regions within the exchanger's cytoplasmic domain, of which the XIP region shown to be involved in the rate and extent of Na^+-dependent inactivation (I_1). Although the XIP region is highly conserved amongst NCX isoforms, distinct I_1 phenotypes exist. To better define the role of this region, we constructed chimaeric NCX1:NCX2.1 proteins with their respective XIP regions interchanged as well as amino acid substitutions within the XIP region to examine the more subtle aspects of phenotypic differences between NCX1.3 and NCX2.1. Mutant exchangers were expressed in *Xenopus* oocytes, and outward Na^+-Ca^{2+} exchange activity was assessed using the giant, excised patch clamp technique. Substitution of the XIP region of NCX1.4 with the corresponding region from NCX2.1 caused an apparent loss of I_1 whereas a reduction in the extent of inactivation and a 15-fold increase in the rate of recovery from I_1 were observed in the NCX1.3 - XIP2 chimaera. Similarly, substitution of charged amino acids within the XIP region in NCX1.3 caused a slight increase in the rate of recovery, equivalent to that observed for NCX2.1. Thus, non-conserved residues in the XIP region may be essential in maintaining the structural stability of the Na^+-dependent inactive state of NCX1. Furthermore, the XIP region must interact with other regulatory domains of the protein, such as the mutually exclusive exon, thereby contributing to the structure-function relationship as well as the regulatory phenotype of each Na^+-Ca^{2+} exchanger variant and isoform.

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The Role of Phospholamban Cysteines in the Activation of the Cardiac Sarcoplasmic Reticulum Calcium Pump by Nitroxyl

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Phospholamban (PLN) is an integral membrane protein that regulates the Ca^{2+} pump (SERCA2a) in cardiac sarcoplasmic reticulum (CSR). Phosphorylation of PLN in response to β-adrenergic stimulation enhances cardiac inotropy by increasing CSR Ca^{2+} uptake. Nitroxyl (HNO), a new candidate drug therapy for congestive heart failure, improves overall cardiovascular function by increasing Ca^{2+} release and re-uptake in CSR through a direct interaction with RyR2 and SERCA2a, respectively. Using insect cell ER microsomes expressing SERCA2a +/− PLN (WT and Cys → Ala mutant) we have shown that activation of SERCA2a by HNO is PLN-dependent and entails covalent modification of PLN cysteines. Although HNO stimulates SERCA2a activity by uncoupling PLN from SERCA2a, the role of the cysteine residues in the activation mechanism is not completely understood. We propose that HNO, a thiol oxidant, modifies one or more of the three PLN cysteine residues (C36, C41, C46), affecting the regulatory potency of PLN toward SERCA2a. Examples include intra-molecular disulfide cross-links within single PLN molecules or inter-molecular disulfide cross-links between PLN molecules or PLN and SERCA2a. To test this hypothesis, we have constructed a series of PLN mutants containing single, double and triple cysteine substitutions (alanine replacing cysteine). Each of these mutant PLNs will be co-expressed with SERCA2a in insect cells and cell microsomes will be treated with Angeli's salt (an HNO donor) to determine which cysteine residue(s) are essential for activation monitored by enzyme assay and fluorescence spectroscopy of SERCA2a. The results show that intermolecular PLN disulfides play a minor role in activation by HNO. Studies with the Cys → Ala mutations will be useful in determining which cysteine pairs in PLN contribute to intramolecular disulfide cross-links leading to the relief of PLN inhibition and SERCA2a activation.

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Site Directed Mutagenesis of Human GLTP: Role of Tryptophan Residues

