. Individual molecules at the cell surface are localised while simultaneously the surface topography is mapped. The molecules are distinguished by their discrete dynamic fluorescent behaviour, defined orientation and intensity.

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12B-4
FLUORESCENCE CORRELATION SPECTROSCOPY IN SMALL CYTOSOLIC COMPARTMENTS DEPENDS CRITICALLY ON THE DIFFUSION MODEL USED

Arne Gennerich, Detlev Schild

Fluorescence correlation spectroscopy (FCS) is a powerful technique for measuring low concentrations of fluorescent molecules as well as their diffusion constants. In the standard case, fluorescence fluctuations are measured in an open detection volume defined by the confocal optics. However, if FCS measurements are carried out in cellular processes that confine the detection volume, the standard FCS model leads to erroneous results. In this presentation we introduce a modified FCS model that takes into account the confinement of the detection volume. Using this model, FCS measurements give reliable results even in small cytosolic compartments. In addition, we show the first FCS measurements in dendrites of cultured neurons, thereby verifying our FCS model.

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12B-5
ANOMALOUS DIFFUSION INSIDE LIVING CELL NUCLEI INVESTIGATED BY FLUORESCENCE CORRELATION SPECTROSCOPY

Malte Wachsmuth, Waldemar Waldeck, Jörg Langowski

We have investigated spatial variations of the diffusion behavior of the green fluorescent protein mutant EGFP (F64L/S65T) and of the EGFP-β-galactosidase fusion protein in living cells with fluorescence correlation spectroscopy (FCS). The fluorescence fluctuations in cell lines expressing EGFP are caused by molecular diffusion as well as a possible internal and a pH-dependent external protonation process of the EGFP chromophore. The latter processes result in two apparent nonfluorescent states that have to be taken into account when evaluating the FCS data. The diffusional contribution deviates from ideal behavior and depends on the position in the cell. The FCS data can either be evaluated as a two component model with one fraction of the molecules undergoing free Brownian motion with a diffusion coefficient approx. 5 times smaller than in aqueous solution, and another fraction diffusing one or two orders of magnitude slower. This latter component is especially noticeable in the nuclei. Alternatively, we can fit the data to an anomalous diffusion model where the time dependence of the diffusion serves as a measure for the degree of obstruction, which is large especially in nuclei. Possible mechanisms for this long tail behavior include corralling, immobile obstacles, and binding with a broad distribution of binding affinities. The results are consistent with recent numerical models of the chromosome territory structure in the cell nucleus.

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12B-6
THREE-DIMENSIONAL OPTICAL POLARIZATION TOMO-GRAPHY OF SINGLE MOLECULES

Michael Prummer, Bert Hecht, Urs P. Wild

Single molecule microscopy has so far shown details of directed[1] and random[2] rotational as well as translational motion in biological systems. Fluorescence resonance energy transfer on the other hand has proven to be a molecular ruler with Å accuracy[3]. In both cases knowledge about the orientation of the transition dipole moments of fluorescent molecules in three-dimensions is required for an unambiguous interpretation of experimental results.
We have applied the concept of tomography to polarization microscopy of single fluorescent molecules to determine their complete three-dimensional orientation[4]. The method relies on the fact that the emission intensity of a fluorophore is proportional to the projection of its absorption dipole moment onto the electric field vector of the exciting laser beam. By successive illumination from different directions of incidence with well-defined polarization states the distribution of absorption dipole orientations of lipid labels (DiI), immobilized in a polymer, is determined. The wide-field approach in total internal reflection geometry allows for the observation of translational and rotational motion of many molecules in parallel on a timescale of seconds.
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Empedocles et al, Nature 399, 126 (1999)

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12B-7
TWO-PHOTON EXCITED FLUORESCENCE CORRELATION SPECTROSCOPY (FCS): PERSPECTIVES FOR INTRACELLULAR SINGLE MOLECULE ANALYSIS

Katrin Heinze, Michael Jahnz, Petra Dittrich, Flaminia Malvezzi, Andre Koltermann, Petra Schwille

Confocal FCS has proven to be a very versatile and valuable method to investigate dynamic processes involving ultra-low concentrations of fluorescent molecules in the microsecond to millisecond time regime. Parameters to be determined with high sensitivity include concentrations, diffusion coefficients, intramolecular and intermolecular kinetic rate constants, e.g. for molecular association and recognition or enzyme activity. Most FCS applications to date have been performed in aqueous buffer solution, while accessing the cellular environment bears several experimental difficulties, like photobleaching of the fluorophores and enhanced background, e.g. by scattering and cellular autofluorescence. We show that these difficulties can be overcome by proper instrumentation, e.g. employing two-photon excitation in the near IR, and that intracellular measurements on a single molecule scale become feasible. Several examples are given for molecular diffusion and active transport through diffraction limited focal spots, as well as for internal dynamics of fluorescent particles, in mammalian and plant cells. Mobility parameters can be determined over several orders of magnitude. Anomalous subdiffusion of fluorescent probes that might be explained by environmental heterogeneity and/or interaction with cellular structures, is found in various compartments, in particular on plasma membranes.

