1907-Pos Heme-Protein Interactions in Horse Heart Ferricytochrome c Induced By Changes of Ionic Strength and Anion Binding to Protein Surface Charges

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Board B23

We have measured the absorbance and electronic circular dichroism (ECD) of horse heart ferri-cytochrome c for the Soret and 695 nm charge transfer band as a function of hydrogen phosphate and sodium acetate concentrations. The absorption and ECD profiles of the charge transfer band broaden substantially and the respective integrated intensities increase with rising hydrogen phosphate concentration. Concomitantly, the characteristic couplet displayed by the B-band changes significantly. Increasing the acetate concentration causes a shift and an increase in intensity on the low energy side of the charge transfer absorbance band, whereas the corresponding ECD remains nearly unaffected. In addition, only small changes were obtained for the B-band couplet. Our results indicate that an ionic strength increase (via increasing acetate ion concentration) causes some changes in the Fe-Met80 linkage, probed by the charge transfer band, without a substantial modification of the heme environment. However, the binding of hydrogen phosphate ions causes more significant structural changes, which most likely involve a strengthening of the Fe-Met80 bond, which increases the redox potential. Additional structural variations might also involve Phe82, and the hydrogen bonding network in the heme pocket. In contrast to the current belief of anion binding to the protein's binding domain for cytochrome c oxidase, our results show that the structural variations that occur in the heme pocket are most likely of functional significance.

1908-Pos NO Migration and Binding in Myoglobin

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Board B24

Fourier transform infrared spectroscopy is a powerful tool for the investigation of protein-ligand interactions in heme proteins. From the variety of ligands that bind to the heme iron, nitric oxide (NO) and carbon monoxide (CO) are particularly attractive, as their bond stretching vibrations give rise to strong mid-infrared absorption bands that can be measured with exquisite sensitivity and precision using photolysis difference spectroscopy at cryogenic temperatures. These stretching bands are fine-tuned by electrostatic interactions with the environment and, therefore, the ligands can be utilized as local probes of structure and dynamics. We have measured infrared spectra of the stretching absorption on NO in the heme-bound and photodissociated states of ferrous and ferric nitrosyl myoglobin (MbNO) and a few site-specific Mb mutants. In the NO-bound state, conformational heterogeneity was inferred from the appearance of multiple bands, arising from different electrostatic interactions with active site residues, most importantly, His64. In ferrous MbNO, a primary photoproduct site was found similar to the B site of MbCO, as indicated by a characteristic NO stretching spectrum. In ferric MbNO, the His64 side chain appears to interfere with trapping of NO in this site; only a very weak photoproduct spectrum was observed in Mb variants in which His64 was present. Upon extended illumination, the photoproduct spectrum changed in a characteristic way, indicating that NO readily migrates to a secondary docking site C, the Xe4 cavity, in which the ligand performs librational motions on the picosecond time scale. This docking site may play a role in the physiological NO scavenging reaction. Surprisingly, NO cannot be trapped at all in secondary docking site D of MbCO, the Xe1 cavity.

Membrane Proteins - I

1909-Pos Single Cell Studies of Cadherin Adhesion Dynamics

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Board B25

The pre-steady state kinetics of cadherin-mediated intercellular adhesion mediated by cadherin was studied by micropipette manipulation measurements. The resulting binding probability versus contact-time profile reveals two binding states. There is a first, fast binding stage that results in a low probability binding state. This is followed by a lag and then slower increase to a high probability binding state. This biphasic profile differs from the simple rise to a limiting plateau predicted for simple binding between proteins via a single binding site. Measurements with the truncated extracellular region of C-cadherin did not change the biphasic kinetic profile. This indicates that the initial binding kinetics is independent of the cytoplasmic domain. Further studies with mutants lacking different domains showed that the third domain (EC3) of the extracellular region is required for both the two-stage kinetic mechanism and the slow forming second, high probability binding state. Mutating the critical Trp2 residue also abolished the two-stage kinetic process. Studies with domain deletion mutants further mapped the fast, first step to the outer two cadherin domains (EC12). This behavior is inconsistent with the assumed binding model for cadherins in which the proteins are postulated to bind via a single binding site in the N-terminal EC1 domain of the extracellular region.

