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Characterization of Prestin Oligomerization and Diffusion At the Single Molecule Level

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Prestin is the putative motor protein that drives outer hair cell electromotility. Several groups have shown evidence of prestin-prestin interactions and prestin oligomerization using biochemical techniques and optical imaging. Furthermore, studies have demonstrated that alterations of cholesterol in the cell membrane, which modify prestin function, affect membrane fluidity and prestin mobility. However, the role prestin-prestin interactions or oligomerization play in electromotility and the molecular motifs that mediate these interactions are unknown. It is also unknown whether the effects of cholesterol modifications on prestin function are due to changes in prestin mobility or changes in prestin interactions. Ensemble optical measurements of prestin self-association cannot provide the molecular level details of prestin-prestin interactions, nor can they distinguish the oligomeric states the interactions produce. Likewise fluorescence recovery after photobleaching cannot elucidate non-Brownian modes of diffusion which could indicate interactions with the cytoskeleton. We have thus developed our ability to detect individual prestin-citrine molecules using single molecule fluorescence (SMF) microscopy. We have applied SMF to measure the distribution of intensities emitted by individual prestin clusters in the HEK cell membrane. The distribution displays peaks spaced at multiples of the unitary intensity which one would expect for a distribution of non-interacting fluorescent emitters. We have resolved the stoichiometries up to tetramers, however the data makes clear that higher populations also exist. We have also explored the effect of membrane cholesterol depletion on the oligomerization of prestin using SMF microscopy to asses whether this treatment dissociates prestin oligomers or simply removes a bulk population of intact oligomers from microdomains. We will further utilize total internal reflection fluorescence microscopy and site-directed labeling to measure prestin diffusion at the single molecule level and explore the effects of cholesterol depletion and cytoskeletal inhibitors.

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Molecular Dynamics Simulations of the Rotary Motor F0 Under External Electric Fields Across the Membrane

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The membrane-bound component F_0, which is one major component of the F_0F_1-ATP synthase, works as a rotary motor and plays the central role of driving the F_1 component to synthesize ATP. We have conducted MD simulations of a b_2-free F_0 in the POPC lipid bilayer for tens of nanoseconds with two different protonation states of the key residue *c*Asp61 in the absence of electric fields and under electric fields of ± 0.03 V/nm across this membrane. Principal component analysis revealed that the motions of F_0 are clustered by the protonation state of Asp61. Analysis of the residue-pair Pearson correlation coefficient showed that electric fields reduced the correlated motion between the *a*-*c* subunits. When all Asp61 were protonated, the Ala24 and Ile28 of the N-terminal helix rotated clockwise about its axis by 30°. This rotation induced by electrostatic interaction between N-terminal helix and c_{12} unit may lead the conformational changes in the C-terminal helix which are important for the rotation of *c* ring. Hydrogen bonds networks analysis to the residues on three proton pathways is applied to see whether the hydrogen bonds formed between them. The intrinsic pK_a values of Asp61, Lys203 and Glu219 are supported by the experimental data. The deuterium order parameter (S_{CD}) profile calculated by averaging all the lipids was not very different from that of the pure bilayer, which agrees with recent ^{2}H solid-state NMR experiments (Kobayahi *et al*, Biophys. J., 94, 4339, 2008). However, by delineating the lipid properties according to their vicinity to F_0, we found S_{CD} and lateral diffusion of lipids followed a shell-dependent behavior. These findings may not only be useful to understand the dynamics of F_0, but shed some light to rationalize its extraordinary energy conversion efficiency.

