We present studies of interactions between proteins of the efflux pump of Pseudomonas aeruginosa: MexB (the rotor of the pump connecting the interior of the bacterium and the periplasm), OprM (a pipe connecting the periplasm to the outside of the bacterium) and perplasmic MexA. The mode of interaction, the size of protein complexes and their stoichiometry were determined. In particular we show that MexA and OprM interact only if they are incorporated in opposite bilayers. The population of this complex reaches its maximum when the bilayers are separated by a distance of about 200 A, which is the thickness of the Pseudomonas aeruginosa periplasm. The stoichiometry of the above complex will be presented as well as results describing Mexa-MexB and MexB-OprM associations.
We will thus demonstrate the versatility of our system, which is well suited to study the associations of membrane proteins in a biologically relevant environment.

265-Pos
Studying Membrane Protein Thermodynamics Using a Steric Trap
Tracy M. Blois, Heedeok Hong, Tae H. Kim, James U. Bowie.
University of California, Los Angeles, Los Angeles, CA, USA.
An understanding of the molecular forces that specify a protein's structure is essential for many of the central quests of structural biology. While there has been a larger effort to understand soluble protein folding, we know very little about membrane protein folding energetics. We are developing a novel method to study the forces that stabilize membrane proteins in lipid bilayers, which we termed the 'Steric Trap method.' The steric trap method couples protein unfolding to a measurable binding event by exploiting steric repulsion and the high affinity of the streptavidin/biotin interaction. To do this, we introduce two biotin tags on a target protein that are close in space and employ monovalent streptavidin (mSA) as our steric trap. A single mSA can bind without steric hindrance to the folded protein, but a second mSA can only bind when the protein unfolds due to steric overlap. Thus, the binding affinity of the second streptavidin provides a measure of unfolding free energy because binding is coupled to unfolding. We have developed this method on the water-soluble protein, dihydrofolate reductase (DHFR). When two biotin-labeling sites were rationally designed to be close to one another in space and near the enzyme active site of DHFR, enzymatic activity was reversibly abolished upon incubation with a molar excess of monovalent streptavidin. Incubation with a stabilizing ligand shifted streptavidin binding curves, confirming that the steric trapping can quantitatively detect changes in protein stability. Results on our application of the steric trap method to membrane proteins will be presented. The steric trap method could be a powerful tool for measuring protein association affinities, studying unfolding energetics and investigating membrane protein unfolded states in the context of membrane environments.

266-Pos
Measuring the Thermodynamic Stability of Strong Protein-Protein Interactions in Lipid Bilayers Using a Steric Trap
Heedeok Hong, Tracy M. Blois, James U. Bowie.
University of California, Los Angeles, Los Angeles, CA, USA.
Protein-protein interactions within cell membranes play crucial roles in the assembly of membrane proteins and cell signaling. Although thermodynamic analysis of binding affinity is essential for understanding the stability, specificity, and function of membrane proteins, these measurements can be difficult to make for high affinity interactions in lipid bilayers. To address this problem, we have developed a steric trap method, which couples the dissociation of a membrane protein complex to another measurable binding event. The method postulates that a concomitant binding of two bulky monovalent streptavidins (mSA) to a doubly biotinylated protein complex occur only when the protein is dissociated due to the steric hindrance. This leads to an attenuated binding affinity of the second mSA, which is directly correlated to the stability of a target interaction. We tested the method using a glycophorinA transmembrane domain fusion to staphylococcal nuclease (SNGpA), which forms a stable dimer in various lipid environments. Equilibrium binding of mSA to the enzymatically biotinylated SNGpA exhibited two distinctive phases, which corresponds to the tight first mSA binding and the weaker second binding in decyl maltoside (DM) micelles and palmitoyloleoyl phophatidylcholine (POPC) bilayers. The stability of GpA dimer extracted from the second binding event at different micellar concentrations yielded the dissociation constants (K_d) of 10^{-8}~10^{-7} M, which agree well with the previous results. The stability of GpA dimer is enhanced in POPC bilayers by ~4 orders of magnitude at comparable mole fractions. The difference free energies between wild-type and destabilized mutants in both systems correlate with the equilibrium sedimentation data measured in C_8E_5 micelles. Our results suggest that the steric trap method provides a powerful tool to study the strong protein-protein interactions in lipid bilayers.