1910-Pos Sorting Signal of *Escherichia coli* OmpA is Modified by Oligo (R)-3-Hydroxybutyrate

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Board B26

Escherichia coli outer membrane protein A (OmpA) is a well-established model for the study of membrane assembly. Previous studies have shown that the essential sequence for outer membrane localization, known as the sorting signal, is contained in a segment of the eighth β-strand, residues 163–171. Sequential digestion of OmpA, purified from outer membranes or inclusion bodies with cyanogen bromide and *Staphylococcus aureus* GluC, yielded peptide 162–174(LSLGVSYRFGQGE). Western blot and chemical assays indicated that the peptide was covalently modified by oligo-(R)-3-hydroxybutyrate (cOHB), a flexible, amphipathic oligoester. MALDI/MS was consistent with modification of peptide 162–174 by up to ten R-3-hydroxybutyrate (HB) residues. Western blot analysis of mutants of the peptide, using anti-OHB IgG, indicated that cOHB modification was not inhibited by the single mutations S163G, S167G, Y168F, R169N or R169D; however, cOHB was not detected on peptides containing the double mutations S163G:S167G S163:V168G, L162G:S167G, and L164G:S167G. MALDI/MS/MS of double mutant S163G:S167G confirmed the absence of cOHB-modification. The results suggest that cOHB may be attached to one or both serines, and point to the importance of the flanking hydrophobic residues. Modification by cOHB may play a role in outer membrane targeting and assembly of OmpA.

1911-Pos Identification And Dissection Of Ca(2+)-binding Sites In The Extracellular Domain Of Ca(2+)-sensing Receptor

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Board B27

Ca2+-sensing receptors (CaSRs) represent a class of receptors that respond to changes in the extracellular Ca2+ concentration ([Ca2+]o) and activate multiple signaling pathways. A major barrier to advancing our understanding of the role of Ca2+ in regulating CaSRs is the lack of adequate information about their Ca2+-binding locations, which is largely hindered by the lack of a solved three-dimensional structure and rapid off rates due to low Ca2+-binding affinities. In this paper, we have reported the identification of three potential Ca2+-binding sites in a modeled CaSR structure using computational algorithms based on the geometric description and surface electrostatic potentials. Mutation of the predicted ligand residues in the full-length CaSR caused abnormal responses to [Ca2+]o, similar to those observed with naturally occurring activating or inactivating mutations of the CaR, supporting the essential role of these predicted Ca2+-binding sites in the sensing capability of the CaSR. In addition, to probe the intrinsic Ca2+-binding properties of the predicted sequences, we engineered two predicted continuous Ca2+-binding sequences individually into a scaffold protein provided by a non-Ca2+-binding protein, CD2. We report herein the estimation of the metal-binding affinities of these predicted sites in the CaSR by monitoring aromatic-sensitized Tb3+ fluorescence energy transfer. Removing the predicted Ca2+-binding ligands resulted in the loss of or significantly weakened cation binding. The potential Ca2+-binding residues were shown to be involved in Ca2+/Ln3+ binding by high resolution NMR and site-directed mutagenesis, further validating our prediction of Ca2+-binding sites within the extracellular domain of the CaSR.

1912-Pos Examination Of Ligand Binding And Multimerization Of Small Multidrug Resistance Protein EmrE In Detergent Using Small Angle Neutron Scattering

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Board B28

Escherichia coli multidrug resistance protein E (EmrE) is a member of the small multidrug resistance protein family and is believed to span the inner membrane of E. coli as a four transmembrane alpha-helix protein. As its name implies, this small protein confers host resistance to a broad range of quaternary ammonium compounds (QAC) and interchelating dyes via proton motive force. The exact multimeric state or states of EmrE protein during transport and ligand binding is not well understood and has often yielded conflicting results that are specific to the conditions of study. To explore this relationship further, organic solvent extracted EmrE from E. coli membranes was solubilized in the detergent, sodium dodecyl sulphate (SDS), at varying protein concentrations. Low concentrations of EmrE solubilized in SDS demonstrated that it predominates in a monodispersed state by size-exclusion high pressure liquid chromatography (SEC-HPLC). Upon increasing EmrE concentrations, a variety of multimeric states can be observed by Tricine SDS-PAGE analysis. To explore the ligand binding and multimeric arrangement of SDS-EmrE further, small angle neutron scattering (SANS) was used to probe the conformations of the EmrE multimers in SDS. SANS analysis demonstrated that large aggregates induced or formed by EmrE were observed in the SDS solution and their structures were altered in the presence of the ligand tetraphenyl phosphonium (TPP). Results from these analyses suggest that ligand binding may also occur in the presence of EmrE multimers and the conformation changes in the presence of ligand.

1913-Pos Daunorubicin interaction with MsbA

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Board B29

MsbA, a lipid transporter of ABC (ATP-binding cassette) superfamily in Escherichia coli exports lipid A core moiety of LPS (lipopolysaccharide) from the cytosolic to the periplasmic side of the inner membrane. ABC family members hydrolyze ATP to transport lipids, sugars, amino acids, and most notably drugs. Homologs of MsbA such as p-glycoprotein and LmrA transport a wide spectrum of cytotoxic molecules. Many of these molecules including the anthracycline daunorubicin stimulate the ATPase activity of MsbA. In this work, we use spectroscopic tools to directly investigate the interaction of daunorubicin with MsbA. Daunorubicin quenches tryptophan fluorescence in a concentration dependent manner. The quenching is inhibited by prior trapping of MsbA in the closed state by addition of AMP-PNP. Spin labels introduced at specific sites in helix 3 quench daunorubicin fluorescence. The quenching is reversed by addition of AMP-PNP suggesting that it is the result of daunorubicin binding to MsbA. These results are consistent with daunorubicin binding directly to MsbA and identify residues involved in this interaction.