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12B-8
CHARACTERIZING SINGLE-MOLCULE PROPERTIES BY MULTI-PARAMETER FLUORESCENCE SPECTROSCOPY AND FÖRSTER RESONANCE ENERGY TRANSFER

C. Seidel, S. Berger, C. Eggeling, J. Schaffer , E. Schweinberger, J. Widengren, R. Goody, O. Kensch, P. Rothwell

Multi-channel detection in a confocal epi-illuminated microscope is a new tool in single-molecule spectroscopy that allows the simultaneous registration of all fluorescence information (intensity, lifetime, anisotropy and spectral range) on a signal burst, where each burst reflects a single molecule freely diffusing in the focus. This information can be used in high-quality analytics and studies of conformational dynamics of individual, single and double labeled DNA-molecules in solution. Direct access to the time trajectories of the different fluorescence parameters is obtained by sliding burst analysis of the registered bursts. The construction of more-dimensional frequency histograms of the fluorescence parameters found in the trajectories on the single molecule level and selective analysis of these species gives detailed view on the molecular energy landscape and the associated molecular structures. The simultaneous acquisition of all four fluorescence parameters on the single molecule level is presented by Förster fluorescence resonance energy transfer (FRET) studies on double-labeled DNA molecules and HIV-1 Reverse Transcriptase/DNA complexes. Perspectives for monitoring biomolecular interactions and dynamics are discussed.

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12C-1
SPM OBSERVATION OF MODIFIED DNA ADSORBED ON A GOLD SURFACE

Minoru Takeuchi[1], Takao Ishida[2], Mitsuru Ishikawa[1]

Resolution of the base composition in DNA by scanning probe microscopy (SPM) is long expected to revolutionize massive analysis of genome information. However, even if the microscope attains atomic resolution of DNA, there remains a persistent problem. DNA has a double helical structure, in which both phosphate and ribose cover up bases in the form of two spiral shells. These shells prevent the SPM tip from approaching the bases.
To address this problem, we introduced an intercalation reagent, which is known to unwind the double helix through stacking between the bases. We noticed that when DNA is subjected to the reagent, the spiral shells straighten out on one side, while the bases form a parallel ladder on the other side. We adsorbed the unwound DNA on a gold surface with the stretches of the phosphate and ribose shells facing down to the gold surface, and with the bases facing up toward open space. This configuration allows the SPM tip to directly approach the exposed bases.
In determining whether the intercalated DNA has the structure we expected in the adsorbed condition, we observed the DNA with atomic force microscopy and also measured X-ray photoelectron spectra. Details of the model and analyses will be presented and discussed.

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12C-2
ATOMIC FORCE MICROSCOPY STUDY OF BIOMOLECULE IMMOBILISATION UPON OXIDISED POLYSTYRENE SURFACES

Sean A. Gillespie, David C. Cullen

Functional modification of polymer surfaces is required in a number of analytical fields, where covalent immobilisation of biological receptor molecules is required – such as, immunodiagnostics, biosensors and DNA chips. In particular, polystyrene has no functional groups and these must be generated by one of a number of physical or chemical surface modification techniques. Presented here is (i) a method for the oxidation of polystyrene surfaces to generate carboxyl moieties and (ii) a biophysical study of the immobilisation process at key stages, using atomic force microscopy, contact angle and protein immobilisation measurements. A number of important immobilisation variables have been investigated such as oxidation conditions, polystyrene composition and polystyrene format. Following oxidation, significant variations in surface topography and oxidation of polystyrene at sub-micrometer size scales were observed – resulting in very different patterns of protein coverage and activity. These features are characteristic of both oxidation method and polystyrene composition, demonstrating the importance of these considerations when modifying polystyrene surfaces – particularly when a well-defined and reproducible 'bioanalytical' surface is required.

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12C-3
DECOMPOSITION OF ATOMIC FORCE MICROSCOPY IMAGES USING THE SCALING INDEX METHOD

Ferdinand Jamitzky, R. W. Stark, G. E. Morfill, W. M. Heckl

The scaling index method was used in order to analyze atomic force microscopic images of double stranded DNA (plasmid PUC 8) adsorbed to mica. With this algorithm the image can be decomposed into constituents of different dimensionality, i.e. point-like, thread-like, and area-like structures, and residual noise. Special focus is on the evaluation of this method for the future automated analysis of binding events of DNA-specific proteins.

