oligosaccharide head group for hydrolytic cleavage by the hydrolase, HexA. Mutations in GM2AP or HexA lead to an accumulation of GM2 in the lysosomes, causing lysosomal storage diseases such as Tay Sachs or the AB variant of Sandhoff's disease. Our lab is utilizing site directed spin-labeling (SDSL) with electron paramagnetic resonance (EPR) spectroscopy to probe the conformational changes and the membrane bound orientation of GM2AP. This protein contains eight naturally occurring cysteine (CYS) residues involved in four disulfide bonds. With site directed mutagenesis, a ninth CYS residue is introduced as the reporter site for spin labeling . A series of 10 single CYS mutants have been generated. To validate the EPR results, the mass spectrometry protocol described here was developed to characterize spin-labeled GM2AP samples. For mass spectrometry measurements, either biotin-linked maleimide (BM) or 4-maleimide tempo (4MT) were used to modify and trap available CYS residues in a thioesterbond. The remaining eight native CYS residues, which are disulfide bonded, are then reduced and modified with iodoacetamide. Samples were analyzed by high performance nano-liquid chromatography electrospray ionizaton Fourier-transform ion cyclotron resonance mass spectrometry (FT-ICR MS) equipped with a 14.5 T magnet. FT-ICR spectra of enzymatically digested BM-labeled or 4MT-labeled GM2AP were utilized to determine to which CYS residue is modified with the maleimide moeity. Those sites labeled with acetamide are inferred to have been disulfide bonded. The fragment that contains the maleimide moeity tells us, which CYS residue (and how many CYS residues) is accessible for reaction with the spin label for EPR studies.

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Purification And Reconstitution Of The Connexin43 Carboxyl Terminus Attached To The 4Th Transmembrane Domain In Detergent Micelles

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In recent years, reports have identified that many eukaryotic proteins contain disordered regions spanning greater than 30 consecutive residues in length. In particular, a number of these intrinsically disordered regions occur in the cytoplasmic segments of plasma membrane proteins. These intrinsically disordered regions play important roles in cell signaling events, as they are sites for protein-protein interactions and phosphorylation. Unfortunately, in many crystallographic studies of membrane proteins, these domains are removed because they hinder the crystallization process. Therefore, a purification procedure was developed to enable the biophysical and structural characterization of these intrinsically disordered regions while still associated with the lipid environment. The carboxyl-terminal domain from the gap junction protein connexin43 attached to the 4th transmembrane domain (TM4-Cx43CT) was used as a model system (residues G178-I382). The purification was optimized for structural analysis by nuclear magnetic resonance (NMR) because this method is well suited for small membrane proteins and proteins that lack a well-structured three-dimensional fold. The TM4-Cx43CT was purified to homogeneity with a yield of ~6 mg per liter from C41(DE3) bacterial cells, was reconstituted in the anionic detergent 1-palmitoyl-2-hydroxy-sn-glycero-3-[phospho-RAC-(1-glycerol)], and circular dichroism and NMR were used to demonstrate that the TM4-Cx43CT was properly folded into a functional conformation by its ability to form a-helical structure and associate with a known binding partner, the c-Src SH3 domain, respectively.

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A Refinement Protocol to Define the Structure, Topology and Depth of Insertion of Membrane Proteins using Hybrid Solution/Solid-state NMR Restraints

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To fully describe the folding space and ultimately the biological function of membrane proteins (MPs), it is necessary to determine their interactions with lipid membranes. This intrinsic property of MPs referring to as structural topology is not possible to resolve using x-ray crystallography or solution NMR. Here, we present a hybrid solution and solid-state NMR approach for the simultaneous determination of the structure, topology, and depth of insertion of MPs. Distance and angle restraints obtained from solution NMR of MPs solubilized in detergent micelles are combined with backbone orientational restraints (15N chemical shift anisotropy and 15N-1H dipolar couplings) derived from solid-state NMR in orientated lipid bilayers (PISEMA) into a hybrid objective function. In addition, a supplementary energy term, Ez (insertion depth potential), is used to ensure the correct positioning of helical MPs domains with respect to a virtual membrane. The hybrid objective function is optimized using a two-stage simulated annealing protocol and is implemented into XPLOR-NIH software for general use. To validate the hybrid method, the effects of chemical shift tensor orientations, principal tensor values, and dipolar constant magnitudes on the structural ensemble are determined.