267-Pos
Monitoring and Optimizing Detergent Concentration For Membrane Protein Crystallization While Following Protein Homogeneity
Larry J.W. Miercke[1], Rebecca A. Robbins[1], Mimi Ho[1], Andrew Sandstrom[2], Rachel K. Bond[1], Robert M. Stroud[1].
[1]UCSF, San Francisco, CA, USA, [2]University of Chicago, Chicago, IL, USA.
Detergent concentration is critical to growing quality membrane protein crystals. Since the optimal detergent concentration lies just below the detergent phase boundary, the starting detergent concentration must be minimized for initial crystallization trials. However, the majority of Pure, Homogenous and Stable targets contain excessive levels of detergent micelles upon concentration using molecular weight cut-off filters. Size Exclusion Chromatography (SEC) and a Tetra Detector Array (refractometer, viscometer, light scattering, and UV detectors; TDA) are now being successfully utilized to monitor and optimize detergent concentration while assaying PDC homogeneity during purification and concentration for crystallization. In doing so, the oligomeric state, size, shape and the detergent:protein ratio of the Protein Detergent Complex (PDC) is measured.
Five different membrane proteins using 3 different detergents (OG, DDM, and FC14) and 4 different methods will be presented where detergent was successfully minimized while maintaining PDC homogeneity. Methods utilized where ultra filtration (centrifugal and high-pressure molecular weight cut-off filters) plus SEC and dialysis, changing detergent isomer, Ni-NTA and ion exchange chromatography.
Detergent micelle SEC retention volume, dn/dc, Rh, IV, mass and behavior on different molecular cut-off filters and formats are all being measured using TDA. As expected, there is a direct correlation of measured excess micelle concentration to crystal phase separation and diffraction quality. Unexpectedly, free micelles in the presence of PDCs tend to be highly retained on cut-off filters which would freely pass a pure detergent micelle system; therefore, when measuring whether a micelle is retained or passed through by specific molecular cut-off filters and formats, it must be measured using a PDC system and not just buffered detergent controls.

268-Pos
A Semi-Quantitative Analysis of Detergent Exchange For Integral Membrane Proteins
Zexuan Li, Fang Sun, Yue Hu, **Yufeng Zhou**.
Yale University Medical School, New Haven, CT, USA.
Biophysical and biochemical studies of membrane proteins often require the protein to be analyzed in a detergent different from the one used for purification. Detergent exchanges are often achieved through size exclusion chromatography. Despite the widespread usage of this approach, it is not clear how one can determine the exchange efficiency, and how two different detergents interact during the chromatography process. Here we seek to semi-quantitatively analyze the process of detergent exchange using thin layer chromatography. We choose a bacterial potassium channel KcsA as our model protein, and studied the exchange of this protein in various non-ionic and zwitterionic detergents.

269-Pos
Biochemical Definition of 'harsh' Vs. 'mild' Detergents For Membrane Protein Solubilization
Vincent G. Nadeau[1,2], Arianna Rath[1], Charles M. Deber[1,2].
[1]Hospital for Sick Children, Toronto, ON, Canada, [2]Department of Biochemistry, University of Toronto, Toronto, ON, Canada.
Selection of a solubilizing detergent for membrane proteins is typically based on its ability to maintain the native structure and/or function of the molecule of interest. Descriptors of detergents as 'harsh' or 'mild' in terms of their propensity to denature membrane protein structures may act as a qualitative guide to this process, but the basis of the variable effects of detergents on native membrane protein folds is not yet fully explained. Previous work by our group suggested that the ability of the 'harsh' detergent sodium dodecylsulfate (SDS) to denature a series of wild-type and mutant model helical membrane-soluble 'hairpin' (helix-loop-helix) proteins depends on their level of detergent binding, as manifested by significant variability in their electrophoretic mobilities on SDS-PAGE, and in circular dichroism (CD) spectra and hydrodynamic radii [Rath, Nadeau et al., *PNAS USA* 106, 1760-65 (2009)]. Here we have extended this work to the characterization of a corresponding library of hairpin proteins in sodium perfluorooctanoate (SPFO), a surfactant considered to be non-denaturing. The 'hairpin' library has been developed from helix-loop-helix constructs from transmembrane segments 3 and 4 from the cystic fibrosis transmembrane conductance regulator (CFTR). We find that SPFO-solubilized 'hairpins' exhibit significantly less variability vs. their SDS-solubilized counterparts in their electrophoretic mobilities, CD spectra, and hydrodynamic radii. In addition, SPFO favours more stable helical structure while binding hairpins

in larger amounts than does SDS across hairpin sequences. Pyrene excimer fluorescence data indicate that SPFO additionally retains more tertiary contacts vs. SDS for all hairpin sequences tested. Solubilization in SPFO therefore appears to favour protein-protein over lipid-protein interactions. Our overall results imply that the 'harshness' of a detergent is proportional to its protein sequence specificity upon binding, and consequent tendency not to disrupt intra-protein contacts during micelle formation.