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12C-4
COMBINED SCANNING ELECTROCHEMICAL-ATOMIC FORCE MICROSCOPY: A NEW APPROACH FOR IMAGING MEMBRANE TRANSPORT

Julie V. Macpherson, Patrick R. Unwin

The ability to identify active sites, and quantitatively measure local rates of transport through membranes and tissues impinges on many biophysical and physiological processes.
We have developed a combined scanning electrochemical microscope (SECM) – atomic force microscope (AFM) that permits simultaneous topographical and electrochemical (amperometric and potentiometric) measurements at surfaces. Several types of probe tips have been developed that permit imaging both in air and under fluid. These include Pt-coated silicon nitride probes and Pt microwires that have been flattened and etched.
The SECM-AFM technique is illustrated with model studies on transport (diffusion or migration) of electroactive solutes through polycarbonate membranes containing pores of diameter 100 nm–1 μm. This technique enables active transport pathways to be identified - from the current response - and correlated directly with surface topography.
The prospects for using SECM-AFM on biological samples will be illustrated with high resolution permeability measurements of solutes within cartilage and skin.

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12C-5
HIGH RESOLUTION PHASE IMAGING OF PROTEIN CRYSTALS IN TAPPING-MODE AFM: A METHOD FOR THE ANALYSIS

Martin Stark, Clemens Möller, Daniel J. Müller, R. Guckenberger

We present a method for the analysis of phase imaging data in tapping-mode atomic force microscopy (AFM). In this mode, the cantilever is excited to oscillate close to its resonance, and the oscillation amplitude is held constant to generate the topographical image. The phase shift between excitation and response of the cantilever provides a signal related to energy dissipation in the tip-sample contact, and thus allows to extract information about local sample properties.
As model system, purple membrane of Halobacterium salinarum is investigated with tapping-mode AFM in buffer solution additionally recording the phase shift. The obtained resolution of 1 nm is essential for the interpretation of the data because it is in the range of the tip size and the lateral structure length of purple membrane. The presented analysis of phase images accounts for contributions of topography and contact volume: the phase image is decomposed in a contribution of the derivative of the topography in scan direction and a contribution of the contact volume mediating the interaction. To extract the interaction itself we suggest a relation plot of the decomposed phase versus contact volume. In the case of purple membrane the analysis reveals differences in the interaction of the tip with the protein from that with the lipid matrix.

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12D-1
MANIPULATION OF CELL FUNCTION USING NANO-STRUCTURED PATTERNS OF SINGLE RECEPTORS

Vanessa Z.-H. Chan, Joachim P. Spatz, Martin Möller

Cell position and function has traditionally been studied using microsized patterns of adhesive and nonadhesive regions. With such patterns, migration of the cell from the nonadhesive regions to the regions that promote cell adhesion is observed. This technology has been taken a step further through the formation of *nano*sized scaled patterns which allow the study of the interaction of single proteins with the cell membrane and its influence to the formation of patterns within the cell cytoskeleton. Using electron beam lithography and block copolymer micelles, metal islands with diameters ranging from 4 nm to 30 nm were precisely patterned with spacings of several microns onto silicon and glass substrates. Nanosized patterns of proteins were then produced by tethering the proteins to the metal islands through alkanethiol groups. By culturing cells modified with green fluorescent proteins on these substrates, the interaction of the cell with the nanostructured substrate can be studied through optical microscopy.

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12D-2
R&D ASPECTS OF THE DYNAMIC MARKING (DM)
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Cells in suspension can be labeled with superparamagnetic crystals which are linked to the cell surface by suitable antibodies.
A major application of this technique is the sorting of the marked cells due to external DC fields.
Still the DM techniques seem to be unknown.
One of the objectives of the new DM technique is an increased registration selectivity of marked objects.
By applying or not applying dynamic magnetic fields to the labeled objects, the observer can either move or not move the marked objects in particular ways. The fields can be described by Maxwells first equation. Therefore, in connection with these special external fields the superparamagnetic nm-particles become Dynamic Markers enabling the marked objects to perform controlled movements. These movements can be viewed under the microscope. This allows to observe single, dynamically marked objects among a large number of unmarked objects, an aspect important in cancer research.
Another aim of the DM is to utilize the special movement abilities and the very fast speeds attainable by magnetically labeled particles. These aspects can be useful in directing antibodies or vesicles containing drugs for target orientated drug supply.

