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A Functional, Pentameric Form of Phospholamban Is Required For Two-Dimensional Crystallization With the Sarcoplasmic Reticulum Calcium Pump

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A Functional, Pentameric Form Of Phospholamban Is Required For Two-Dimensional Crystallization With The Sarcoplasmic Reticulum Calcium Pump
Phospholamban physically interacts with the sarcoplasmic reticulum calcium pump (also known as SERCA) and regulates contractility of the heart in response to adrenergic stimuli. We have studied this interaction using electron microscopy of large two-dimensional crystals of SERCA in complex with phospholamban. In our original work, phospholamban oligomers were found interspersed between dimer arrays of SERCA and a three-dimensional model was constructed to show potential interactions between the two proteins. In the present study, we have examined the effects of phospholamban phosphorylation and mutation on the formation of two-dimensional co-crystals with SERCA. Phospholamban phosphorylation at Ser^{16} and a well-characterized loss-of-function mutation (Asn^{34}-to-Ala) significantly reduced crystal formation. More importantly, projection maps calculated from these crystals revealed that the densities attributable to phospholamban become disordered, suggesting a reduced interaction with SERCA. In contrast, a pentameric gain-of-function mutant (Lys^{27}-to-Ala) significantly enhanced crystal formation. These latter crystals were used to calculate an improved projection map from frozen-hydrated crystals to a resolution of 8 Å. We conclude that the oligomeric state of phospholamban in the crystals is a pentamer, and that phosphorylation and mutation of phospholamban alter physical interactions in the crystals in a manner that is consistent with a functional association with SERCA. Combined, the data suggest that the pentameric state of phospholamban is not simply an inactive storage form.

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Towards the Development of Rationally Designed Phospholamban Mutants For Treatment of Heart Failure

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PLN is the endogenous inhibitor of the sarco(endo)plasmic reticulum Ca2+-ATPase (SERCA), the integral membrane enzyme responsible for 70% of the Ca2+ shuttling into the SR, inducing cardiac muscle relaxation in humans. Dysfunctions in SERCA:PLN interactions have been implicated as having a critical role in cardiac disease. Inspired by the success of S16E, a pseudo-phosphorylated form of PLN which successfully reduced the progression of the cardiac failure in murine models and large animals upon delivery via rAAV gene therapy, I wish to further develop paradigms to rationally design therapeutic mutations based on the foundation of biophysical data known about the system, thereby ''tuning'' phospholamban structural dynamics to directly affect SERCA function. The present study utilizes a combination of NMR spectroscopy and coupled-enzyme assays to investigate the functional contribution of the structural dynamics of both loss-of-function (LOF) and naturally occurring malignant mutants of phospholamban, and draw correlations between the structural dynamics of the inhibitor to the activity of its target enzyme. These studies further develop the model by which the control of enzyme function is performed by altering the structural dynamics of a small inhibitor can then be translated to other membrane enzymes, such as the Na/K-ATPase.

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Phospholamban Topology As a Regulator of Sarcoplasmic Reticulum Ca2+ATPase Function

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Sarcoplasmic reticulum Ca^{2+}ATPase (SERCA) transports Ca^{2+} ions from the cytosol of cardiomyocytes into the SR, making it a crucial regulator of cardiac muscle function. SERCA is inhibited by phospholamban (PLN), a membrane-spanning protein consisting of a transmembrane (TM) helix connected to an amphipathic helix by a short loop. Phosphorylation of PLN at Ser16 relieves the inhibition of SERCA but does not dissociate the PLN-SERCA complex. Owing to their pivotal role in cardiac muscle function, SERCA and PLN have received much attention as therapeutic targets. In one such study, a pseudo-phosphorylated S16E mutant of PLN was successfully used to relieve the effects of heart failure in hamsters (*Nat. Med*., 2002, 8, 864-871). Ongoing efforts in our lab are aimed to design new PLN mutants that bind tightly with SERCA, but relieve inhibition (i.e., mimic Ser16 phosphorylation).
A recent study refined the structure and topology of PLN in a lipid bilayer environment using a hybrid solution and solid state NMR approach (***PNAS***, 2009, 106, 25, 10165-101670). The tilt angle of the PLN TM helix in DOPC/DOPE bilayers was determined to be 24o with respect to the bilayer normal. In this study, we reconstituted PLN into magnetically aligned bicelles and used PISEMA experiments to show that the topology of PLN is strongly dependent on hydrophobic mismatch, since mutations in the PLN sequence and changes in the bilayer composition alter the TM helix tilt angle. Taken with results from our lab that correlated PLN structural dynamics to inhibitory function on SERCA (***J. Biol. Chem***., 2007, 282, 51, 37205-14), the differences in topology presented in this work introduce a new dimension in the study of PLN mutants that could be crucial for understanding the complex structural features of PLN that are important for SERCA regulation.