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Mechanism of Targeting the a Kinase Anchoring Protein AKAP18δ To the Membrane

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Many proteins bind phospholipids too weakly to direct membrane association on their own. Localization studies nevertheless, reveal membrane anchoring. Membrane anchoring is furthermore considered to be crucial for their function. AKAP18δ, for example, is part of the signalling cascade which regulates the plasma membrane abundance of the water channel aquaporin-2. The cascade requires both proteins to colocalize in intracellular membranes. In contrast, membrane affinity appears to be rather low as suggested by high sequence homology to the preferentially cytoplasmic AKAP18γ, and the lack of palmitoylation or myristoylation sites which are believed to tailor the homologous proteins AKAP18α and AKAP18β to the membrane. Coincidence detection of a putative binding domain with large net positive charge to negatively charged lipids and specific recognition of a membrane anchored protein (e.g. phosphodiesterase PDE4D) may explain specific membrane targeting of AKAP18δ. Oligomerization of AKAP18δ would also result in an increased membrane affinity by providing several binding sites. To distinguish between both hypotheses, we monitored binding of purified wild type AKAP18δ and AKAP18δ fragments to planar lipid bilayers using fluorescence correlation spectroscopy. Protein binding to both charged and uncharged membranes did not require accessory proteins. Analysis of membrane diffusion constant revealed the existence of oligomers, confirming thereby the second hypothesis.

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The Electrostatics of VDAC: Implications in Selectivity and Gating

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Voltage-dependant anion channels (VDAC) are transmembrane proteins found in high abundance in the outer mitochondrial membrane of sacchromyces cerevisiae and all higher eukaryotes. VDAC is gated, or opened and closed, by changes in voltage across the membrane as well as pH, and it is thought to mediate the transfer of metabolites such as ATP, ADP, and NADH between the cytoplasm and intermembrane space. Apoptotic regulating proteins interact directly with VDAC to modulate the mitochondrial membrane potential and control the release of cytochrome c during apoptosis. Additionally, altered VDAC permeability has been associated with cancer and cardiovascular disease. Recently the x-ray crystal structure of VDAC from mouse (mVDAC1) was solved at 2.3 Å resolution making it possible to study the molecular workings of this channel in unprecedented detail. Our lab is using computational methods to explore the biophysical properties of the channel. I will discuss the results from continuum electrostatics calculations that show that the channel is selective for anions, which suggests that the x-ray structure is in the open state. This claim is buttressed by Poisson-Nernst-Planck (PNP) calculations that predict a high single channel conductance indicative of the open state. Furthermore, we performed in silico mutagenesis on residues shown to be involved in selectivity, and the changes in the calculated free energy profiles are consistent with experimental changes in selectivity. I will end by discussing how we used membrane potential calculations to rule out a proposed gating mechanism of the channel.

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Mapping the SecA-SecY Interaction Interface Using in Vivo Photocrosslinking

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In *Escherichia coli* (*E. coli*) the major secretory pathway for proteins is the ''general secretion pathway'' or ''Sec-dependent pathway''. Its major components include the SecA ATPase, the SecB chaperone, and SecYEG: the channel-forming complex. Once bound to SecYEG, the preprotein substrate, and ATP, SecA undergoes an ATP-driven conformational cycle that drives the step-wise translocation of proteins. While cytosolic SecA specifically recognizes and binds SecYEG, the specific residues by which these two proteins interact are not known. A study from Mori and Ito (2006) analyzed SecY-SecA interaction by using an in vivo site-directed cross-linking technique developed by Schultz and co-workers. In this experimental system, an *amber* suppressor tRNA and a tyrosyl-tRNA synthetase from *Methanococcus jannaschii* are genetically engineered to allow for the charging of the tRNA with *p*-benzoyl-phenylalanine (pBpa), a photo-reactive phenylalanine derivative. They showed that pBpa residues introduced into the second, fourth, fifth, and sixth cytoplasmic domains of SecY could be crosslinked to SecA.

We have chosen a similar approach to Mori and Ito, but we have designed sites of in vivo pBpa incorporation into SecA based on an in vivo SecA membrane topology study performed by Jilaveanu et al. (2006). The amber positions in SecA were chosen based on residues of SecA that were accessible to small molecule labeling from the exterior side of the membrane-indicating proximity to the SecYEG channel. Our results demarcate novel sites of SecA interaction with SecY. Our data provide in vivo support for the biological relevance of the recent SecA-SecYEG X-ray structure (Zimmer et al., 2008), but they also

demonstrate the importance of both the SecA N-domain as well as its C-domain in stabilizing the interaction in vivo.

