TonB-binding region of these transporters, called the Ton box, undergoes dramatic structural changes from an immobile to a mobile configuration upon substrate binding. However, this change is not observed in the crystal structures of BtuB. When SDSL is performed with protecting osmolytes such as polyethylene glycols (PEGs), which are contained in the buffers used in crystallization, this order-disorder transition of the Ton box is abolished in BtuB. Here we find that the substrate-dependent Ton box transition in FecA is also modulated by PEGs, and we show that the five residues N-terminal to the Ton box of BtuB are in conformational exchange between folded and unfolded states. Upon addition of PEGs, this equilibrium is shifted towards the folded state. Larger molecular weight PEGs produce a larger apparent shift in the conformational free energy, consistent with the finding that PEGs are excluded from protein surfaces as a result of steric interactions. The observation of conformational exchange in the N-terminus provides an explanation for differences seen between the *in surfo* and the *in meso* crystal structures of BtuB and suggests that each of these structures represents one conformational substate among a family of substates that are normally sampled by the protein. This work illustrates how SDSL and osmolytes may be used to characterize and quantitate conformational equilibria in membrane proteins.

260-Pos

Proximity of the EGF Receptor Kinase Domain To the Plasma Membrane

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Understanding the activation mechanism of the epidermal growth factor receptor (EGFR) is of fundamental importance in anticancer drug development. Crystal structures establish the two kinase domains in the receptor dimer bind to and activate each other by an allosteric mechanism. However, information about the conformation of the juxtamembrane (JM) region and the proximity of kinase domains to the membrane in live cells is lacking. Electrostatic potential calculations suggest that both the positively charged N-terminal JM region and a positive face of the kinase domain could bind to the negatively charged inner leaflet of the plasma membrane. The objective of this report is to investigate the proximity of the kinase domain of EGFR to the plasma membrane. We truncated the EGFR after the kinase domain and genetically tagged it with the monomeric yellow fluorescent protein (mYFP). We measured FRET between a plasma membrane target cyan fluorescent protein (PMT-CFP) and the truncated EGFR- mYFP (tEGFR-mYFP). The observed FRET is consistent with the hypothesis that the kinase domain is located close to the membrane in the absence of ligand. Addition of epidermal growth factor (EGF) produced a decrease in FRET, which indicates the kinase domain may dissociate from the membrane during activation.

261-Pos

Distinct Topologies For the HIV-1 Transmembrane Glycoprotein gp41 C-Terminal Tail on Cellular and Viral Lipid Membranes

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The HIV-1 envelope (Env) transmembrane protein, gp41, is typically considered a type I membrane protein with an extracellular N-terminus, a single membrane-spanning domain, and a C-terminus forming a ~150 residue intracytoplasmic tail. However, published studies indicate an alternative or dynamic topology for portions of the C-terminal tail (CTT) that results in exposure of CTT segments on the membrane surface. To distinguish between these alternative models, we evaluated the accessibility of a reference CTT sequence, the ''Kennedy epitope'' (KE), in viral and cellular membranes to map CTT topology relative to the lipid bilayer. KE accessibility in cell-associated Env was defined by reactivity of native or VSV-G epitope-tagged KE to specific monoclonal antibodies (MAbs), measured by FACS of intact cells. In parallel, KE exposure in virion-associated Env was characterized by MAb binding to intact virions as measured in immunoprecipitation and surface plasmon resonance (SPR) spectroscopy assays. FACS analyses of live cell-associated Env demonstrated significant reactivity of the KE with MAbs to native or VSV-G epitope-tagged KE. No reactivity was observed in cells expressing Env with the VSV-G epitope substituted into a cytoplasmic domain. In contrast to cellular Env, KE in the context of virions failed to react with MAbs directed to the native KE sequence, as measured both by immunoprecipitation and SPR assays. However, MAbs specific for a membrane-proximal sequence bound virions in both assays. Together, the results of these accessibility assays indicate that the KE sequences of gp41 are accessible to antibody binding in cell surface-expressed, but not virion-associated, Env. These observations suggest that the CTT may assume distinct topologies (reflected in KE exposure) that depend on the membrane environment (viral/cellular) and that parts of the CTT may be (transiently) exposed on the membrane surface.