1914-Pos Identification Of The Calmodulin Binding Domain Of Connexin43

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Board B30

Calmodulin (CaM) has been implicated in mediating the Ca2+-dependent regulation of gap junctions. This report identifies a CaM-binding motif comprising residues 136–158 in the intracellular loop of Cx43. A 23-mer peptide encompassing this CaM-binding motif was shown to bind Ca2+-CaM with 1:1 stoichiometry by using various biophysical approaches, including surface plasmon resonance, circular dichroism, fluorescence spectroscopy and NMR. Far UV circular dichroism studies indicated that the Cx43-derived peptide increased its a-helical contents on CaM binding. Fluorescence and NMR studies revealed conformational changes of both the peptide and CaM following formation of the CaM:peptide complex. The apparent dissociation constant of the peptide binding to CaM in physiologic K+ is in the range of 0.7–1 microM. On binding of the peptide to CaM, the apparent Kd of Ca2+ for CaM decreased from 2.9 +/− 0.1 microM to 1.6 +/− 0.1 microM, and the Hill Coefficient nHill increased from 2.1 +/− 0.1 to 3.3 +/− 0.5. Transient expression in HeLa cells of two different mutant Cx43-EYFP constructs without the putative Cx43 CaM-binding site eliminated the Ca2+-dependemt inhibition of Cx43 gap junction permeability, confirming that residues 136–158 in the intracellular loop of Cx43 contains the CaM binding site that mediates the Ca2+-dependent regulation of Cx43 gap junctions. Our results provide the first direct evidence that CaM binds to a specific region of the ubiquitous gap junction protein Cx43 in a Ca2+-dependent manner, providing a molecular basis for the well characterized Ca2+-dependent inhibition of Cx43-containing gap junctions.

1915-Pos 195 Pt Nmr: Interactions Of The Cancer Drug Cis-platin With Membranes And Mgst1, A Integral Membrane Detoxification Protein

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Board B31

The microsomal Glutathione-transferase (MGST1) is an integral membrane protein, which catalyses the conjugation of glutathione (tripeptide GSH) with xenobiotics; a process essential for cells to remove and detoxify e. g. carcinogens. While this glutathione system plays an essential role in healthy cell survival, glutathione has been shown to have a pivotal role in the development of acquired drug resistance. It prevents successful chemotherapies against a range of cancer types, therapies often based on cisplatin based drugs. These Pt compounds are initially quite effective, they become non-effective e.g. during the treatment of prostate cancer (very common 10000 new cases/a in Sweden) which progresses into a non-curable form during therapy. To understand the molecular mechanism behind the activity of Pt drugs and their inhibition by the human defense system, we use an solid state NMR approach (complemented by liquid NMR) to elucidate for cis-platin (diamino-dichloroplatinat II):

(i) conversion of cis-platin complex into an diamino-diaqua-complex, essential for its membrane passage into the cell interior.
(ii) lipid membrane - drug interactions: binding to cell membrane surface, solubility and membrane transport.
(iii) Pt drug binding to MGST1 enzyme, followed by glutathione conjugation into more water soluble compounds.

1916-Pos Implementation of Hybrid Solution/Solid-state NMR Restraints into Structural Refinement of Membrane Proteins

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Board B32

Membrane Proteins (MPs) constitute ~30% of all genomes and ~70% of drug targets. Despite their importance, structure determination of MPs lags far behind that for soluble proteins due to experimental difficulties. Solution and solid-state NMR methods

are complementary techniques to obtain atomic information on MP structure, dynamics, and topology. Here, we present a hybrid method for structural refinement of MPs which uses restraints from both NMR techniques.

Traditionally, only solution NMR data such as NOE and dihedral angles are implemented in structure calculation. Together with the solution NMR data, chemical shift anisotropy and dipolar coupling from solid-state NMR (PISEMA experiment) are introduced as harmonic restraints in the hybrid approach. A target function of both solution and solid-state NMR restraints is formulated. The target function can be optimized using torsion angle dynamics in Xplor-NIH to generate a hybrid solution/solid-state structure ensemble.

The hybrid method is applied to monomeric and pentameric PLN (phospholamban), a 52-residue integral MP that regulates sarco (endo)plasmic reticulum Ca-ATPase (SERCA) function in cardiac muscle. Using NOEs, dihedral angles, hydrogen bonds and PISEMA restraints, a solution/solid-state ensemble containing both structural and orientational information is obtained. The hybrid ensemble also shows improved precision over the solution NMR ensemble.