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Increasing Chloride Conductance Through the SecY Complex By Mutagenesis or Trivalent Cations

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The SecY complex or channel is responsible for translocation of proteins across the bacterial inner membrane. The complex maintains a seal for small molecules by means of a plug domain and a hydrophobic pore, consisting of six isoleucine residues arranged in a ring. When these pore residues are mutated into asparagine, a specific conductance for small monovalent anions, like chloride, is observed. Here, we show that an enhanced chloride conductance is also observed when bulky phenylalanine residues are introduced into the pore ring. The increased conductance is accompanied by an increase in protein translocation. Chloride conductance was also observed upon addition of trivalent aluminum cations, which are suspected of binding to negatively charged residues near the lateral gate of SecY.

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Stability of Vesicles With Incorporated Aquaporin Z Under Various Physiochemical Conditions

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Stability of Vesicles with Incorporated Aquaporin Z Under Various Physiochemical Conditions

Aquaporins are integral membrane proteins that can transport large amounts of water molecules across the cell membrane by facilitated transport. One aquaporin can transport over 3 billion water molecules per second. Among many different types of aquaporins, we are using *E. coli* Aquaporin Z that is known as a highly specific water channel. This prokaryotic origin aquaporin is easy to modify genetically and it is possible to produce it in large amounts. By means of nanobiotechnology we attempt to develop aquaporin-incorporated membranes that can act as energy efficient water filtration membranes. To fabricate the aquaporin incorporated filters, it is important to characterize the aquaporin endurance against various chemical and physical conditions, in order to know what type of nanofabrication technologies can be used in the final membrane production. In our membrane concept, aquaporin incorporated vesicles are the main building blocks because thermodynamically vesicles are stable and aquaporin retains its functionality when incorporated into vesicles. In this research, we have tested aquaporin-incorporated vesicle functionality with respect to various physiochemical influences such as pH, high temperature, long time UV exposure, high pressure, oxidative stress and several solvents at various concentrations. Subsequently, we have characterized the effect of the applied physiochemical conditions by stopped flow light scatter (SFLS) to check if the water permeability of the vesicles was changed or killed by the various treatments.

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Atomistic Model For the Outward-Facing State of Lactose Permease and Quantification of Ligand Binding

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The Major Facilitator Superfamily (MFS) is an important class of membrane transporters whose members are found in unicellular organism to complex systems like the human central nervous system. Lactose Permease (LacY) of E. coli, an MFS protein that transports various sugar molecules across plasma membrane, has been studied as a prototype of secondary transport proteins. Though the structure of LacY in the cytoplasmic open state (inward-facing) has been determined, the atomic-level details of the periplasmic open structure (outward-facing) are unknown. Using a two-step hybrid simulation approach that involves Self-Guided Langevin Dynamics (SGLD) simulations with an implicit membrane followed by molecular dynamics simulations with an explicit membrane, we have obtained an opening of LacY on the periplasmic side that is consistent with the Double Electron-Electron Resonance (DEER) experiments (Smirnova et al., PNAS, 2007). The inward-facing state is stabilized by several helix-helix hydrogen bonds involving side chains of the residues N245, S41, E374, K42 and Q242 and mutations in these residues may destabilize these interactions and help crystallize the outward-facing state. We believe that this hybrid simulation approach can be extended to determine the unknown structures and mechanisms of other secondary active transporters. In addition, sugar binding to LacY has been investigated and our simulations support the alternating access model of sugar transport (same binding site accessible from either side of the membrane). More extensive studies on binding involved alchemical free energy perturbation calculations on αβ-(Galp)2 and ββ-(Galp)2 to better understand the anomeric binding effect. Our methods are tested by comparing the computed binding free energy values for NPG (p-nitrophenyl α-D-galactopyranoside) with the experimental values (Nie et al., JBC, 2006). This work will aid in understanding the effect of substrate structure and affinity to LacY.

