dramatically upon the introduction of mutations that bias EmrE orientation. While early mutations, especially those surrounding the first transmembrane helix, appear capable of biasing the initial orientation, the effects of later mutations can be explained by altering the rate of misfolding events in accordance with the positive-inside rule. Positive charges thus appear to act as folding signals, enhancing proper folding and insertion when positioned in agreement with the positive-inside rule, and enhancing misfolding when placed in violation.

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Developing a Universal Steric Trapping Strategy for Studying Folding and Stability of Helical Membrane Proteins

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"Steric trapping" is a method that links binding of monovalent streptavidin (mSA) to unfolding of a biotinylated protein (MP). It allowed the measurements of high affinity protein–protein interactions and thermodynamic stability of polytopic helical MPs in a native environment, which had been difficult to achieve using more conventional methods. In the current steric trapping framework, a conformation-sensitive chromophore or an enzymatic activity in a target MP is required for monitoring mSA-induced unfolding. This target-specific approach is a limiting factor that hinders its application to various MP systems, *i.e.* MPs in a misfolded conformation, MPs with an assembly role, or MPs without convenient unfolding readout. To further advance the steric trapping strategy for more general application, we have developed novel tripartite probes possessing a thiol-reactive group, a biotin group and a fluorescent or paramagnetic group for sensitization of unfolding or binding of mSA. We applied the new strategy to investigating the stability and unfolding mechanism of an intramembrane protease GlpG. By combining FRET between fluorescently labeled GlpG and quencher-labeled mSA as a measure of mSA binding and the proteolytic activity of GlpG as a measure of unfolding, we proved the thermodynamic coupling between binding and unfolding, and determined the thermodynamic stability and unfolding rate of GlpG in a native micellar environment. While the stabilities were similar independent of the location of biotin pairs, the unfolding was 20 times faster when the biotin pair was placed near the proteolytic active site. This result suggests a subdomain-organization of the helical bundle architecture of GlpG. Steric trapping may serve as a useful tool for elucidating the local versus global flexibility of helical MPs.

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Studying Membrane Protein Folding by Molecular Dynamics Simulations

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Membrane protein folding is a process of fundamental importance in biophysics and structural biology. Despite major advances in our understanding of translocon-mediated insertion of membrane proteins, many aspects of the underlying biophysics remain unclear.

Computational methods provide a powerful tool for understanding membrane protein folding. As an initial model for a translocon tunnel, we are using a simplified pore system to investigate systematically the effects of this on helix formation. This approach is being used in parallel with enhanced sampling methods to accelerate the folding reaction. As test systems we have focused on two membrane proteins: the influenza M2 channel protein which contains a single transmembrane (TM helix plus a C-terminal amphipathic helix, and the seven TM helix protein bacteriorhodopsin, For bacteriorhodopsin we can successfully fold all seven helices inside our simple model of a translocon, starting from a fully extended conformation. For influenza M2, we observe the two helices (TM and amphipathic) folding to a state that is consistent with NMR structures.

We wish to develop this model further to include the crucial step of helix insertion into the membrane. In this context we are also exploring coarse-grained approaches to help us address time-scales accessible to simulations.

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Identifying the Oligomerization State of DegP in the Absence and Presence of Substrate

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Bacterial outer membrane proteins (OMPs) are synthesized in the cytoplasm, cross the inner membrane, and are then transported across the periplasm before folding into the outer membrane. Similar pathways are present in both mitochondria and chloroplasts. While unfolded outer membrane proteins (uOMPs) are prone to aggregation, they are able to avoid this off pathway reaction with the help of periplasmic chaperones and proteases. The primary protease that interacts with uOMPs is DegP. DegP is a member of the high temperature requirement A (HTRA) protease family and has been implicated in both the Cpx and σ^E stress response pathways. DegP sequesters and degrades uOMPS when they accumulate in the periplasm. It has been suggested that DegP functions by transitioning from an inactive hexameric state to an active cage-like oligomeric state of either 12 or 24 subunits when substrate is present. In order to further investigate the relationship between the oligomeric state of DegP and the presence of substrate, we performed sedimentation velocity experiments with and without various uOMPs. This allows us to identify the oligomeric populations of DegP at biological concentrations. Results suggest that DegP has significant populations of multiple oligomeric states even in the absence of substrate. This work contributes to our understanding of OMP biogenesis by identifying the nature of uOMP interactions with an important pathway component.