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12D-3
3D-MICROSCOPY IN DIELECTRIC FIELD CAGES
Torsten Müller, Christoph Reichle, Gabriele Gradl, Petra Klein, Günter Fuhr

We represent a new fluorescence microscope compatible chip design with fluidic inlet and outlet consisting of two funnels and three dielectric field cages (DFC) on a circuit board in the size of a typical microscope slide. Individual cells were transported in integrated micro channels of 40 µm height by hydrodynamic flow using syringe pumps and aligned by "funnel" electrodes. A glass substrate of 150 µm allows high resolution optical detection and the use of the chip with all objectives (aperture up to 1.3).
Two planes of 4-electrodes glass plates spaced with a 40 µm polymer spacer forms a 3D octode. Electrodes were driven with high frequency fields in aqueous solutions. Based on negative dielectrophoresis micro objects were repelled symmetrically from the electrode edges and caged in the centre. The induced dielectric forces are in the range up to 5 pN. Dielectrophoretically caged single cells were fluorescently stained (ConA, Transferrin) and were analysed using fluorescence microscopy and confocal techniques (CLSM and FCS). We discuss some restrictions and some new possibilities of DFCs for 3D-microscopy and present the dielectric field cages as an non-invasive instrument for: (i) contact-free handling of cells and micro particles in typical size of 5–25 µm; (ii) holding of objects against a streaming velocity up to 100 µm/s; (iii) precise holding of micro objects in xy-direction of ±0.5 µm and in z-direction of ± 1 µm; (iv) alteration of position/rotation of objects in all 3 axes.

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12D-4
COMBINING PHOTONIC AND MECHNICAL NANOMANIPULATION FOR THE COLLECTION OF BIOLOGICAL MATERIAL
Robert W. Stark, Stefan Thalhammer, Javier Rubio Sierra, Wolfgang M. Heckl

The combination of mechanical nanomanipualtion by atomic-force microscopy (AFM) and UV-laser ablation techniques offers a direct approach to manipulate biological specimens on different scales ranging from millimetres down to a few nanometres. Additionally, both techniques provide the possibility to extract smallest amounts of material for subsequent (bio-)chemical analysis.
Combining both instruments requires an integrated setup with custom designed geometry which allows simultaneously to investigate the specimen with light-microscopic techniques (i.e. fluorescence imaging), atomic-force microcopy and UV-Laser Manipulation.

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12D-5
LASER-BASED ISOLATION OF CELLS AND CELL CLUSTERS FOR VIRUS SPECIFIC PCR ANALYSIS
Stefan Thalhammer, Anja Kölzer, Gerhard Frösner, Wolfgang Heckl

Laser-based microdissection provides a direct approach for the isolation of cell clusters, single cells and even cell components. We present a noncontact method, *laser pressure catapulting*, for the isolation of single cells and cell clusters from tissue sections. The laser precisely cuts around the selected area leaving a micron sized gap. The isolated specimen are subsequently ejected from the slide by single laser shots onto a collection device. The isolated material can be used for further genetic analysis. The experiments were performed with tissue sections infected with TTV. TTV is a newly described circular single-stranded DNA virus.

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12D-6
MANIPULATING THE MECHANICAL BEHAVIOUR OF SINGLE GFP-ACTIN FIBROBLAST CELLS ON NANOSTRUCTURED SURFACES

Joachim P. Spatz[+], Alexandre Micoulet[+], Vanessa Chan[+], Albrecht Ott*

The chemical-physical properties of synthetic macromolecules and peptides have been used to modify surfaces on the molecular level. These surfaces were used to manipulate the adhesion and motility properties of GFP-actin fibroblast cells based on single receptors allowing the cells to be probed with a pattern made of single receptors. The interaction of cells with such surfaces were studied by applying a uniaxial force to an individual cell. Conditions of constant stress or constant strain allowed the determination of the dynamic reaction of a cell to an external, mechanical stimulation. By using GFP-actin fibroblast cells, the dynamic development of actin filaments were monitored in-situ during defined external stimulation.

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12E-1
MOLECULAR HETEROGENEITY OF PROTEINS BY TWO-PHOTON EXCITED FLUORESCENCE SPECTROSCOPY

Giuseppe Chirico[#], Sabrina Beretta[#], Stefano Bettati[§*], Andrea Mozzarelli[$]

We have investigated in solutions and in silica gels the fluorescence emission of fluoresceine-labeled betalactolobulin and of pyridoxal 5'-phosphate molecule (PLP) bound at O-acetylserine sulfhydrylase-A (OASS from Salmonella typhimurium) as a coenzyme. By employing two-photon excitation we are able to investigate the emission of the proteins, detecting few (≅10) molecules at the time. In the case of Oass, the analysis of the photon counting histograms and of the fluorescence decay indicate that two species, with different quantum yield and lifetime are present. The lifetimes, but not the details of the internal photo-dynamics, are similar to those measured by single photon excitation, and the relative number fractions of the two species are measured.
In the case of the fluoresceine labeled betalactoglobulin, preliminar results will be shown regarding different responses of the protein behavior to external perturbation, such as pH, ionic strength and denaturating agent, when in solution and entrapped in silica gels.