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Glycolipid transfer proteins (GLTPs) are small, soluble, ubiquitously expressed proteins that selectively accelerate the intermembrane transfer of glycolipids in vitro. Mammalian GLTPs (209 aa) are intrinsically fluorescent by virtue of having three tryptophans and ten tyrosines. The crystal structure of human GLTP (glycolipid-bound form) reveals the importance of W96 in the glycolipid liganding site where its aromatic indole ring acts as a stacking platform that facilitates hydrogen bonding of the initial ceramide-linked sugar with Asp48, Asn52, and Lys55. To gain insights into W96 functionality and to define the role of the other two Trp residues (i.e., W85 & W142), three GLTP Trp mutants (W96Y, W85Y-W96F, W96F-W142Y) were constructed by QuikChange™ site-directed mutagenesis, overexpressed (pET-30) in E. coli, purified by metal ion affinity and FPLC size exclusion chromatography, and characterized by glycolipid transfer activity measurements and by fluorescence and CD spectroscopy. Compared to wtGLTP, the single Trp mutant, W96Y, retained 65% activity; whereas the double Trp mutants, W85Y-W96F & W96F-W142Y, retained 22% and 110% activities. Quenching with acrylamide and potassium iodide at physiological ionic strength resulted in linear Stern-Volmer plots, suggesting accessibility of emitting Trp residues to soluble quenchers and consistent with wtGLTP native folding. However, CD measurements revealed significant differences in the secondary structure of W85Y-W96F-GLTP compared to wtGLTP; whereas W96F-W142Y-GLTP and W96Y-GLTP retained native secondary structure. We conclude that the negative consequences of conservative mutation of Trp 85 suggest a crucial role in proper folding of GLTP; whereas, the tolerance of Trp96 and Trp142 for conservative, but not radical, mutation is consistent with specialized roles in GLTP function, i.e. glycolipid liganding and membrane interaction. [Support: NIH/NIGMS GM45928 & GM34847, NIH/NCI CA121493, The Hormel & Mayo Foundations].

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Effects Of Gap Junction Blockers On The $P2x_7$ Receptor

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Peritoneal macrophages express the $P2X_7$ receptor, which opens a pore in the membrane after long exposure with ATP, allowing passage of molecules up to 900 Daltons. It has been argued that activation of $P2X_7$ receptor leads to opening of an independent pore entity, not structurally related to the $P2X_7$ receptor. Based on results with connexin knock outs and pharmacological manipulation with known gap junction blockers, some groups have included connexins and pannexins, the gap junction-forming proteins in vertebrates, as reliable candidates to provide for the large permeation pores associated with $P2X_7$ activation. In the present study we performed electrophysiological (whole cell patch clamping recordings) and permeabilization assays (optical analysis and FACS analysis) in which both efficacy and specificity of some gap junction blockers were tested at conditions of putative $P2X_7R$ activation by ATP. ATP generated a current in a nA levels that was blocked by well know P2X blockers as BBG, KN-62 and oxidized ATP, in contrast the junction blockers did not interfere with these effect. More than that, the up take assays showed similar results to the patch clamp experiments, none of the junction blockers was able to block the up take of ethidium bromide or propidium iodide. Our results indicate that well-known gap junction pharmacological blockers do not interfere with current generation or dye uptake after activation of $P2X_7$ receptor. Taken together, our data strongly suggest that the high permeability pore evident at prolonged $P2X_7$ activation does not correspond to connexin or pannexin hemichannels in peritoneal macrophages.

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Qm Simulation Of Binding Site In P-type Atpases

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P-type ATPases are enzymes that stablish cation gradients across biologcal membranes where ions are transported using the energy from hydrolysis of ATP. The Na,K-ATPase and SERCA1a transport 3 Na+/ 2 K+ and Ca2+ , respectively, against a concentration gradient for each ATP molecule hydrolyzed. The two enzymes have very high sequence homology and show structural similarities in the binding site of the ions. This raises questions concerning the selectivity of the different structural basis and how each protein select its specificion. The present study aims at characterising this selectivety.

In order to understand the specificity of the enzymes, we investigate the coordination site in the two crystal structure of SERCA1a(PDB ID: 1SU4) and Na+/K+ ATPase(PDB ID: 3B8) by constructing models of the active site. The models are constructed using analogs of the amino acids from the first and second solvation shell. We optimise the geometry of the models by constraining the C-alpha atoms using semi-empirical methods such as PM6 and AM1. The energies and geometries are investigated further using ab initio, restricted Hartree Fock, and density functional theory with the B3LYP functional using the basis set 6-31G(d,p)*. We substitute Ca2 ± ions into the binding pocket of the Na+/K+ ATPase model to investigate how the specificity is related to the coordination environment. Furthermore, the protonation state of side chains coordinating the ions are investigated in order to estimate the influence of pH changes on the binding of ions in the two pumps.