The hybrid solution/solid-state methods can be easily implemented with other structural restraints and applied to different MP systems, aiding the structure determination of MPs by solution and solid-state NMR.

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1917-Pos Snapshots Of Disulfide Bond Formation: NMR Solution Structure Of The Integral Membrane Enzyme DsbB

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Board B33

Disulfide bond formation is a critical biological process for the folding and stability of many proteins. In prokaryotes, this process is catalyzed by DsbA and DsbB and their homologs. DsbA is a soluble protein that functions as the primary oxidant for proteins in the periplasm of *E. coli* whereas DsbB is an integral membrane protein that reoxidizes DsbA and is itself reoxidized by various quinones. In order to understand the mechanism of *de novo* disulfide generation, we have characterized the structure of DsbB and its interaction with DsbA and ubiquinone.

For structure determination, we have introduced point mutations to stabilize DsbB in a reaction intermediate containing an interloop disulfide bond which quenches the apparent μs-ms timescale dynamics of wildtype DsbB, resulting in high quality spectra. 98% of the backbone resonances have been assigned using TROSY based triple resonance methods at 600 MHz. TALOS derived dihedral angle restraints and sequential NOEs have been used to determine the local secondary structure elements. The global fold has been determined using paramagnetic relaxation enhancement (PRE), NOESY spectra from Ile, Leu, Val methyl protonated sample, and residual dipolar couplings (RDCs). The backbone r.m.s.d. for the 14 lowest energy structures is 0.8Å.

Measuring DsbB chemical shift changes upon titration with DsbA allowed us to define two separate DsbA interaction motifs on DsbB. Unusual chemical shifts of atoms around the interloop disulfide bond suggest that the local electrostatic potential at the N-terminus of thesecond transmembrane helix stabilizes and polarizes this disulfide bond, helping to prevent the back reaction. By measuring NOEs between ubiquinone and DsbB, we were able to identify the quinone binding pocket on DsbB and design mutagenesis experiments to characterize the DsbB-quinone interaction.

1918-Pos Preparation Of Labeled Cannabinoid Receptor CB2 For NMR Structural Studies

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Board B34

The peripheral cannabinoid receptor, CB2, a heptahelical G protein-coupled membrane receptor, has become one of the most sought after pharmaceutical targets. Structural studies on CB2 would provide valuable information for development of novel specific ligands targeting this receptor. NMR experiments on CB2 require labeling with the stable isotopes, 2H, 13C, 15N. In order to study CB2 at functional conditions, reconstitution of the purified receptor into liposomes is desirable.

CB2 was expressed in *Escherichia coli* as a fusion with maltose-binding protein and several affinity tags and its functional activity confirmed by ligand binding. The fermentation protocol was adapted to expression in minimal media supplemented with stable isotope-labeled nutrients. Procedures suitable for production of mg-quantities of labeled CB2 were developed. Currently fermentation conditions are further optimized to reduce expenses for isotope-labeled materials.

The receptor was purified by Ni-NTA and Strep-tactin affinity chromatography in the presence of detergents and reconstituted into a lipid matrix consisting of monounsaturated phosphatidylcholine and phosphatidylserine (4/1, mol/mol). The ability of CB2-containing proteoliposomes to activate G-proteins in response to agonist binding was studied as a function of detergent, lipid, and CB2 concentration.

1919-Pos Membrane Reconstitution of Functionally Refolded Human Neuropeptide Y Receptor Type 2

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Board B35

The human neuropeptide Y receptor type 2 is an integral membrane protein, which belongs to the G protein-coupled receptor family. Its ligand, the 36 amino acids neuropeptide Y (NPY), plays among others an important role in the regulation of food uptake. To obtain milligram quantities of protein, the receptor was overexpressed in *E. coli* as inclusion bodies using high-density-fermentation with a yield of more than 100 mg protein per liter medium. The refolding *in vitro* takes place in the presence of non-denaturating detergents well above the critical micelle concentration, so that the folded receptor is kept in micellar solution. From this micellar state the receptor was functionally reconstituted into liposomes and the detergents were removed by dialysis.

To determine ligand binding and the concentration of correctly folded receptor, affinity assays were performed, such as fluorescence measurements and competitive ligand binding assays with ^{3}H-labeled NPY as tracer. The radio labeled ligand binding assay was adapted to the application of micelles and used to confirm specific binding in comparison with active receptors expressed in mammalian cells. With fluorescence measurements we were able to show quenching of the ligand/receptor complex of up to 20% of the total fluorescence signal. This technique was also used in titration experiments with NPY to determine ligand affinity with a KD value of around 26 nM for the receptor in micelles. In contrast, the KD value for the receptor into lipid membrane was around 4 nM. Further in micellar solution, only around 10% of the receptor was folded correctly, while up to 100% of the receptor was active after reconstitution. The reconstituted receptor was stable for at least 25 days.