Membrane Protein Function I

270-Pos

Anion Translocation in a Brush-Like Nanopore: Simulations of the Outer Membrnae Protein OprP

Prapasiri Pongprayoon, Oliver Beckstein, Chze Ling Wee, Mark Sansom.
University of Oxford, Oxford, United Kingdom.

The outer membrane protein OprP from *Pseudomonas aeruginosa* forms an anion-selective pore, especially selective for phosphate ions. The protein is homotrimeric, with each pore lined by three positively charged loops (L3, L5, and T7) folded into its lumen. OprP plays a key role in high-affinity phosphate uptake under the condition of phosphate starvation. To better understand the mechanism of phosphate-selective permeation, we employed three simulation techniques: (i) equilibrium molecular dynamics simulations (MD); (ii) steered MD (SMD); (iii) umbrella sampling to calculate a potential of mean force (PMF) for phosphate and chloride ions. The PMFs reveal a deep energy well midway along the OprP channel. Two adjacent phosphate-binding sites (W1 and W2), each with a well depth of ~8kT, are identified close to the L3 loop in the most constricted region of the pore. The transfer of phosphate between sites W1 and W2 is correlated with changes in conformation of the sidechain of K121, which serves as a 'charged brush' to facilitate phosphate passage between the two subsites. The PMF for chloride has also been computed and can be compared with that of phosphate. Our simulations suggest that OprP does not conform to the conventional picture of a channel with a relatively flat energy landscape for permeant ions, but rather resembles a membrane-inserted binding protein with a high specificity that allows access to a centrally located binding site from both the extracellular and the periplasmic spaces.

271-Pos

Regulation of Channel Function Due To Coupling With a Lipid Bilayer

Md. Ashrafuzzaman[1], J. Tuszynski[1,2].
[1]Cross Cancer Institute, University of Alberta, Edmonton, AB, Canada,
[2]Department of Physics, University of Alberta, Edmonton, AB, Canada.

Regulation of membrane protein functions due to hydrophobic coupling with lipid bilayer is investigated. Binding energy between lipid bilayer and integral ion channel with different structures has been calculated considering 0th or 1st, 2nd, etc. order terms in the expansion of the screened Coulomb interaction V_{sc}(r)=integral of $d^3kExp\{i\mathbf{k}.\mathbf{r}\}V_{sc}(\mathbf{k})$ with V_{sc}(r) being the inverse Fourier transformation of the screened Coulomb interaction in Fourier space $V_{sc}(\mathbf{k})=V(\mathbf{k})(1+f(n,T)V(\mathbf{k}))^{-1}$ for bilayer thickness (d_0) channel length (l) mismatch (d_0-l) to be filled by none or single, double etc. lipids, respectively. $V(k)$ is the direct Coulomb interaction (in Fourier space) between channels and lipids on the bilayer, $f(n,T)=n/2k_BT$, n is the lipid density, T is absolute temperature and k_B is Boltzmann's constant. We find that the hydrophobic bilayer thickness channel length mismatch d_0-l induces channel destabilization exponentially while negative lipid curvature (c_0) linearly. Lipid charge appears with dominant effects in case of higher mismatch. Experimental parameters related to gramicidin A (gA) and alamethicin (Alm) channel dynamics in black lipid membranes inside NaCl aqueous phases are consistent with theoretical predictions. Our experimental results (with others) show that average gA channel lifetime decreases exponentially with increasing d_0-l but linearly with increasing negative c_0. The Alm channel formation rate and relative free energy profiles between its different conductance levels follow identical trends as predicted by our theoretical results. This study provides a general framework for understanding the underlying mechanisms of membrane protein functions in biological systems.

272-Pos

Properties of Liposomes With Complex Lipid Mixture

Markus Schwiering, Antje Brack, Heinz Decker, **Nadja Hellmann**.
Institute for Molecular Biophysics, Mainz, Germany.

Although the alpha-toxin from *S. aureus* was the first pore-forming toxin identified, its mode of interaction with membranes is still not fully understood. The toxin forms heptameric pores on cellular and artificial membranes. The observation that artificial membranes are permeabilized by this toxin indicates that no protein receptor is mandatory. Efficient permeabilisation is only possible in presence of cholesterol and sphingomyelin, which could be interpreted as a preference of the toxin for raft-like structures. However, the extent of oligomer formation as monitored by pyrene-fluorescence depends in a complex way on the lipid-composition of the liposomes which in our studies contain different amounts of eggPE, brainPS, eggSM and Cholesterol. Thus, we employed thin-layer chromatography in order to check whether the lipid composition as found finally in the liposomes correspond to the original mixture in chloroform. The results show that in case of extruded vesicles the deviation from the original mixture is not significant, but that in case of GUVs completely different relative amounts of the different lipid components can be obtained. Thus any comparison of liposome properties or toxin/liposome interactions between different liposome types has to be done very cautiously if these types of mixture are employed.
We thank the DFG (SFB 490) for financial support, S.Bhakdi and A.Valeva for production of the toxin and helpful discussions and G. Gimpl for help with fluorescence microscopy.