1671-Pos Board B515

Substrate Specificity And Peptide Bond Selectivity In Rhomboid Intramembrane Proteases

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Rhomboid intramembrane proteases catalyze the transmembrane domain cleavage of single-pass membrane proteins. This activity is crucial for the activation of epidermic growth factor receptor ligands Gurken, Keren and Spitz in D. melanogaster. How rhomboids recognize their substrates and select which peptide bond to cleave is not understood. We have studied the substrate specificity and peptide bond selectivity of purified rhomboids from E. coli, P. aeruginosa, D melanogaster and H. sapiens using chimeric substrates containing the transmembrane domains of Gurken, Keren and Spitz. Analysis of the proteolytic products by mass spectrometry reveals that cleavage occurs in the membrane water interface at sites that are shared by both eukaryotic and prokaryotic rhomboids. Mutagenesis of the substrates reveals a helical amino acid motif that is crucial for substrate recognition and peptide bond selection. With insight from computational data a model for the substrate-enzyme complex will be presented.

1672-Pos Board B516

Biophysical Properties of Transmembrane Segment 6 of E.coli MntH Transporter

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The Natural Resistance-Associated Macrophage Protein (Nramp) family of secondary active divalent metal ion transporters plays an important role in a variety of biological processes, such as metal ion homeostasis, in virtually all living organisms. Due to its structural and functional homology with eukaryotic Nramps, the E. coli transporter MntH (Proton-dependent Manganese Transporter) represents a prototypic model to advance understanding of structure-function relationship in Nramp family. Synthetic peptides corresponding to the transmembrane (TM) segments of membrane proteins could serve as a suitable alternative model for studying the structure and interaction of the membrane protein TM domains with biological membranes. In this study the synthetic peptide corresponding to the sixth transmembrane segment (TMS6) of E. coli MntH and its two mutants, in which the His211 residue was substituted by arginine or alanine, were used. TMS6 was previously shown to contain two functionally important histidine residues. The H211A mutation preserves bacterial sensitivity to metal ions and facilitate H+ uptake in the presence of metal ions. In contrast, H211R does not induce metal sensitivity (1,2). The secondary structures of TMS6 and its mutants were determined in model membranes and membrane-mimicking organic environments, using CD spectroscopy. The conformation of the peptides exhibited ordered α and β conformations in these milieus. Furthermore, patch clamp measurements demonstrated that TMS6 was able to form multi-state ion channels in the presence of manganese as a physiological substrate of MntH. The mutant H211R does not show any channel-like activity and with the mutant H211A the ion channel activity was rarely observed.

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1673-Pos Board B517

Solid State NMR of Membrane Proteins: Towards Complex Structural and Functional Information for Bacterial ABC Class Importers

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Despite the wealth of protein structural data available today, membrane protein structural characterization continues to pose a significant challenge in structural biology. Choosing the membrane mimetic is a challenge and often detergent micelles are employed. However, detergents are prone to induce distortions in the protein structure. An emerging technique, solid state nuclear magnetic resonance (NMR), provides a path to structural characterization using an environment similar to the native one, liquid crystalline lipid bilayers.

Static solid state NMR experiments on proteins determine the orientation of the peptide planes with respect to the magnetic field. All ^{15}N-^{1}H dipolar couplings and anisotropic ^{15}N chemical shifts observed in two dimensional separated local field experiments (PISEMA) lie within a butterfly shape in the spectrum. Special patterns called PISA wheels arise for uniformly aligned protein samples which directly reflect the orientation of protein secondary structure. Using these wheels the tilt angle of each helical axis from the magnetic field and membrane normal can be determined without complete structure determination. Consequently, a single data set allows for characterization of secondary structure in the membrane mimetic as well as providing a set of high resolution peptide plane orientations that can be used directly in structural refinement.