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Solid State NMR Observation of the Geometry of Kinked Protein Helices

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Oriented sample solid state nuclear magnetic resonance (ssNMR) is a technique for characterizing the structure of membrane proteins in a nearly native environment. 15N anisotropic chemical shift and 15N-1H dipolar couplings in two dimensional separated local field ssNMR spectra provide precise restraints on the orientation of peptide planes with respect to the membrane normal in alpha helical protein structures. These restraints can be utilized in structure determination and provide a straightforward technique for characterization of helical orientation using PISA wheel analysis. PISA wheel analysis provides the tilt angle and rotational orientation of helical structures. This analysis breaks down for helices with a kink, because the tilt angles to the membrane normal can be determined but the 3D geometry of the kink and how it relates to the membrane environment is not known from the PISA wheel analysis.
Here, we present the detailed mathematical analysis of the geometry of the kink and how it relates to the ssNMR spectra. This analysis will be applied to the M2 proton channel from the influenza A virus. The kinked helical system will be modeled as two idealized helices with a single pair of modified phi/psi angles. The kink angle is calculated as a function of the modified phi/psi angles. The PISA wheel analysis yields the rotation and tilt angles for each idealized section of helix. These values are used to derive a relationship between the tilt and rotation angles and the kink phi/psi angles. The result links the ssNMR data to the structure of a kinked helix. Specifically important to the structure of membrane proteins is the known relationship of the structure to the membrane normal. This information can then be used in structural refinement using ssNMR and other structural data.

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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 during virus infection and inhibited by drugs Amantadine and Rimantadine. Previously determined structures for the membrane domain of M2 protein in detergent micelles have shown different conformations of TM helices with respect to channel axis i.e. tilt as well as relative orientation than structures solved in lipid bilayer environment. We report a new three dimensional structure of closed channel state of M2 protein residue (22-62) in native like PC:PE bilayer that encompasses all functionally relevant domains for proton channel activity.
In order to obtain high resolution structure in bilayer environment, M2(22-62) was expressed and purified from E. Coli. membrane and reconstituted in liposomes. Liposomal assays demonstrated fully active and amantadine-sensitive channels with an average proton uptake of 21.1 ± 1.9 H+/tetramer/s. Multidimensional Solid State NMR experiments performed on uniform 15N labeled and amino acid specific labeled M2(22-62) reconstituted in a DOPC:DOPE(4:1) lipid bilayer generated precise orientational restraints for amide bond vectors and peptide planes for each residue to determine three-dimensional structure. Helical tilt and rotation were calculated using dipolar couplings, chemical shift wave and polarization inversion slant angle (PISA) wheel analysis. Tetramer assembly and membrane equilibration was performed using molecular dynamics simulations. Structure shows four-helix bundle with TM and amphipathic helices tilted at ~34 degrees and ~105 degrees to channel axis, respectively with a tight turn joining two helices. Although the oligomeric state of the channel is stabilized due to the interactions of amphipathic helices as previously reported, membrane interaction and rotational orientation of amphipathic

helices is significantly different with bulky hydrophobic residues buried deep in the membrane. Salient features of the structure will be reported in light of proton transport mechanism.

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Site-Directed Spin-Label EPR Studies Report on Drug-Induced Conformational Change of Influenza a M2 Protein

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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 have obtained residue-specific environmental parameters in the presence of the antiviral drug amantadine to gain information about the drug bound state of M2 in POPC/POPG lipid bilayers. Power saturation studies of spin-labeled peptides reconstituted in a DOGS-NTA(Ni)-containing bilayers report on the accessibility of spin labels to nickel(II) chelated at the aqueous-lipid interface.

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HIV-1 Matrix Binding to Model Membranes Investigated By Neutron Reflectivity: Electrostatics and Binding Orientation

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The N-terminal Matrix (MA) domain of the HIV-1 Gag protein is responsible for binding the membrane during viral assembly. A basic patch of residues localized in the MA domain confers a strong electrostatic component to this binding interaction. Through mutagenesis the putative binding interface of MA has been mapped out, but not directly determined by experimental measurements. We present neutron reflectivity measurements that resolve the one dimensional scattering length density profile of MA bound to a lipid membrane. The model membrane system used maintained the anionic surface charge density of the native viral membrane. Molecular refinement using atomic structures of MA suggests an orientation of the protein on the membrane consistent with previous mutagenesis and electrostatic modeling studies. Remarkably the MA protein maintains this orientation without the presence of the post-translational myristate group. Furthermore MA is found to only peripherally penetrate the membrane headgroups by 4.8 ± 1.2 Å allowing only amino acid side chains to contact the lipid headgroups. Our results find that electrostatic interactions are sufficient to preserve the correct binding motif of MA with the viral membrane.