1920-Pos Air -stable Supported Bilayer And Membrane Protein Microarrays

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Board B36

Cell membranes are essential for the integrity and function of cells. Each cell membrane is specialized to contain specific proteins and lipid components that are unique for a particular cell or organelle. Since a major percentage of drug targets are membrane bound, it is important to develop high throughput screening techniques, such as membrane protein microarrays. While supported lipid bilayers, with the incorporation of membrane proteins, have been successfully developed in the last twenty years, they are too fragile to survive multiple steps of solution incubation, washing, and air exposure usually required between processing steps. Balancing the stability and fluidity of lipid bilayers is highly challenging. Here we report a successful strategy to stabilize supported lipid bilayers (SLBs) based on a combination of covalent tethering and electrostatic attractiion. The resulting SLBs show remarkable stability and remain intact after repeated cycles of air exposure, drying, and rehydration. More importantly, nearly complete lipid mobility is maintained in these stable SLBs. We successfully incorporated transmembrane proteins into these stable SLBs in an array format and demonstrated their potential for functional screening, such as posttranslational modification and functional domain identification.

1921-Pos Dielectric Spectroscopy of Single Cells: Towards Tag-free Detection of Proteins and Their Interactions *In Vivo*

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Board B37

Investigation of protein localization, activity, and interactions in living cells is most often done by detecting the light emitted by optically excited fluorescent tags attached to the proteins of interest. Despite its widespread use and incontestable usefulness, fluorescence tagging may perturb the activity of the proteins under study. This presentation begins with an overview of recent advances in the development of a tag-free method for detection of membrane proteins from dielectric measurements on suspensions of cells. Then one particular direction of development is presented, which has emerged from studies in our laboratory on dielectric spectroscopy of single cells in microfluidic channels. Microfluidics offers numerous possibilities for manipulation of cells and their external medium, including selection of cells expressing a protein of interest and simplified procedures for electrode polarization correction, which poses particular challenges in bio-dielectric studies.

1922-Pos SEIDAS Or How To Perform Voltage-clamp Experiments On Membrane Proteins With Structural Sensitivity?

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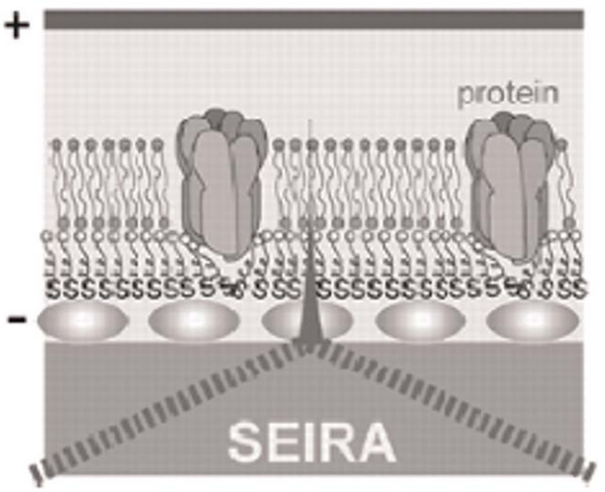
Board B38

Electrophysiological techniques, like patch-clamp, unravelled many functional aspects of ion channels but usually suffer from poor structural sensitivity. We have developed Surface Enhanced Infrared Difference Absorption Spectroscopy (SEIDAS) to probe potential-induced structural changes of a protein on the level of a monolayer. A novel concept is introduced to incorporate membrane proteins into solid supported lipid bilayers in an orientated way via the affinity of the His-tag to the Ni-NTA terminated gold surface. Full functionality of the surface tethered cytochrome c oxidase is demonstrated by cyclic voltammetry after binding of the natural electron donor cytochrome c. General applicability of the methodological approach is shown by tethering photosystem II to the gold

surface. In conjunction with hydrogenase, the basis is set towards a biomimetic system for H2-production. Recently, we succeeded to record IR difference spectra of a monolayer of membrane protein under voltage-clamp conditions. This approach opens an avenue towards mechanistic studies of voltage-gated ion channels with unprecedented structural and temporal sensitivity.