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12E-2
TWO-PHOTON EXCITATION OF PHOTOSENSITIZERS AND MOLECULAR FLUOROPHORES: FLUORESCENCE EXCITATION SPECTRA AND SUBCELLULAR LOCALIZATION PATTERNS

L. Kelbauskas, W. Dietel, Th. Feurer, F. Ewald, R. Wolleschensky

Two-photon fluorescence excitation (TPE) spectra are presented for some photosensitizers (e.g. porphyrines) and fluorescence cell probes in the excitation wavelength range 480 nm $< \lambda <$ 1600 nm. The fluorescence excitation spectra of the cell probes were compared for both one- (OPE) and two-photon (TPE) excitations in order to determine valuable cell probes for the two-photon laser scanning microscopy (TPLSM). The excitation was performed using ~100 fs pulses of an optical parametric amplifier pumped by a Ti:sapphire multi-pass amplifier. As expected, the fluorescence emission intensities were found to be proportional to the square of the excitation intensity and the TPE emission spectra are independent on the excitation wavelength.
Spatially resolved fluorescence measurements on different human carcinoma cell lines were performed employing the confocal LSM technique in order to determine the subcellular localization sites and the way of the internalization of some sensitizers by cells. A comparison between the fluorescence patterns and signal-to-noise ratio for the TPE and the OPE is made. The way of the uptake of amphiphilic sensitizers by cells was followed using the OPE and the TPE.

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12E-3
TWO PHOTON FLUORESCENCE SPECTROSCOPY AND IMAGING OF TRYPTOPHAN CONTAINING PROTEINS

Markus Lippitz[1], Wolfgang Erker[2], Heinz Decker[2], Thomas Basché[1]

The binding of oxygen to hemocyanins is an example for cooperative behaviour in proteins. The intrinsic fluorescence intensity of tryptophans signals the amount of bound oxygen. Single molecule detection is expected to give information about the oxygenation distribution and fluctuation which should lead to deeper insights in the cooperativity of complex proteins. Difficulties of excitation in the UV can be avoided by nonlinear two photon excitation.
In a first step, we investigated the photophysics of tryptophan ensembles with one and two photon excitation. In the latter case, the sample is excited by a Titanium:Sapphire pumped frequency doubled OPO, producing 200 fs pulses in the range of 550–600 nm. The fluorescence intensities and spectra are recorded with a PMT and a CCD camera. Surprisingly, the fluorescence quenching efficiency of tryptophans depends on the excitation process. This is shown by acrylamid induced quenching of tryptophan solutions and by the binding of oxygen to hemocyanins. We suppose this effect is due to two photon enhanced ^1L_b fluorescence.
As a model for hemocyanins, we used latex spheres labeled with avidin which contain in total 350 tryptophans. These spheres are easily detected in a two photon scanning microscope. From these results we anticipate the future detection of single hemocyanins which contain 148 tryptophans.

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12E-4
PROXIMITY DETECTION IN TWO-PHOTON MICROSCOPY

Emmanuel Beaurepaire, Jérôme Mertz, Bruno Keramsi, Serge Charpak, Martin Oheim

We investigated the benefits of a detector located close to the objective for two-photon fluorescence imaging in scattering tissue. Fluorescence imaging of thick samples benefits from 2-photon excitation, due to the intrinsic confinement of fluorescence excitation, greater depth penetration of infrared light and whole-field detection. Yet, in the limit of photon diffusion, it is appropriate to consider a distribution of incoherent pointsources located at the surface rather than a single point source in the object plane. This distribution gives rise to a divergent cone of light when imaged with the microscope's objective. Thus, only a fraction of the photons captured by the objective are incident on the detector.
To quantitate this effect, we placed a point-source of light at different depths and used Monte-Carlo simulations to calculate the spatial and angular distributions of photons arriving at the surface. Ray tracing then provided an estimate for the detected fraction of photons. Unlike when imaging ballistic photons, the detection efficiency decays rapidly with increasing depth. This effect is more pronounced for isotropic scatters and larger separation distances between objective and detector. We directly compared the collected signal of a conventional whole-field detector and a home-built proximity detector for two-photon fluorescence imaging of the rat olfactory bulb *in vivo*. We show that the 'proximity gain' can be up to 300% when imaging 600 μm below the pia.