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Single Molecule Studies of *E-ColiF$_1$F$_o$ ATP Synthase* in Lipid Bilayers

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While rotation of *F_1 ATPSynthase* has been well characterised at the single molecule level over the past twelve years, direct observation of coupled rotation of *F_1F_o ATP Synthase* in energised lipid bilayers is still fraught with difficulties. One of the biggest challenges is the creation and preservation of a stable and energisable lipid bilayer, so that the proteins can be inserted and remain functional in its native environment. Such a setup must also be coupled with a high resolution microscopy technique, in order to allow direct single molecule observations. In this study, we report the use of the *droplet-on-hydrated-support bilayers* (*DHBs*) technique, by Heron et. al. , to observe both fluorescence labelled and gold labelled *EColiF$_1$F$_o$ATPase*, which have been inserted into the lipid bilayer. This was done using *Total Internal Reflection Microsopy* (*TIRF*) and a novel Dark Field Microscopy setup. The 2-dimensional diffusional constant of the protein ensemble in the lipid bilayer was characterised by tracking the position of the proteins over time. Furthermore, we have also attached the F_o portion of the protein to a functionalised substrate sitting below the bilayer and thereby inhibiting its movement. The F_1 portion is, in turn, labelled with gold beads and free to rotate above the bilayer. This configuration would allow the F_o to behave as the stator while the F_1 as the rotor, so that coupled rotation can be observed. The next part of this on going work would thus be to provide either ATP or a proton motive force to drive the rotation of the protein in hydrolysis and synthesis mode. We hope to report these findings in the coming conference.

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Exploring Conformational Changes in the RbsABC Transporter Using EPR Spin Labeling

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ATP Binding Cassette (ABC) transporters are transmembrane transporters that use the energy released by ATP hydrolysis to transport a wide array of substrates. They are found in all kingdoms of life, and are complicit in various genetic conditions, such as cystic fibrosis, macular degeneration, and multi-drug resistance. The E. coli ribose transporter (RbsABC) is a multisubunit ABC transporter complex with a periplasmic ribose binding domain, a transmembrane domain dimer, and a cytoplasmic nucleotide binding domain. The ribose transport complex has been shown to assemble and disassemble into distinct combinations of the subunits based on the presence of cofactors (ATP and analogues, ADP, orthovanadate, and magnesium), suggesting a series of steps for how the subunits associate and subsequently transport ribose.

To further explore the conformation of the subunits in response to these different sets of cofactors, cysteine mutations were introduced to allow the addition of EPR spin labels. These labeled mutants will be used to determine whether subunits are bound. Additionally, double mutants will be used to elucidate conformational state of subunits.

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Investigation of the Mobile Regions of GM2 Activator Protein Using Continuous Wave and Pulsed Electron Paramagnetic Resonance and Fluorescence Spectroscopy

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GM2AP is an 18kDa protein that is involved in the catabolism of the ganglioside GM2. GM2AP is thought to bind GM2 in intralysosomal vesicles and present the oligosaccharide head group for hydrolytic cleavage. Mutations in GM2AP lead to an accumulation of GM2 in the lysosomes, causing the lysosomal storage diseases Tay Sachs or the AB variant of Sandhoff's disease. The crystal structure of GM2AP revealed the protein in five different crystal forms, with large differences in the diameter and area of the opening to the lipid binding cavity differences can be attributed to the flexibility of the mobile loops. Site directed spin-labeling combined with continuous wave and pulsed electron paramagnetic resonance methods has been used to investigate the intramolecular distances between the mobile loop regions of GM2AP. Distance profiles are obtained with and without the physiological ligand GM2 as other phospholipids.