negatively charged CTT of tubulin penetrates into VDAC pore, reaching through the channel at application from both sides of the membrane and interacting with high affinity with the positively charged channel lumen. We used the VDAC-tubulin specific interaction feature to probe orientation of VDAC in a planar membrane and extrapolate the results to the mitochondria outer membrane. We found that after *in vitro* phosphorylation by PKA or GSK3β cytosolic kinases the tubulin binding to VDAC (from rat liver mitochondria) becomes highly asymmetrical. When tubulin was added to the *cis* side of the membrane (side of VDAC addition) k_{on} was more than 10 times higher than with tubulin added to the *trans* side. Untreated VDAC interacts symmetrically with tubulin. Considering putative PKA and GSK3b phosphorylation sites on the cytosolic loops 3, 5 and 7, we conclude that these loops face *cis* side in the VDAC reconstitution system. Our preliminary data show that some of the specific antibodies raised against different VDAC peptides, compete with tubulin-VDAC binding when added to the membrane bathing solution and therefore could be employed to probe VDAC orientation and positioning of the loops. Recent VDAC three-dimensional structures are compared with the functional data of VDAC-tubulin binding.

1079-Plat

Electrostatic Properties of VDAC Channel: Structure Vs. Selectivity

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The measurement of ionic selectivity of large channels has been demonstrated as a useful tool for exploring their structure and their electrostatic properties. This is true even in channels whose crystal structure has been resolved down to a few angstroms by X-ray or NMR techniques. The comparison of structural data with measurements of channel conductance and reversal potential performed under a variety of experimental conditions adds valuable information about the role and/or position of key ionizable residues in the protein channel. The recently published 3D structure of the mitochondrial channel, VDAC, has challenged a large body of experimental evidence accumulated over the years about the channel functional properties. Here we analyze several experiments performed with VDAC (isolated from mitochondria of N. Crassa and rat liver) where the net charge of the channel has a key role. We discuss in detail whether measurements of selectivity, conductance, gating and tubulin-induced partial blocking of the channel are basically consistent with the same picture of the channel structural and electrostatic properties.

1080-Plat

Role of the N-Terminal Moiety in VDAC Isoforms

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VDAC or mitochondrial porin is the most abundant transmembrane protein in the mitochondrial outer membrane. Similarly to bacterial porins that are formed of a typical transmembrane β-barrel, the mitochondrial VDAC structure has been also found to form a β-barrel [1-3]. Interestingly the VDAC N-terminal 20 amino acids fold as an amphipathic α-helix [1-3]. We have synthesized the human VDAC1 (HVDAC1) N-terminal peptide Ac-AVPPTYADLGK-SARDVFTK-NH2 (Prn2-20) and determined its structure by CD and NMR. Our studies show that the Prn2-20 peptide exists as an unstructured peptide in aqueous solvent but it is structured in a hydrophobic environment [4]. Transfection of eukaryotic cells with recombinant humanVDAC1 devoid of N-terminal amino acids reduced the number of COS cells with depolarised mitochondria. These mutants showed an unaffected mitochondrial targeting [4].
Two more VDAC isoforms exist in mammals. 3D structural prediction showed that they have high probability to assume the same structure than VDAC1. N-terminal deletion experiments were performed in VDAC2 and VDAC3 and the effect of such N-terminal ablation was compared.
The overall picture emerging from our experiments is that the VDAC N-terminal peptide plays a role in the proper function of this protein during cell life events.
[1] Hiller et al (2008) Science 321, 1206; [2] Bayrhuber et al PNAS (2008) 105, 15370; [3] Ujwal et al (2008) 105, 17742; [4] De Pinto et al Chembiochem (2007) 8, 744.
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1081-Plat

Investigating VDAC1 Orientation *In Vivo*

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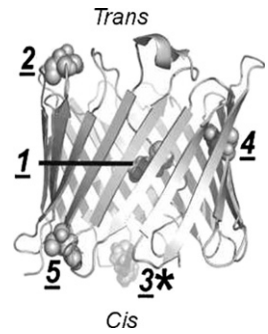
VDAC is an important and abundant ion channel in mitochondrial outer membranes but its structure remained unclear until recent structures of human VDAC1 revealed a 19 stranded β-barrel. Nevertheless there are still unresolved questions concerning the orientation of VDAC in the membrane and its fine structure. Starting from a bacterial OMP perspective we employ topology predictions based upon bacterial porins studies and orientation determination using measurements of antibody epitope exposure. We probe here the topology and orientation of yeast scVDAC1 *in vivo*. Five FLAG epitopes were independently inserted into scVDAC1 and their surface exposure in intact and disrupted mitochondria detected by immunoprecipitation. Functionality of the FLAG inserts was confirmed by measurements of respiration. Two epitopes suggest that scVDAC has its C terminus exposed to the cytoplasm whilst two others are more equivocal and, when combined with published data, suggest a dynamic behaviour.



References.