1923-Pos Characterization of the Cysteine-free Inner Membrane Transferase Protein ArnT from *S. typhimurium*

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Board B39

Widespread antibiotic resistance in bacteria has necessitated the identification of new targets for the design of novel antimicrobial drugs. Cationic antimicrobial peptides kill infecting cells by permeabilizing the membrane after binding to the negatively charged lipids on the bacterial outer surface. One of the proteins discovered to be specifically involved in *Salmonella typhimurium* and *Escherichia coli* resistance to the antibiotic polymyxin is the inner membrane protein ArnT, which confers resistance to polymyxin through the modification of lipid A, a major component of the outer surface of Gram-negative bacteria. ArnT transfers a neutral aminoarabinose moiety onto one or both of the negative phosphate groups of lipid A before localization to the outer membrane. The reduced surface charge prevents cationic peptides such as polymyxin from electrostatically recognizing and then killing the bacterium. We previously published the first expression, purification and functional analysis of ArnT from *S. typhimurium*. Our studies showed that ArnT is highly α-helical and described a new *in vivo* functional assay. The studies presented here report on the further characterization of purified ArnT. We have determined that all eight of the native cysteines in *S. typhimurium* ArnT are reduced and therefore not involved in disulfide bonds. We have also created a cysteine-free protein that is both structurally and functionally intact as characterized by circular dichroism and *in vivo* activity assays. These studies provide an essential foundation for analysis of ArnT structure and function using mutagenesis and biophysical techniques. Further, site-directed spin labeling EPR spectroscopy has been utilized in preliminary studies of the secondary structural motifs of ArnT.

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1924-Pos The Trimeric Periplasmic Chaperone Skp of E. Coli Forms 1:1 Complexes with Outer Membrane Proteins via Hydrophobic and Electrostatic Interactions

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Board B40

The interactions of outer membrane proteins (OMPs) with the periplasmic chaperone Skp from E. coli are not well understood. We have examined the binding of Skp to various OMPs of different origin, size, and function. These were OmpA, OmpG, and YaeT from E. coli, the translocator domain of NalP from Neisseria meningitides, FomA from Fusobacterium nucleatum, and the voltage-dependent anion selective channel, human isoform 1 (hVDAC1) from the outer membrane of mitochondria. Binding of Skp was observed for bacterial OMPs, but neither for mitochondrial hVDAC1 nor for soluble bovine serum albumin. Trimeric Skp formed 1:1 complexes, OMP·Skp3, with bacterial OMPs, independent of their size or origin. The dissociation constants of these OMP·Skp3 complexes were all in the nanomolar range, indicating that they are stable. OMP binding to Skp3 was pH-dependent and was not observed when either Skp or OMPs were neutralized at basic or at acidic pH. When the ionic strength was increased at neutral pH, the free energies of binding of Skp to OmpA or OmpG were reduced. Electrostatic interactions were therefore necessary for the formation and the stabilities of the OMP·Skp3 complexes. Skp efficiently shielded the tryptophans of the transmembrane strands of OmpA against fluorescence quenching by acrylamide. Lipopolysaccharide (LPS) is a major component of the outer membrane of Gram-negative bacteria. LPS bound to the OmpA·Skp3 complex at low stoichiometries and a ternary complex was formed. Acrylamide quenching of fluorescence indicated that in this complex, the tryptophans of the hydrophobic transmembrane domain of OmpA were located closer to the surface than in the binary OmpA·Skp3 complex. This may explain the previous observation that folding of Skp-bound OmpA into lipid bilayers is facilitated in presence of LPS.

1925-Pos Electrophysiological Characterization Of A Substrate-specific Bacterial Outer Membrane Protein

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Board B41

The Gram-negative bacteria such as *Pseudomonads aeruginosa* do not possess porins, and therefore they have a poorly permeable outer membrane for low-molecular weight substrates. The transport of the majority of small molecules in *Pseudomonads* is mediated by the substrate-specific channels of the OprD family. Here, we characterized OprD as a prototype of this large family. The crystal structure of OprD is now solved (Biswas et al., 2007), and reveals a monomeric 18-stranded β-barrel with a narrow constriction (~5.5 Å) made by two loops (L3 and L7) and the barrel wall. We confirmed the structure by performing single-channel electrical recordings with the wild-type OprD protein and its mutants. The wild-type OprD protein conductance is 28 ± 1 pS, in accordance with previous studies (Huang and Hancock, 1996), and asserting the constriction observed in the crystal structure. The R131G and ΔL3 mutations further validated the crystal structure. Based upon the wild-type OprD structure, the R131G mutant would have a dual pore. As expected, the R131G conductance increased (46 ± 2 pS). The single-channel electrical conductance of the ΔL3 channel, in which the residues 130–135 were replaced by two glycines, was substantially increased by comparison with the wild-type OprD channel (88 ± 7 pS). The current OprD structure, along with this preliminary electrophysiology study opens opportunities for further experiments to discern the role(s) of the individual residues in the pore for OprD substrate binding and transport. It is expected that such future studies would shed light on the mechanism involved in the substrate specificity within this large outer membrane protein family.