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Theoretical Prediction of Mutations Improving Thermal Stability of Adenosine A2a Receptor

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G protein-coupled receptors (GPCRs) are physiologically important membrane proteins possessing seven transmembrane domains, which are responsible for signal transduction pathways. Therefore, they form the most important target for drug design. However, their mass production and structure determination by the X-ray crystallography are quite difficult to achieve due to the low thermal stability in detergents. Though the stability can be enhanced by introducing mutations into GPCRs, a random search accompanying a heavy experimental burden is currently employed to obtain mutations leading to sufficient enhancement. In the present study, through mutations for the antagonist-binding structure of the adenosine A2a receptor, we investigate how to predict the mutants which lead to enhanced thermal stability using our free-energy function (FEF) recently developed for membrane proteins. The FEF comprises two components: the energetic term, which is focused on the energy decrease arising from formation of intramolecular hydrogen bonds, and the entropic term, which originates from the translational displacement of hydrocarbon groups constituting nonpolar chains of the lipid bilayer. After calculations of the FEF for all mutants, we have chosen some candidate mutants whose thermal stability would be most improved, and then their stabilities are experimentally examined. The findings are as follows. The success rate of the prediction focused on the entropic term alone is about 1/3 that is much higher than that reached by the trial-and-error prediction. This result implies that the entropic effect of hydrocarbon groups is critical for the structural stability of GPCRs. Moreover, when the energetic term is also considered, the success rate is improved to 1/2. Since the calculation of the FEF can be accomplished quite rapidly, we can theoretically examine a large number of mutations.

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Bioinformatic Methods for the Rapid Identification of Thermostabilizing Mutants

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Membrane proteins are often of particular difficulty to study, frequently limited by stability in detergent solution. Taking advantage of the available genomic and cell culture data, presented are two bioinformatic methods to quickly identify a limited set of amino acids or positions which likely underlie the thermal adaptation of a given protein family. This set then provides a small number of mutants to screen for stabilization and are used to demonstrate a significant increase in thermostability of an example membrane protein.

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Interaction of the Phage Endolysin PlyC with Model Membranes

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Endolysins are bacteriophage-encoded peptidoglycan hydrolases expressed during the late stages of a phage replication cycle that function to lyse the

bacterial cell wall, thus enabling progeny phage release. When exogenously added, these enzymes lyse the peptidoglycan of Gram-positive pathogens, resulting in osmotic lysis and cell death. One particular endolysin, PlyC, has a distinctive ability to translocate eukaryotic membranes and retain killing activity in the intracellular environment, making it a particularly interesting target molecule for the development of novel therapeutic approaches. However, the protein-membrane interactions and the cell penetration processes remain unknown. Here, we investigate molecular-scale aspects of such membrane interactions using sparsely-tethered lipid bilayer membranes (stBLMs), a robust planar biomimetic lipid membrane model.

Applying complementary surface-sensitive techniques such as surface plasmon resonance, electrochemical impedance and neutron reflectometry, we demonstrate the first steps towards a mechanistic understanding of how the PlyC binding domain, PlyCB, initiates membrane translocation. Our data reveals that while the interaction of PlyCB with purely zwitterionic membranes is negligible, the protein strongly interacts with anionic membranes that contain phosphatidylserine (PS) above a well-defined concentration threshold. In contrast, PlyCB affinity for other anionic lipids tested is low, suggesting specificity for PS rather than non-specific ionic interactions. Furthermore, the PlyCB point mutant R66E that lacks the ability to translocate membranes has likewise lost affinity for PS. With Neutron reflection we identified two distinct PlyCB membrane-association modes. Depending on PS membrane concentration, PlyCB is either peripherally associated or membrane-spanning. Because the outer leaflet of eukaryotic membranes is largely zwitterionic, our findings imply that PlyC induces and/or recognizes PS exposure during cellular uptake. In addition, these results show how lipid membrane surface charge density and composition play a critical role for PlyC internalization.

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Structural Basis of Phosphoinositide (PIP) Recognition by the TIRAP PIP-Binding Motif

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Toll-like receptors (TLRs) provide early immune system recognition and response to infection. TLRs activated by pathogens consequentially initiate a cytoplasmic signaling cascade though adaptor proteins, the first being the modular TIR domain-containing adaptor protein (TIRAP). TIRAP contains a C-terminal TIR domain, which is responsible for association with TLRs and other adaptors including the myeloid differentiation primary response gene88 (MyD88) protein. Membrane recruitment of TIRAP is mediated by its N-terminal PIP-binding motif (PBM). Upon ligand-mediated activation, TLRs are recruited to the PIP-enriched regions where TIRAP resides. At these sites, TIRAP recruits MyD88 to the membrane by bridging MyD88 to activate the TLR signaling pathway. To understand the mechanism of membrane targeting of TIRAP and the basis for its regulation, we functionally and structurally characterized its PBM using experimental and computational studies. TIRAP PBM adopts a folded conformation in membrane mimics, such as dodecylphosphocholine micelles, and binds PIPs. Structural rearrangements of TIRAP PBM were influenced by membrane interaction, with monodispersed PIPs inducing helical structure in the peptide. In contrast, monodispersed phosphatidylinositol and inositol trisphosphate did not promote structural changes in TIRAP PBM. NMR spectra reveal that TIRAP PBM binds PIPs in a fast exchange regime with a moderate affinity through two conserved basic regions. Solution NMR structure of TIRAP PBM shows a central short helix, and paramagnetic studies indicate that this region is close to the micelle core. Molecular dynamics simulations studies indicated that TIRAP PBM diffused to and interacted with a model membrane composed of palmitoyl oleoyl phosphatidylcholine and phosphatidylinositol 4,5-bisphosphate. Thus, we propose that two sets of basic residues contact both the head group and acyl chains of PIPs, whereas the central helix is responsible for membrane insertion.