The hybrid method is applied to monomeric and pentameric PLN (phospholamban), a integral MP that regulates sarco(endo)plasmic reticulum Ca-ATPase (SERCA) function in cardiac muscle. The hybrid conformational ensemble defines the structure, topology and depth of insertion of PLN in lipid bilayer simultaneously. This ensemble is compared with solution NMR structures in DPC micelle obtained using conventional solution NMR data (NOEs, J-couplings) and residual dipolar coupling as orientational restraints.

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Solution and Solid-State NMR Analysis of Phosphorylated and Pseudo-Phosphorylated Phospholamban

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Phospholamban (PLN) is a 52 residue transmembrane protein that regulates the Ca2+ ATPase (SERCA) of muscle cells. At low μM Ca2+ concentration, PLN binds to SERCA and decreases the rate of uptake of Ca2+ into the sarcoplasmic reticulum (SR) thereby promoting muscle relaxation. This inhibitory activity is reversed by phosphorylation of PLN at Serine-16 or Threonine-17 in response to ß-adrenergic stimulation. Phosphorylation of PLN is associated with increased cardiac contractility of muscle cells due to the larger Ca2+ load into the SR.

PLN has become important as a therapeutic target in fighting heart failure, a complex disease associated with impaired cardiac contractility. It has been shown that delivering of phospho-mimiking PLN (Ser-16 → Glu substitution) to failing mice cardiomyocytes significantly improves contractility.

Although functional effects of PLN phosphorylation have been extensively studied, the mechanistic details of how phosphorylated and pseudo-phosphorylated (S16E) PLN interacts with SERCA to reverse inhibition are still unclear. In here we present data on the structural characterization of Ser-16 phosphorylated and S16E monomeric and pentameric phospholamban (AFA-PLN and WT-PLN) in the presence and absence of SERCA as probed by solution and solid-state NMR spectroscopy in detergent micelles and oriented lipid bilayers. For solid-state NMR, SERCA and PLN were reconstituted in planar lipid bilayers and uniaxially aligned on glass plates. Residue-specific information as well as topology of PLN monomer and pentamer was determined by PISEMA experiments.

2223-Pos Board B193

Hybrid Solution and Solid-State NMR Analysis of SERCA/Phospholamban Interactions in lipid membranes: From Structural Dynamics to Function

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Phospholamban (PLN) is a sarcoplasmic reticulum (SR) integral membrane protein that regulates calcium translocation in cardiac muscle. Upon interaction with SERCA (the SR calcium ATPase). PLN decreases the rate of calcium uptake, reducing the apparent affinity of the enzyme for Ca^{2+} ions. This process is reversed by adrenergic stimulation of protein kinase A, which phosphorylates PLN at Ser16, re-starting the muscle contraction cycle. Here, we present the hybrid solution and solid-state NMR structural analysis of PLN in its pentameric (storage), monomeric (active), and SERCA-bound forms in lipid membranes. This knowledge about the structural dynamics PLN under these different stages is used to steer the extent of PLN control on SERCA activity. These findings lay the groundwork for the rational design of PLN loss-of-function mutants to be used in gene therapy.

2224-Pos Board B194

Topology of Phospholamban when Bound to Ca2 ± ATPase by Solid-State NMR

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Calcium cycling in muscle cells drives the relaxation and contraction of both skeletal and heart tissue. The sarcoplasmic reticulum (SR) Ca^{2+}-ATPase is central in the relaxation of the heart, accounting for ~70% of calcium sequestration. Phospholamban (PLN) is a small integral membrane protein regulator of Ca^{2+}-ATPase. Its inhibition of the enzyme is shown in calcium dependence ATPase activity curves, resulting in decreased ATPase affinity for Ca^{2+}. While there have been several successful attempts to gain structural knowledge of the complex between PLN and Ca^{2+}-ATPase, no high-resolution structure exists.