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12E-5
COHERENT ANTI-STOKES RAMAN MICROSCOPY: SELECTIVITY WITHOUT STAINING

A. Zumbusch, Th. Hellerer

Fluorescence microscopic methods based onconfocal microscopy or two photon excitation have become important tools in molecular biology. Despite their undeniable value, some problems inherent to fluorescence excitation persist. These include the phototoxicity of many dyes, their bleaching and not at least the need to overcome a strong background signal from autofluorescence of the sample.
We present a novel microscopic technique with contrast generation based on Coherent Anti-Stokes Raman Scattering (CARS). In this case the resonant excitation of molecular vibrations is exploited for selective imaging and the necessity for staining is removed. In our setup two beams from two different pulsed laser sources illuminate the sample simultaneously. If the frequency difference of the two laser beams coincides with the frequency of a molecular vibration of the sample, a resonantly enhanced four wave mixing signal is observed. By simply tuning the frequency of one of the lasers and raster scanning the sample, structures with different vibrational spectra can be visualized. Other than IR microscopy and conventional Raman microscopy, CARS microscopy offers high sensitivity at low excitation intensities with a three dimensional spatial resolution similar to common two photon microsopy. We will exemplify the capabilities of the method with live cell images.

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12E-6
THE TWO-PHOTON EXCITATION BIOMOLECULES AND DRUGS

Yu. P. Meshalkin

Two-photon excitation of biological molecules and drugs allows: 1) the selective excitation of photosensitizers in pathological cells; 2) photoinactivation of a bacteria and viruses; 3) to activate medical preparations. For these purposes the radiation penetrates through window of a transparency of a biological tissue.
For all applications we need a biological molecule with the large two-photon absorption cross sections (TPA cross-sections).
The TPA cross-sections of organic molecules varies from 10^{-46} to 10^{-52} cm^4 s/ phot mol. The TPA cross-sections of biological molecules and drugs were rarely measured. The results of TPA cross-sections measurements, obtained in our laboratory are presented in the table below.
In measurements of TPA cross-sections by a fluorescent method it is better to used parameter $-\beta$ (efficiency TPEF). The β is equal TPA cross sections multiplied by quantum yield of fluorescence and divided by normalizing factor 10^{-50} (GM-Goeppert-Mayer).
The results of TPA cross-sections measurements, obtained in our laboratory are presented below:
Al-phthalocyanine – 1.27×10^{-49} cm^4 s/phot mol (λ = 1064 nm) – β = 8.89
Procaine – 3.1×10^{-49} cm^4 s/phot mol (λ = 532 nm) – β = 3.1
Adrenaline – 3.2×10^{-52} cm^4 s/phot mol (λ = 532 nm) – β = 3.2×10^{-3}
Coffeine - 5.6×10^{-53} cm^4 s/phot mol (λ = 532 nm)

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12F-1
TWO DIMENSIONAL CORRELATION ANALYSIS OF FTIR DATA USED TO INVESTIGATE PROTEIN STRUCTURAL CHANGES

M-H. Baron, M. Revault, J-P. Forgerit, S. Lecomte, S. Servagent-Noinville

The 2D analysis of the FTIR spectra follows the procedure introduced by Noda (*Applied Spectrosc.* 44, 550, 1990). It allows the correlation of the dynamics of fluctuations of IR signals in a series of spectra measured as a function of one parameter (time or pH). Cross correlation analysis provides 2D spectra that are defined by two independent wavenumbers $\nu 1$ (abscissa) and $\nu 2$ (ordinate). The synchronous representation displays autopeaks on the diagonal reflecting those IR signals that vary as a function of the applied perturbant. Cross peaks are observed for bands which exhibit (at least partially) correlated dynamic behaviour. The asynchronous representation is characterised by missing autopeaks and asymmetric cross peaks which reveal non-correlated (out of phase) behaviour of two bands. Cross peaks, which occur in both the S- and the A- maps, provide information about the temporal order of the spectral changes.
We have investigated the dynamic of structural changes of two different proteins (Cytochrome c_{552} and BSA) adsorbed on hydrophobic supports. From 2D correlation analysis we conclude for time-dependent system (Cytochrome c_{552}) that only the solvation process is enlightened and for pH-dependent system (BSA), that the secondary structural change is correlated to the deprotonation of carboxylic side-chains.

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12F-2
FTIR-LINEAR DICHROISM MEASUREMENTS YIELD STRUCTURAL INFORMATION

Thomas Uliczka, Benedikt Heßling, Klaus Gerwert

FTIR-Linear-Dichroism measurements resolve structural changes for individual residues in membrane proteins. The movement of the S-H group of Cys 183 mutated against Ser 183 during the protonpumping photocycle is determined in the intermediates M and N. The S-H group absorbs at 2556 cm^{-1} in BR and shifts to 2568 cm^{-1} in M and 2572 cm^{-1} in N. The dichroic ratios of these bands are determined. This novel approach allows now to extract structural information out of the FTIR-spectra.