McDonald, B.M et al., (2009). *Febs Letters* **583**: 739–742.
Bayrhuber, M., et al., (2008) *PNAS* **105**: 15370–15375.
Hiller, S et al., (2008) *Science* **321**: 1206–1210.
Ujwal, R. et al., (2008) *PNAS* **105**: 17742–17747.

1082-Plat

Hard Constraints on the Structure of VDAC from Functional Studies

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Three different laboratories have solved the 3D structure of mammalian VDAC1 and obtained essentially the same result, a 19-stranded beta barrel. However, this structure is in serious conflict with published results of many experiments performed over the last 25 years. The 3D structures are of VDAC expressed in E. coli and refolded from inclusion bodies, and, despite the achievement of a uniform population of refolded protein, upon reconstitution into planar membranes, the conductances observed lack the characteristic electrophysiological properties of VDAC. It seems unlikely that the these properties of VDAC are an artifact and the refolded protein represents the native structure because these properties are highly conserved in at least one VDAC isoform from all species tested, from humans to potatoes to fungi to protists. Moreover, one single point mutation can drastically alter each of VDAC's characteristic properties: selectivity, steepness of voltage gating, and single-channel size. The strict evolutionary conservation compels the conclusion that the characteristic properties of VDAC are essential to optimal cell survival. Thus structural constraints deduced from studies on VDAC channels with these characteristic properties must be used as tests for any proposed structure of VDAC whether determined experimentally or theoretically. On this basis, the published 3D structures do not represent the native structure. (Supported by NSF grant: MCB-0641208)

Workshop 1: Applied Single-Molecule Techniques

1083-Wkshp

Single Molecule DNA Sequencing: from Demonstration to Application

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In recent years, single molecule DNA sequencing by cyclic synthesis has progressed from the demonstration stage [1] to a working system with high throughput DNA [2,3], cDNA [4], and direct RNA [5] unbiased sequencing capabilities. In this system, fluorescence microscopy is used to individually monitor tens of millions of immobilized DNA or RNA molecules for incorporation of labeled nucleotides. This process yields read lengths with sufficient sequence information to allow reliable and unique alignment of most tested fragments to a reference sequence, supporting a sequencing method that is amplification-free, fast and cheap. In this presentation, various aspects of single molecule sequencing by cyclic synthesis will be discussed. Low cost and high throughput DNA and RNA sequencing methods will usher in a new era of personal medicine.
1. Braslavsky I, Hebert B, Kartalov E, Quake SR: Sequence information can be obtained from single DNA molecules. Proceedings Of The National Academy Of Sciences Of The United States Of America 2003, 100:3960–3964.
2. Harris TD, Buzby PR, Babcock H, Beer E, Bowers J, Braslavsky I, Causey M, Colonell J, Dimeo J, Efcavitch JW, et al.: Single-molecule DNA sequencing of a viral genome. Science 2008, 320:106–109.

3. Pushkarev D, Neff NF, Quake SR: Single-molecule sequencing of an individual human genome. Nature Biotechnology 2009, 27:847–850.
4. Lipson D, Raz T, Kieu A, Jones DR, Giladi E, Thayer E, Thompson JF, Letovsky S, Milos P, Causey M: Quantification of the yeast transcriptome by single-molecule sequencing. Nature Biotechnology 2009, 27:652–658.
5. Ozsolak F, Platt AR, Jones DR, Reifenberger JG, Sass LE, McInerney P, Thompson JF, Bowers J, Jarosz M, Milos PM: Direct RNA sequencing. Nature 2009, advance online publication.

1084-Wkshp
Nano-Devices for Probing Single Molecules
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By confining molecules to 2-dimensional sheets or zero-dimensional wells, with nanometer-scale dimensions, one can observe individual fluorescently labeled molecules for long times, at high concentrations, and without surface attachment. We present two simple nano-devices that achieve these goals. We use these devices to obtain previously inaccessible information on molecular size, composition, and dynamics.
2-dimensional confinement is achieved near the point of contact between a convex lens and a planar coverslip. The lens-coverslip spacing varies smoothly from zero to many microns as the radial distance from the point of contact is increased. Commercial fused silica optics have surface roughness of approximately 1 nm, so one can select a vertical confinement with nanometer accuracy simply by imaging at a given radius from the point of contact. The lens-coverslip system allows: a) fluorescence imaging of immobilized single molecules in the presence of a micromolar concentration of diffusing fluorophores; b) long-time observations of freely diffusing single molecules in dilute solution, which further allows determination of diffusion coefficients, brightness, and spectral dynamics molecule-by-molecule; and c) direct mechanical measurement of the size distribution in a population of fluorescently labeled molecules.
Zero-dimensional confinement is achieved in nanometer-scale wells in a fused silica coverslip. A solution of fluorophores is washed over the wells, and then the bulk solution is replaced with a fluorinated oil. At most one molecule, or molecular complex, is immobilized in a nanoscale water droplet in each well. As with the lens-coverslip system, the dimple machine allows long-time observations of individual molecules, without surface attachment and in the presence of a high fluorescence background. By counting the number of photobleaching steps in each of several thousand chambers, we determine the *distribution* of stoichiometries in multimeric complexes.