One important feature that differentiates membrane proteins from soluble ones is topology. This is the specific *entanglement* between the membrane protein and the lipid bilayer. Probing this specific orientation of the protein has been best achieved using oriented solid-state NMR experiments such as PISEMA, SAMPI4, and HIMSELF. These experiments correlate an anisotropic ^{15}N (or ^{13}C) chemical shift with a ^{1}H-^{15}N (or ^{1}H-^{13}C) dipolar coupling, allowing for the resolution of backbone restraints with respect to the lipid bilayer normal. To investigate whether topology plays a role in this membrane protein complex, we reconstituted PLN (^{15}N labeled) in the presence and absence of Ca^{2+}-ATPase (purified from rabbit skeletal muscle) into oriented lipid bilayers. Our results show unambiguously and with high reproducibility that PLN's topology is substantially altered upon binding the ATPase. Specifically, we see that the membrane embedded helix (residues 23-52) changes its tilt angle with respect to the bilayer normal from ~23° in the absence of the enzyme to ~40° in its presence. This substantial change in topology might be a necessary attribute of regulation by PLN and potentially a central difference between an active and inactive PLN bound state to Ca^{2+}-ATPase.

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A Solid-State NMR Study on the Structure and Dynamics of the Myristoylated N-Terminus of the Human Guanylate-Cyclase Activating Protein-2

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Guanylate cyclase-activating protein-2 (GCAP-2) is a retinal Ca^{2+} sensor protein. It plays a central role in shaping the photoreceptor light response and in light adaptation through the Ca^{2+}-dependent regulation of the transmembrane retinal guanylate cyclase (GC). GCAP-2 is N-terminally myristoylated and the full activation of the GC requires this lipid modification. The structural and functional role of the N-terminus and particularly of the myristoyl moiety is currently not well understood. In particular, detailed structural information on the myristoylated N-terminus in the presence of membranes was not available. Therefore, we studied the structure and the dynamics of a 19 amino acid peptide representing the myristoylated N-terminus of GCAP-2 bound to lipid membranes (DMPC liposomes) by solid-state NMR.^{13}C isotropic chemical shifts measured in CP HETCOR experiments revealed a random coiled secondary structure of the peptide. Order parameters for Cα, Cβ, and side chain carbon atoms obtained from DIPSHIFT experiments are relatively low suggesting high mobility of the membrane associated peptide. Static ^{2}H solid-state NMR measurements show that the myristoyl moiety is fully incorporated into the lipid membrane. The order parameters of the myristoyl moiety and the DMPC lipid chains are quite similar. Further, dynamical parameters (obtained from ^{2}H NMR relaxation rates) of the peptide's myristic acid chain are also comparable to those of the lipid chains of the host matrix. Therefore, the myristoyl moiety of the N-terminal peptide of GCAP-2 fills a similar conformational space as the surrounding phospholipid chains. However, we did not find a specific hint for a membrane interaction of the amino acids adjacent to the lipid modification.

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A Molecular Gearbox: The Mechanical And Regulatory Complexity Of The Vacuolar ATPase Revealed

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Vacuolar H^{+}-ATPases (V-ATPases) are ATP-driven rotary molecular motors that function as transmembrane proton pumps in all eukaryotic cells. Central roles of V-ATPases in cell physiology include: energising secondary active transport, maintaining acidity in intracellular compartments, and pumping acid out of the cell. Loss of V-ATPase function is associated with forms of kidney disease and inherited deafness; excess or unregulated activity is implicated in diseases such as osteoporosis and in tumour metastasis or multidrug resistance. The V-ATPase is a large ~1 MDa complex of about 30 subunits of at least 13 types. It has a basic architecture comprising 2 domains, with the soluble V_1 domain responsible for ATP hydrolysis and the integral membrane V_0 domain responsible for proton translocation. We have generated the first 3D reconstruction of a native V-ATPase using cryo-electron microscopy of single particles of the pump from tobacco hornworm (*Manduca sexta*). The derived density map, with resolution at 17 Å, reveals a complex network of interactions that both drive and regulate the V-ATPase. In particular, 3 peripheral stators are present, which are linked via a horizontal collar of density that circles 250° around the inter-domain region. In contrast, the related but simpler F_1F_0 ATPase has only 1 stator responsible for connecting the ATP hydrolysing and proton translocation domains. A fourth central stalk forms the V-ATPase axel and is attached to the V_0 membrane domain, but makes minimal contact with the V_1 ATP-hydrolysing region. The definition of the reconstruction is such that previously characterised subunits crystal structures can be accurately fitted into the density map. This provides insight into the organisation of key components directly involved in regulation of activity.