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Mechanism of Action of Salt Adaptation Mutations in Artemia Franciscana

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Nearly all animals maintain a large electrochemical gradient for Na^+ across the plasma membrane. This gradient is generated by the Na-K pump, which exports 3 Na^+ and imports 2 K^+ per ATP molecule hydrolyzed. Ion-coordinating residues in the α subunit are usually conserved, but the brine shrimp (Artemia franciscana) living in extreme saline conditions express a pump with two asparagine to lysine substitutions within the ion binding site region (Jorgensen and Amat (2008) J. Memb Biol. 221:39-49). We used two-electrode voltage clamp on Na^+-loaded Xenopus oocytes to evaluate the effect of the substitutions (N333K and N785K) individually and concurrently on the function of Xenopus Na/K pumps. We studied their effect on activation of pump currents by eternal K^+ and on voltage-dependent conformational changes related to external Na^+ binding (charge movement). The center of the Q-V curves are displaced by ~-80 mV by both individual mutations suggesting a reduced (>10 fold) external Na^+ affinity. Surprisingly the double mutant showed a nearly identical shift in the Q-V, indicating non-additive effects on external Na^+ affinity. Apparent affinity for K^+ in the absence of Na^+ was reduced (~10-fold) by the N785K mutation while N333K and the double mutant had similar affinity to the wild type. These results can be explained with recent structures of the Na/K pump with Na^+ or K^+ bound. N333, outside the ion-binding pocket, forms a hydrogen bond with ion-coordinating N785 in the Na^+ bound conformation. Once the disruption of normal Na^+ coordination by N785K is in place the mutation N333K does not affect Na^+ binding. This contrasts with previous findings regarding internal Na^+ binding. We are investigating intracellular ion dependence in these mutants by measuring currents in patch clamp and enzymatic activity in membrane preparations. Supported by NSF-MCB-1243842 & NIH-NS081570-01.

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Determining Oligomeric Order of a Membrane Protein by Double Electron-Electron Resonance Spectroscopy

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Many different classes of membrane proteins are known to form oligomers in cellular membranes in order to carry out specific cellular functions. Detection and detailed structural characterization of protein oligomers in lipid milieu is by no means a trivial task. Here we demonstrate the use of spin-labeling and Double Electron-Electron Resonance (DEER) spectroscopy to determine the oligomeric order of a membrane protein. Specifically, we investigate oligomerization of a seven-helical membrane photoreceptor Anabaena Sensory Rhodopsin (ASR) from Anabaena sp. PCC7120. Recently, ASR structure has been solved by both x-ray protein crystallography (Science 2004, 306, 1390) and solid-state NMR (Nat Methods 2013, 10, 1007). Here we show that the same spin-labeling sites we employed for paramagnetic relaxation enhancement (PRE) NMR can also be used for DEER experiments. The results demonstrate that DEER restraints can not only differentiate between the dimer (x-ray) and trimer (ssNMR) models that have very different interfaces, but further rule out hypothetical tetramer and other higher order polygon models. The crux of our DEER-based approach relies on taking advantage of the multi-spin effects and analyzing experimental DEER traces by direct fitting to the multispin models. Overall, the observed profound effect of higher order spin correlations on the DEER trace allows for a reliable differentiation between oligomer models. In the specific case of ASR, the DEER trace modeling allowed us to unambiguously discard all but the trimer model. Furthermore, the addition of DEER electron-electron distances to the NMR restraints in the structure calculation protocol improves local RMSD, and allows for refinement of the orientation of helices. Supported by U.S. DOE Contract DE-FG02-02ER15354 to AIS and NSERC Discovery Grants RGPIN-2014-04547 to VL and RGPIN-2013-250202 to LSB.

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Nucleotide-Dependent Membrane Interaction and Dimerization of K-Ras4B

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