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12G-1
QUANTITATIVE CHEMICAL ANALYSIS OF LIPID/ PROTEIN FILMS BY SECONDARY NEUTRAL MASS SPECTROMETRY

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Phosphatidylcholine (PC) and phosphatidylglycerol (PG) are major constituents of the lung surfactant, a surface-active layer that stabilizes lung during respiration. A model system of the surfactant protein C (SP-C), which is essential for surfactant function, and the dipalmitoylated lipids DPPC and DPPG was probed using time-of-flight secondary neutral mass spectrometry (TOF-SNMS) to i) localize the SP-C in a lipid matrix, ii) calculate the total amount of SP-C in the mixed film, and iii) estimate the content of lipid in the fluid and condensed phase.
In contrast to TOF-SIMS, neutral particles, not intrinsic ions, are detected by TOF-SNMS. It is exceptionally suited for detecting elements rather than organic molecules. As an example, the small content of sulfur (three S per SP-C molecule) suffices to visualize the lateral structure caused by inhomogeneously distributed SP-C in a lipid film, whereas phosphorus is typical of phopsholipids. But some organic neutrals like CN and C_3H_8N also give excellent maps of the lateral distribution of SP-C and DPPC.
For the analysis of molecular monolayers, TOF-SNMS can be regarded as completion of methods like x-ray crystallography, electron diffraction, ellipsometry, fluorescence microscopy, Brewster angle microscopy, and TOF-SIMS. Yet little is known about applying SNMS to ordered organic monolayers, but it may turn out to be the most powerful technique to analyze thin films quantitatively.

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12H-1
3-D RECONSTRUCTION OF MACROMOLECULS FROM SMALL-ANGLE SCATTERING DATA BY A MONTE CARLO FITTING PROCEDURE

Hermann Hartmann

Small-angle scattering data are commonly used for a direct determination of model independent parameters of large biomolecules such as the radius of gyration. For the determination of the 3-D structure, however, some a priori knowledge or assumptions about the shape are often used. Moreover, there is not a unique solution of the inverse Fourier transformation. Any resulting structure is only one of the possible solution. An algorithm for reconstructing 3-D models from small angle scattering data should have two essential features: (a) Few or no assumptions about the structure. (b) It should give all the possible solutions. Available programs, using only very limited a priori knowledge use either a superposition of spherical harmonics, best suited for nearly spherical biomolecules [1], or a genetic algorithm [2]. We developed a new procedure based on the Metropolis algorithm which needs only data from a small-angle experiment for a 3D-reconstruction, but further information as the symmetry of the molecule can be applied. Additionally the algorithm gives all possible structures fitting the data, without manual interaction. The procedure has been successfully applied to a protein, KLH, with an overall shape of a hollow cylinder and to a compact protein with a globular structure.(Granted by DFG)
[1] Svergun DI, Barberato C., Koch MHJ. (1995) J. Appl. Cryst., 28: 768–773.
[2] Chacon P, Moran F, Diaz JF, Pantos E, Andreu JM (1998) Biophys J., 74(6):2760–75

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12H-2
INTEGRATION OF GROUP THEORY AND MAXIMUM ENTROPY TO SHAPE RECONSTRUCTION BY SAS

Francesco Spinozzi, Flavio Carsughi, Paolo Mariani

The reconstruction of the particle shape from solution scattering is an important task for understanding the structure-function relationship However, being SAS a low resolution technique, most of the details of the particle structure are lost. In the case of monodisperse particles at infinite dilution, as it is the case in many biological samples analysed in solution by SAS, a well-established method is based on the multipole expansion of the particle shape [Svergun, et. al, *J. Appl. Crtyst.*, 30, 798 (1997)].
In the method here presented [Spinozzi et al, *J. Chem. Phys.*, 109, 10148 (1998)], we improve the multipole expansion method introducing the group theory and the Maximum Entropy to deduce in a more efficient way the shape of the scattering particles. The group theory procedure is used to select the multipole expansion coefficients that agree with the particle symmetry. Moreover, in order to avoid the non-physical meaning of the shape function due to the truncation to a maximum finite rank L, a new Maximum Entropy formulation of the shape function is proposed.
To show the capability of the method, experimental scattering curves have been simulated and analysed. The results obtained by the proposed procedure gave an excellent agreement between the original and reconstructed shapes. Some examples of protein shape reconstruction from true SAXS data show that the presented procedure is able to reveal the effective symmetry of low resolution structure.