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1926-Pos Fluorescence Quenching of Trp41 of the Influenza M2 Proton Channel

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Replication of the influenza A virus is dependent on the proton channel activity of the tetrameric M2 proton channel protein. The proton channel is activated by the low pH environment of the endosomes of infected cells. M2 proton channels are the target of the antiviral drug amantadine. The M2 protein forms a homotetramer, the active state of the proton channel, through strong interactions of the single transmembrane (TM) domain of each subunit. Activation of the channel is regulated by two TM residues, His37 and Trp41. The environment of the Trp41 residues was probed by fluorescence quenching. Recombinant protein was expressed in E. coli with a 6XHis-Tag and purified by nickel affinity chromatography. Reconstitution of the protein in membranes of POPC, POPG and cholesterol (4:1:2) produced liposomes that displayed proton currents and sensitivity to amantadine. The protein had helical content and was oligomerized in detergent micelles and liposome membranes. A single tryptophan M2 mutant protein (W15F) was constructed to study the fluorescence of Trp41. Iodide quenching experiments on liposomes containing W15F M2 protein indicated that the Trp41 side chain exposure to the aqueous quencher was increased at low pH. Amantadine inhibited proton channel activity of the W15F M2 protein, but did not affect the pH induced changes in iodide accessibility of the Trp41 residue. These results demonstrate a conformational change of the protein induced by the activating stimulus of low pH.

1927-Pos The role of the transmembrane domainTrw on R388 bacterial conjugation

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For those integral membrane proteins containing the bulk of the protein mass and the active center outside the membrane, and a single transmembrane domain, the latter is considered to work merely as an anchor, without any major influence on the structure and/or function of the catalytic domain [1].

TrwB is a protein encoded by the conjugative plasmid R388. It plays an essential role in bacterial conjugation. It is an integral membrane protein consisting of 507 residues, that contains characteristic nucleotide triphosphate-binding domains, reminiscent of those of F_1-ATPase α and β subunits. The protein consists of a large (ca. 440 residues) extramembranous domain, and a transmembrane domain comprising the 70 N-terminal residues that includes two transmembrane helices and a small periplasmic domain in between.

In our laboratories TrwBΔN70, a soluble form of TrwB lacking the N-terminal transmembrane segment, was purified [2]. In that purification protocol, TrwBΔN70 behaved as a monomer. However, its crystal structure revealed a molecule with six equivalent protein units. In a more recent publication [3], we reported the purification of native TrwB in monomeric and hexameric forms, in the presence of β-D-dodecylmaltoside (DDM). The availability of TrwB both in

the complete, detergent-solubilized, and in the truncated, water-soluble forms prompted us to perform a comparative study of the properties of both proteins as a previous step to unravel the role of the transmembrane domain of TrwB in bacterial conjugation.

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1928-Pos Influence of Cholesterol on the Lateral Mobility of Na+/K-ATPase Reconstituted on Giant Unilamelar Vesicles

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Na^+/K^+-ATPase is an enzyme present in the majority of the cellular membranes and has a fundamental role in a number of physiological process. The idea that the physical state and the physicochemical properties of the lipidic matrix influence the dynamic and function of integral proteins is well accepted. The existence of segregated lipid domains and the possibility that enzymes will differentially distribute among them add to the effect of the lipid matrix on the protein function an extra degree of complexity. Our previous work showed that minute changes in cholesterol concentration produce abrupt changes in the membrane organization, increasing interdomain surfaces. In addition, we proposed that cholesterol induces a change in hydration at the protein-lipid interface. These changes induce small changes in the pig kidney Na^+/K^+-ATPase's structure and dynamics, acting to fine-tune the enzyme.

In this work, we present preliminary data on the role of cholesterol on the mobility of the protein inside the membrane. We reconstituted pig kidney Na^+/K^+-ATPase labeled with Fluorescein into Giant Unilamellar Vesicles (GUVs) of endogenous lipids containing different concentrations of cholesterol and measured the diffusion coefficient of the enzyme using fluorescence correlation spectroscopy (FCS) with 2-photon excitation. The results are discussed under the scope of the relationship between activity and mobility of the enzyme in the membrane

1929-Pos Thermodynamics of Membrane Protein Insertion/Folding: Fluorescence Correlation Spectroscopy Study of Annexin B12

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Deciphering the stability of membrane proteins in their native environment has been hindered by the lack of both appropriate model systems and adequate experimental techniques. Previously we suggested that annexin B12 (ANX), known to insert across the lipid bilayer at acidic pH in the absence of Ca^{+2}, could serve as such a model, owing (a) to the reversibility of its bilayer insertion and (b) to the absence of aggregation in the inserted and insertion-competent states [Biochemistry 2005, *44*:34042]. Here we used a combination of the experimental and computational techniques to characterize structural and thermodynamic features along the insertion pathway of ANX. First, we applied a java-based web tool, MPEX (http://blanco.biomol.uci.edu/mpex), which utilizes experimental hydrophobicity scales to predict potential transmembrane helices. Second, we tested membrane insertion of potential TM segments using our novel fluorescence lifetime topology method [Analytical Biochemistry 2006, *348*:87]. These topology measurements demonstrate that the insertion proceeds via an interfacial refolded intermediate state, which can be stabilized by high concentrations of anionic lipids. And finally, we applied fluorescence correlation spectroscopy to determine (1) the pH-dependent formation of the membrane-competent ANX and (2) the free energy (ΔG) stabilizing final transmembrane and interfacial intermediate states. Our results indicate that protonation of two residues in the pre-bilayer zone triggers the refolding/insertion process. The ΔG difference between the TM and the interfacial state is rather small (~1-2 kcal/mole) compared to the ΔG of membrane partitioning of ANX ($<$10 kcal/mole). Our results confirm the interface-directed model of spontaneous insertion of non-constitutive membrane proteins and provide an important benchmark for future measurements of ΔG stabilizing the structure of constitutive membrane proteins.