Uniform alignment has been achieved for multiple proteins in our laboratory. Still, studies on larger membrane proteins are required to make solid state NMR a generally applicable technique for membrane protein structure characterization. One excellent example is SugAB, the transmembrane domain of an ABC importer from *Mycobacterium tuberculosis.* These transport proteins contain at least 10 transmembrane helices. This makes SugAB an excellent target for determining the utility of solid state NMR to structurally characterize large membrane proteins.

1674-Pos Board B518

Re-examination of the Role of the Amino-Terminus of SecA in Promoting Its Dimerization and Functional State

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The SecA nanomotor promotes protein translocation in Eubacteria by binding both protein cargo and the protein-conducting channel and undergoing ATP-driven conformation cycles that drive this process. Conflicting reports exist as to whether SecA functions as a monomer or dimer during this dynamic process.Here we re-examine the role of amino and carboxyl termini of SecA in promoting its dimerization and functional state by examining three secA mutants and their respective proteins: SecAD8 lacking residues 2-8, SecAD11 lacking residues 2-11, and SecAD11/N95 lacking both residues 2-11 and its carboxyl-terminal 70 residues. We demonstrate that whether or not SecAD11 or SecAD11/N95 was functional for promoting cell growth depends solely on their vivo levels that appear to govern residual dimerization. Cell fractionation revealed that SecAD11 and SecAD11/N95 were still proficient in membrane association, although they were reduced in the formation of integral membrane SecA. The presence of a modestly higher level of SecAD11/N95 in the membrane and its ability to form dimers as detected by chemical crosslinking were consistent with the higher secA expression level and better growth property of this mutant compared to secAD11. Biochemical studies showed that SecAD11 and SecAD11/N95 displayed identical dimerization defects, while SecAD8 was intermediate between these proteins and wildtype SecA. Furthermore, both SecAD11 and SecAD11/N95 were equally defective in their translocation ATPase specific activity. Our studies show that the non-essential carboxyl-terminal 70 residues of SecA play no role in its dimerization, while increasing truncation of the amino-terminal region of SecA from 8 to 11 residues results in an increasing defect in SecA dimerization and poor in vivo function unless highly overexpressed and also clarify a number of conflicting reports in the literature and support the essential nature of the SecA dimer.

1675-Pos Board B519

Substrate Selectivity in Adic, an E. Coli Inner Membrane Arginine-agmatine Antiporter

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AdiC is a membrane antiporter that transports arginine and its decarboxylation product agmatine across E. coli inner membrane. It plays a key role in the arginine-dependent extreme acid resistance. We overexpressed AdiC in E. coli and reconstituted the purified protein into liposomes. A series of arginine analogs were tested on the transporter. The permeability sequence is as follows: arg / agm > 1,5-diaminopentane, 1,6-diamino-hexane, 1,4-diaminobutane >> argininamide, lysine, ornithine, canavinine. Kinetic analysis results are K_m~ 1mM for 1,5-diaminopentane, ~3 mM for argininamide and lysine, ~15 mM for canavanine.

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Identification of Functionally Important Sites within the Cysteine-Free Inner Membrane Transferase Protein ArnT

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The bacterial inner membrane protein ArnT confers resistance to the antibiotic polymyxin in *Salmonella typhimurium* and *Escherichia coli* through the modification of lipid A, the major component of the outer surface of Gram-negative bacteria. ArnT transfers a neutral aminoarabinose moiety (L-Ara4N) onto the negative phosphate group(s) of lipid A, which significantly reduces the surface charge of these bacteria and thus prevents cationic peptides such as polymyxin from electrostatically recognizing and killing the bacteria. We have previously reported the first expression, purification and functional analysis of ArnT from *S. typhimurium*, and our studies showed that ArnT is highly α-helical and described a new *in vivo* functional assay. In this continuation of the characterization of the ArnT protein, we used the cysteine-specific maleimide-PEG_{5000} to demonstrate that all eight of the native cysteines in *S. typhimurium* ArnT are in the reduced form and therefore not involved in disulfide bonds. In addition, we created a cysteine-free protein that is structurally and functionally intact as determined by circular dichroism and the results of the new *in vivo* growth