273-Pos

Functional Reconstitution Into Liposomes of Purified Human RhCG Ammonia Channel

Isabelle Mouro-Chanteloup[1], Sylvie Cochet[1], Mohamed Chami[2], Sandrine Genetet[1], Nedjma Zidi-Yahiaoui[1], Andreas Engel[2], Yves Colin[1], Olivier Bertrand[1], Pierre Ripoche[1].
[1]INSERM UMR_S 665/INTS/Université Paris Diderot-Paris 7, Paris, France,
[2]C-CINA, Center for Imaging and Nanoanalytics, .E. Müller Institute for Structural Biology, Biozentrum, University of Basel, Basel, Switzerland.

Rh glycoproteins (RhAG, RhBG, RhCG) are members of the Amt/Mep/Rh family which facilitate movement of ammonium across plasma membranes. Changes in ammonium transport activity following expression of Rh glycoproteins have been described in different heterologous systems: yeasts, oocytes and eukaryotic cell lines. However, in these complex systems, a contribution of endogenous proteins to this function cannot be excluded. To demonstrate that Rh glycoproteins by themselves transport NH_3, human RhCG was purified to homogeneity and reconstituted into liposomes, giving new insights into its channel functional properties.

An HA-tag introduced in the second extracellular loop of RhCG was used to purify to homogeneity the HA-tagged RhCG glycoprotein from detergent-solubilized recombinant HEK293E cells. Electron microscopy analysis of negatively stained purified RhCG-HA revealed, after image processing, homogeneous particles of 10 nm diameter with a trimeric protein structure. Reconstitution was performed with sphingomyelin, phosphatidylcholine and phosphatidic acid lipids in the presence of the $C_{12}E_8$ detergent which was subsequently removed by Biobeads. Control of protein incorporation was carried out by freeze-fracture electron microscopy. Particle density was a function of the Lipid/Protein ratio. When compared to empty liposomes, ammonium permeability was increased two and three fold in RhCG-proteoliposomes, depending on the Lipid/Protein ratio (1/300 and 1/150, respectively). This strong NH_3 transport was reversibly inhibited by mercuric and copper salts and exhibited a low Arrhenius activation energy.

This study allowed the determination of ammonia permeability, showing that the apparent $Punit_{NH3}$ per RhCG monomer (around $1x10^{-3}$ $\mu m^3.s^{-1}$) is close to the permeability measured in HEK293E cells expressing a recombinant human RhCG ($1.60x10^{-3}$ $\mu m^3.s^{-1}$), and in red blood cells endogenously expressing RhAG ($2.18x10^{-3}$ $\mu m^3.s^{-1}$). The major finding of this study is that RhCG protein is active as an NH_3 channel and that this function does not require any protein partner.

274-Pos

Tracking Single Protein Translocation Complexes in the Membranes of Living Bacteria

Yves Bollen, Siet van, den Wildenberg, Erwin Peterman.
Vrije Universiteit, Amsterdam, Netherlands.

The Twin Arginine Translocation (Tat) system transports fully folded en sometimes even oligomeric proteins across the inner membrane of bacteria. Its mechanism is largely unknown. Remarkably, a stable translocation complex has not been observed. Instead, the three components of the system, i.e., TatA, TatB and TatC, are isolated from the membrane of Escherichia coli in various complexes of different sizes, which suggests that a complete and active Tat complex is formed only transiently. We have used single particle tracking in living bacteria to gain more insight into the dynamics of the Tat proteins. TatA has been genetically fused tot enhanced Green Fluorescent Protein (eGFP). Living bacteria expressing low levels of TatA-eGFP have been immobilized on glass slides and imaged with a sensitive wide-field fluorescence microscope. Mobile fluorescent spots are observed, and their intensity and location have been tracked by fitting a 2D Gaussian function to successive frames. Analysis of the data shows that diffusion of TatA-eGFP is heterogeneous, and that the average diffusion coefficient of fluorescent TatA particles decreases when excess substrate is expressed. When the electrochemical potential, which is known to drive protein transport via the Tat system, is removed the diffusion coefficient of TatA-eGFP increases again. The latter suggests