2227-Pos Board B197

Towards an atomic model of the Hepatitis C virus p7

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The hepatitis C virus (HCV) p7 protein forms ion channels which are essential for the assembly and secretion of infectious virions, making it an important therapeutic target. Electron microscopy experiments suggest that p7 oligomers may co-exist as hexamers and heptamers. Electrophysiology experiments show that Cu^{2+} has an inhibitory effect on the p7 ion channel and that the amino acid responsible for this inhibition is one histidine in each monomer. These results coupled with the p7 sequence data suggest that the N-terminal helix of p7 lines the transmembrane pore and that this histidine is pore-lining. We combine these results with a previously described hexameric model of the pore derived from transmission electron microscopy and random conical tilt reconstruction to generate improved models of the p7 pore. We discuss the improved models in relation to mutagenesis and potential inhibitor interactions.

2228-Pos Board B198

Structural and Functional Studies of M2 Proton Channel from Influenza A Virus

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M2 protein of influenza A virus forms a homo-tetrameric proton channel involved in modifying virion and trans-Golgi pH for virus infection and inhibited by drugs Amantadine and Rimantadine. The aim of this research is to study the correlation of structure and function of M2 proton channel and mechanism of inhibition by influenza drugs. In order to obtain high resolution structural information in native bilayer like environment, we have expressed and purified intact M2 protein and two peptides M2(22-46) and M2(22-62) as a maltose binding fusion protein from E. Coli. membrane and reconstituted in a DMPC:DMPG(4:1) and DOPC:DOPE(4:1) lipid bilayer. We have applied a range of NMR approaches to study the structure of M2 protein and truncated peptides in apo state as well as complexed with amantadine reconstituted in lipid bilayer and uniformly aligned with respect to external magnetic field. Multidimensional Solid State NMR experiments performed on uniform 15N labeled and amino acid specific labeled protein suggest that helical tilt angle of transmembrane domain in intact M2 protein is smaller compared to that of isolated peptides M2(22-46) and M2(22-62) and oligomeric state of the channel is stabilized due to interactions of amphipathic helices. Using the proteoliposome fusion assay of channel activity, we found that M2 (22-62) (1 mg peptide/8 mg DMPC + 2 mg DMPG/ml H2O, diluted to 3% in the cis chamber) forms active channels in planar lipid bilayers (4 POPE: 1 POPC: 1 POPS: 2 cholesterol in decane). With asymmetric KCl solutions (1 M, pH 8 cis, 0.1 M, pH 6 trans), the time-averaged membrane current was >10-fold higher using proteoliposomes than with protein-free liposomes (N>8) for Vm = ±100 mV. Similar experiments with M2 (22-46) yielded no channel activity (N>80).

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EPR Spectroscopy of the C-terminal Domain of the M2 Protein from Influenza A Virus

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The M2 protein from influenza A is a pH-activated proton channel that plays an essential role in the viral life cycle and serves as a drug target. Using spin labeling EPR spectroscopy we studied a 38-residue M2 peptide spanning the transmembrane region and its C-terminal extension. We obtained residue-specific environmental parameters under both high and low pH conditions for eleven consecutive C-terminal sites. We have also collected data in the presence of the antiviral drugs amantadine and rimantadine. The C-terminal region forms a membrane surface helix at both high and low pH although the arrangement of the monomers within the tetramer changes with pH and drug binding.