262-Pos

Membrane Topology of Hepatitis C Virus Protein NS4B

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Hepatitis C virus (HCV) protein NS4B is a poorly characterized highly hydrophobic integral membrane protein which is associated with membranes of the ER or an ER-derived modified compartment. NS4B induces the formation of intracellular membrane changes that are visible by electron microscopy, the so called membranous web. This membranous web has been postulated to be the HCV RNA replication complex. Since morphogenesis and budding has been suggested to take place in ER modified membranes, a function of NS4B might be to induce a specific ER membrane alteration that serves as a scaffold for the formation of the HCV replication complex. If that were true, NS4B might have a critical role in the HCV cycle. It has been predicted that NS4B possess four / five transmembrane (TM) domains. Two helical elements have been predicted in the C-terminal part of the protein, pointing out to a yet unknown common function of the C-terminal globular part. Additionally, RNA binding properties through a nucleotide-binding motif have recently been reported, as well as a new membrane association segment in the N-terminal portion of the protein. Many questions remain unanswered about the NS4B topology but due to the highly hydrophobic nature of the protein, a detailed structure determination using experimental techniques will not be obtained in the near future. In order to define the membrane topology of NS4B we have analyzed the spatial distribution of the spatial hydrophobicity of thirty-one sequences of protein NS4B pertaining to different strains representing the seven major genotypes of HCV. Our study shows that NS4B has at least five and possibly six TM domains.

263-Pos

Evolutionary Conservation of Phospholipid-Binding Sites in Membrane Proteins

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Membrane proteins evolved to function in a diverse phospholipid environment. Experimental evidence suggests that some phospholipid molecules are recruited by membrane proteins as co-factors or ligands that tightly bind to specific sites on the protein surface and play important functional roles. In this work we have assessed the evolutionary conservation of phospholipid-binding sites in several alpha-helical and beta-barrel membrane proteins. We first identified the membrane protein surface residues and residues that are in close contact with co-crystallized phospholipids in the x-ray structures of adrenergic receptor, photosynthetic reaction center, Kcsa potassium channel, formate dehydrogenase and ferric hydroxamate uptake receptor using methods of computational geometry such as Delaunay triangulation and alpha shape. We next collected orthologous cDNA sequences for every protein and used posterior probability analysis of evolutionary selection pressure measured as ω-ratio with the aid of PAML package to identify phospholipid-facing residues under strong purifying selection pressure. We show that protein residues interacting with co-crystallized phospholipids are collectively more conserved than the rest of the phospholipid-facing residues with statistically significant p-values in the range 10e-7 - 10e-3. Additionally, we found that every phospolipid-binding site on the membrane protein surface contains from 3 to 5 residues that experience strong purifying selection pressure similar to the functionally important buried residues.

264-Pos

Transmembrane Protein Association in a Biomimetic Medium

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A plethora of membrane proteins studies have been performed on micellar systems containing detergent solubilised proteins. A major drawback of these systems is their rather poor mimicry of biological membranes. Therefore, development of new biologically relevant membrane systems is justified.
We illustrate an original approach combining fluorescence recovery after fringe pattern photobleaching (FRAPP) with the use of an optically isotropic, extremely fluid phase of bilayers (the sponge phase). In this biologically relevant system, well suited for spectroscopic studies, the spacing between two adjacent bilayers and the thickness of the bilayers can be easily and very precisely tuned. So when the membranes are sufficiently separated, the only possible interactions occur between proteins embedded in the same bilayer, whereas when the membranes come together, interactions between proteins embedded in adjacent membranes may also occur.

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
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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
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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
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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
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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
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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