1085-Wkshp
Physics and Engineering of Biological Molecular Motors
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No Abstract.

1086-Wkshp
Selectivity Mechanism of the Nuclear Pore Complex Characterized by Single Cargo Tracking
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The Nuclear Pore Complex (NPC) is the selective filter that facilitates all exchange between the cytoplasm and the nucleus in eukaryotic cells, allowing small molecules to passively diffuse through, while larger cargos require specific transport receptors to translocate. How NPCs achieve their exquisite selectivity remains unclear. We have developed a single molecule assay based on small (18 nm diameter) protein-functionalized Quantum Dots (QDs) for studying (with a mean spatial precision of 6 nm and a temporal resolution of 25 ms) the motion of single cargos as they approach, translocate, and exit the NPC. Optical tracking of single QD cargos reveals the individual steps involved in the import reaction. There is a size-selective cargo barrier in the cytoplasmic moiety of the central channel. The majority of QDs are rejected early rather than spending long times partitioned in the channel. Translocation is not governed by simple receptor-NPC binding interactions; rather, the central channel behaves in accordance with the 'selective phase' model. Finally, in the absence of Ran, cargos still explore the entire volume of the NPC, but have a dramatically reduced probability of exit into the nucleus, suggesting that NPC entry and exit steps are not equivalent and that the pore is functionally asymmetric to importing cargos. The overall selectivity of the NPC appears to arise from the cumulative action of a cascade of filters, only the last of which is irreversible.

1087-Wkshp
The Nano-Positioning System - A Fret-Based Tool for Macro-Molecular Structural Analysis
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Single-Pair Fluorescence Resonance Energy Transfer (FRET) experiments reveal structural and dynamic information about macro-molecules by monitoring the change in FRET efficiency between fluorescent dyes attached to a macromolecule. The Nano-Positioning System (NPS) developed recently [1] uses data from several of such experiments to infer the position of a dye attached to protein sites unresolved by x-ray crystallography.
While triangulation, the basic underlying principle, is not new and has already been reported in this or a similar context [2,3], the NPS applies probabilistic data analysis to the problem. That allows us to calculate the distribution of possible dye positions in a simple and objective way without relying on ad-hoc procedures, while at the same time we account for various error sources that usually accompany FRET measurements, for instance dye orientation effects. We have applied the NPS to determine the position of the nascent RNA [1] as well as to map the pathway of the non-template and upstream DNA in yeast RNA polymerase II elongation complexes [4].
[1] A. Muschielok, J. Andrecka, A. Jawhari, F. Brückner, P. Cramer & J. Michaelis, Nat. Meth. 5, 965–971 (2008).
[2] M. Margittai, J. Widengren, E. Schweinberger, G.F. Schroöder, S. Felekyan, E. Haustein, M. König, D. Fasshauer, H. Grubmüller, R. Jahn, and C. A. M. Seidel, PNAS 100, 15516–15521 (2003).
[3] J.L. Knight, V. Mekler, J. Mukhopadhyay, R.H. Ebright, and R.M. Levy, Biophys. J. 88, 925–938 (2005).
[4] J. Andrecka, B. Treutlein, M.A. Izquierdo Arcusa, A. Muschielok, R. Lewis; A.C.M. Cheung, P. Cramer, and J. Michaelis, NAR doi:10.1093/nar/gkp601 (2009).

Workshop 2: Complementary Methods for Studying Membrane Protein Structure

1088-Wkshp
The Role of Detergents and Lipids in Membrane Protein Crystallography
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No Abstract.

1089-Wkshp
NMR Structural Studies of Membrane Proteins in Lipid Micelles and Lipid Bilayers
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Integral membrane proteins regulate major cellular processes in health and disease, including transport, signaling, secretion, adhesion, pathogenesis, and apoptosis, and therefore, represent important targets for structural and functional characterization. Membrane protein structures and functions are regulated by their physical interactions with the surrounding lipids, and NMR is unique in its ability to provide high-resolution information in lipid environments that closely resemble the cellular membranes. Solid-state NMR experiments with proteins in oriented bilayers, and solution NMR experiments with proteins in weakly oriented micelles, provide high-resolution orientation-dependent restraints, which can be combined for protein structure determination and refinement. As previously observed for helical membrane proteins, the NMR spectra of outer membrane barrels in lipid bilayers exhibit characteristic patterns that reflect both protein structure and intra-membrane orientation. Results are presented for mammalian and bacterial α-helical and β-stranded membrane proteins. The NMR structures characterized in lipids provide insights to their distinct functions.
(This research was supported by the National Institutes of Health.)

1090-Wkshp
Using Circular Dichroism (CD) and Synchrotron Radiation Circular Dichroism (SRCD) Spectroscopy to Study Membrane Proteins
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Circular dichroism (CD) spectroscopy can provide valuable information on membrane protein structures, including determination of secondary structures of intact proteins and domains, detection of conformational changes associated with binding ligands and different functional states, examination of environmental effects and intermolecular interactions associated with complex