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12H-3
TIME-RESOLVED MEAN SQUARE DISPLACEMENTS OF PROTEIN – WATER HYDROGENS

Wolfgang Doster, M. Diehl, W. Petry, Claude Pfister, H. Schober

The potential of inelastic neutron scattering to explore structural fluctuations and hydration dynamics of proteins and its relation to computer simulations is discussed. The method probes the single particle motions of the nonexchangeable hydrogens. The exchangeable hydrogens can be masked by deuterium which exhibits a much lower scattering cross-section. Dihedral torsional transitions, rotational jumps of side-chain methyl groups and water-plasticized collective displacements comprise the most important contributions to the fluctuation spectrum of myoglobin and other proteins. The assignment is based on the elastic scattering function, reflecting the spatial distribution of accessible states. Temperature-dependent studies of hydrated proteins reveal a dynamical transition, connected with the onset of collective motions. The transition is triggered by fast fluctuations of protein-water hydrogen bonds, which can be suppressed by dehydration or vitrification in a glassy matrix. Using a moment analysis of the density correlation function we obtain time-resolved mean square displacements of protein and hydration water hydrogens.

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12H-4
SHAPING STRUCTURES OF TRANSCRIPTIONAL ACTIVATOR PROTEINS IN SOLUTION

J. Günter Grossmann, Ju-Fang Chang, Andrew Sharff, Ben Luisi

The description of molecular shape is important in the characterization and analysis of protein-protein and protein-nucleic acid interactions. We have used synchrotron X-ray scattering as a very effective technique for obtaining low-resolution structural details of proteins involved in transcriptional activation in solution. There has been significant progress with the development of an *ab initio* method for shape restoration in terms of spherical harmonics from scattering data alone. Here it will be demonstrated that the low-resolution molecular shape (>15Å) determined from solution X-ray scattering data can provide valuable information on domain orientation and dispositions of two transcription factor proteins (TFIIB and VP16). The protein envelopes can be used as direct comparison of protein conformation in the crystalline state (it is possible to identify protein domains and their relative positions within the calculated shape profile) and also to check the compatibility with results deduced by NMR of separated individual protein domains. Furthermore, the scattering studies confirm the lack of a defined conformation for the transcriptional co-activator Bob1 on its own in solution. Upon the recruitment of Bob1 into a specific ternary complex a partial protein-folding process is suggested.

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12J-1
THIOL-SPECIFIC SPIN LABELING OF HUMAN PLASMA LDL

Marina Kveder, Greta Pifat, Bojana Vukelic, Slavko Pecar, Milan Schara

Since Gotto introduced the electron paramagentic resonance (EPR) method in studying the structure of lipoprotein particles, spin labeling of the lipid phase of LDL has been extensively explored. However, the spin labeling procedure for apoproteinB (apoB) in LDL was not sufficiently selective nor specific. The technique of site-directed spin labeling is still not applicable to this system. The aim of this study was the investigation of more specific spin labeling procedure of the protein component in LDL which could help the interpretation of the EPR spectral features in terms of the structural characteristics of apoB. This protein is made up of 4536 amino acids, of which 25 are cysteine residues: 16 are present as disulfides and 9 are in the reduced form. The later are important as inhibitors of LDL oxidation and present suitable points for spin labeling. We have used methanethiosulfonate-based spin label (MTS-SL) which offers superior properties in the specific covalent modification of protein thiols. Namely, it has been shown that MTS-SL reacts rapidly and solely with thiol groups so that the rate of amine modification is negligible.We now present the labeling procedure and interpretation of the resulting EPR spectra thus extending the possibilities for a detailed characterization of human plasma LDL by the EPR method.

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12J-2
ANALYTICAL SOLUTION TO THE LIPARI-SZABO MODEL BASED ON THE REDUCED SPECTRAL DENSITY APPROXIMATION OFFERS A NOVEL PROTOCOL FOR EXTRACTING MOTIONAL PARAMETER

Christian Renner, Luis Moroder, Tad Holak

Most NMR dynamics investigations of peptides and proteins use the Lipari-Szabo model for interpretation of measured relaxation rates. An analytical solution to the Lipari-Szabo model is derived for isotropic overall tumbling. The parameters of the original Lipari-Szabo model, the order parameter S^2 and the effective internal correlation time τ_e, are calculated from two values of the spectral density function. If additionally the spectral density value J(0) is known, the exchange contribution R_{ex} term can also be determined. The overall tumbling time τ_c has to be determined in advance, for example from T_1/T_2 ratios. The required spectral density values are obtained by reduced spectral density mapping from T_1, T_2 and NOE measurements. Our computer simulations show that the reduced spectral density mapping is a very good approximation in almost all cases in which the Lipari-Szabo model is applicable. The robustness of the analytical formula to experimental errors is also investigated by extensive computer simulations and is found to be similar to that of the fitting procedures. The derived formulas were applied to the experimental ^{15}N relaxation data of ubiquitin. Our results agree well with published parameter values of S^2 and τ_e, which were obtained from standard fitting procedures. The analytical approach to extract parameters of molecular motions may be more robust than standard analyzes and provides a safeguard against spurious fitting results.

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