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1930-Pos A FRET Study on the Functional Reconstitution of the Human Serotonin 5-HT_6 Receptor Using Synthetic Transmembrane Peptides

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Autonomous reconstitution of the seven transmembrane (TM) domains of the human serotonin receptor subtype 6 (5-HT_6) in micelle and lipid bilayer environments is described. Peptides representing the seven α-helical TM domains were synthesized and reconstituted in both detergent micelles and liposomes. Assembly of the seven TM (7TM) peptides was monitored by fluorescence resonance energy transfer (FRET) between donor and acceptor probes labeled at the amino termini of the second and fourth TM-peptides, respectively. FRET analysis suggests that the 7TM assemblies exist as ordered assemblies, and binding studies showed that reconstituted 7TM-peptides in liposomes selectively bind to free serotonin and serotonin-conjugated magnetic beads, yielding a dissociation constant of 0.83 ± 0.14 μM. These results show that the seven individual TM domains of a rhodopsin-type G protein coupled receptor (GPCR) can spontaneously assemble in liposomes into a conformation that mimics a native structure, and further demonstrate that specific interactions between TM helices play a critical role in the folding and stabilizing of GPCRs. Furthermore, the autonomous assembly of 7TM-peptides can be applied to the screening of agonists for GPCRs that are difficult to manipulate.

1931-Pos The Single Channel Water Permeability Coefficient Of Aquaporin-1 Revisited

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Board B47

It is generally accepted that water passes aquaporin-1 (AQP1) at a rate that is close to the diffusion limit. Permeability coefficients of 6 and 11 x 10^{-14} cm^3/subunit/s were derived from swelling experiments carried out with overexpressing oocytes or with reconstituted liposomes. The actual AQP1 transport rate might have been several-fold higher, due to known limitations in accuracy of membrane protein concentration assessment by biochemical or histochemical means. In addition, the unstirred layers in the oocyte system have to be taken into account, and there are variations in the reconstitution efficiency into liposomes. Since such an observation would change the current molecular picture of how water is transported trough single file channels, we now have combined fluorescence correlation spectroscopy (FCS) and scanning electrochemical microscopy to determine the single channel permeability coefficient. FCS was used to count the AQP1 copies reconstituted into planar lipid bilayers and ion sensitive microelectrodes were used to detect water flux induced changes in solute concentration adjacent to these bilayers. The derived value of (4 ± 0.5) x 10^{-14} cm^3/subunit/s is in reasonable agreement with previously published data.

1932-Pos Dependence Of Single Water File Transport Rate On The Solvation Environment At The Channel Mouth

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According to atomistic molecular dynamics simulations, the loss of solvation forms the main energetic barrier for water permeation in gramicidin like channels . However, the subsequent conclusion about the invariance of transport rate on channel length is at odds with the experimentally found exponential increase in single channel water permeability as the channel shortens . The observed length dependence may be caused by

(i) water binding to specific accommodation sites in a fully occupied pore or
(ii) liquid vapour oscillations in the channel, i.e. reduced water density in the pore.

Whereas hypothesis

(i) predicts that the re- and dehydration of water in the entry/exit regions negligibly contribute to the total energy barrier, hypothesis
(ii) envisions that the transition step at the channel mouth and the channel permeation step contribute in a comparable fashion to the total permeation resistance.

We now have measured water permeability of gramicidin-A molecules bearing a hydrophobic group (tert-Butyl-diphenylsilyl) at the C-terminus by imposing an osmotic gradient across the hosting lipid bilayer and detected tiny concentration shifts of an impermeable solute in the immediate membrane vicinity. The lower transport rate of the derivative suggested a significant increase in water solvation energy. Exactly the same conclusion was drawn from single channel water permeability measurements carried out after the removal of charged lipid headgroups or the phosphate moieties of the lipids. Thus, both the solvation environment at the channel mouth and channel length are important determinants of single channel water permeability suggesting that liquid vapour oscillations reduce the density of water molecules in the channel.

Membrane Proteins - II

1933-Pos Gating of the Protein Translocation Channel SecY

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