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Oligomerization of Transmembrane Alpha-Helices Modulated By C-terminal Boundary Residues

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In studies of the structural biology of membrane proteins, the success of strategies based on the ''divide-and-conquer'' approach, where peptides are used to model the individual transmembrane (TM) α-helices of membrane proteins, depends upon the correct identification of the membrane-embedded TM α-helix amino acid sequence within the full-length protein. In the present work, we examine the effects of excluding or including TM boundary residues on the intrinsic properties of the TM2 α-helix of myelin proteolipid protein (PLP). Using protein gel electrophoresis, circular dichroism, and fluorescence resonance energy transfer in the membrane-mimetic detergent sodium dodecylsulfate (SDS) to study parent sequence KKKK-^{61}AFQYVIYGTASFFFLYGALL-LAEG^{89}-KKKK - along with analogs containing an additional wild type Phe-90, Phe-90 and Tyr-91, and a hydrophobic mutant Leu-90 - we demonstrate that the removal of a single amino acid from the C-terminus of this TM segment is sufficient to change its intrinsic properties, with TM2 61-89 displaying only a monomeric form, but with principally dimers arising for the other three peptides. The findings suggest that deletion of critical C-terminal residue(s) tends to re-position the helix terminus toward the membrane-aqueous interface, and emphasize the potential influence of boundary residues on TM properties when utilizing peptides as models for TM α-helices. These finding may implicate a role for such residues in membrane protein folding and assembly.

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Structure, Dynamics and Topology of the N-terminus and First Transmembrane Segment of APJ

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APJ is a G-protein coupled receptor expressed in the cardiovascular system, central nervous system and several other tissues. Activation of APJ by the peptide ligand apelin has defined roles in cardiovascular regulation, in glucose metabolism and in tumour growth. Transmembrane proteins such as APJ are difficult to study due to expression, solubility and refolding difficulties. For this reason we have produced a fragment of APJ containing the functionally essential N-terminal region and first transmembrane helix of the receptor (APJ55). Through a combination of circular dichroism (CD) and nuclear magnetic resonance (NMR) spectroscopy we have characterized APJ55. CD spectroscopy indicates that APJ55 only properly refolds in specific detergents, with the anionic detergents sodium dodecylsulphate and 1-palmitoyl-2-hydroxy-sn-glycero-3-[phospho-rac-(1-glycerol)] (LPPG) being the best. NMR spectroscopy has provided an initial structure of APJ55. As a complement to this structure, relaxation studies and paramagnetic spin label titration demonstrated the dynamics and topology of APJ55 in the LPPG micelle. Finally the structure of APJ55 has been placed into the context of full length APJ using a homology model. APJ55 provides a new system to probe apelin-APJ interactions and is a basis for study of additional regions of APJ.

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CD and EPR Structural Studies on the KCNE1 Protein in a Lipid Bilayer

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KCNE1, also known as minK, is a membrane protein responsible for modulating the KCNQ1 voltage-gated potassium ion channel in the human heart. Previous *in vivo* electrophysiological studies have shown that KCNQ1 loses its functionality in the absence of KCNE1, showing that KCNE1 is an essential protein for proper heart function (Sanders et al., Biochemistry 2007 46:11459-11472). Though KCNE1 has been extensively studied in micelle detergent systems, little work has been done to study the protein in an actual lipid bilayer-membrane system. Our current research uses biophysical techniques such as circular dichrosim (CD) spectroscopy and electron paramagnetic resonance (EPR) spectroscopy to characterize and compare KCNE1 proteins in various micelle and lipid bilayer environments using both qualitative and quantitative methods. Our CD spectroscopy experiments have shown that KCNE1 undergoes a change in secondary structure when removed from a micelle environment and placed in a lipid bilayer. We have used EPR spectroscopy to show that the dynamic properties of KCNE1 also change when taken out of micelles and inserted into lipid bilayers. Calculations have been done to quantify these differences in the structural and dynamic properties observed for KCNE1 in micelles and lipid bilayers.

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Accessory Alpha-Helix of Complexin I Can Displace VAMP2 Locally in the Complexin-Snare Quaternary Complex

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The calcium-triggered neurotransmitter release requires three SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) proteins: synaptobrevin 2 (or VAMP2) on the synaptic vesicle and syntaxin 1 and SNAP-25 at the presynaptic plasmas membrane. This minimal fusion machinery is believed to drive fusion of the vesicle to the presynaptic membrane. Complexin, also known as synaphin, is a neuronal cytosolic protein that acts as a major regulator of synaptic vesicle exocytosis. Stimulatory and inhibitory effects of complexin have both been reported, suggesting the duality of its function. To shed light on the molecular basis of the complexin's dual function, we have performed an EPR investigation of the complexin-SNARE quaternary complex. We found that the accessory α-helix (amino acids 27-48) by itself has the capacity to replace the C-terminus of the SNARE motif of VAMP2 in the four-helix bundle and makes the SNARE complex weaker when the N-terminal region of complexin I (amino acids 1-26) is removed. However, the accessory α-helix remains detached from the SNARE core when the N-terminal region of complexin I is present. Thus, our data show the possibility that the balance between the activities of the accessory α-helix and the N-terminal domain might determine the final outcome of the complexin function, either stimulatory or inhibitory.

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Osmolytes Modulate Conformational Transitions in Solvent-Exposed Regions of Two Outer Membrane Proteins

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Electron paramagnetic resonance (EPR) spectroscopic studies using site-directed spin labeling (SDSL) have been used to investigate local structure and conformational exchange in different regions of two *E. coli* outer-membrane TonB-dependent transporters: BtuB and FecA. It is known that the