View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Elsevier - Publisher Connector

results obtained for backdoor phosphorylation of the enzyme in the presence of Pb^{2+} ions.
In conclusion, we demonstrate that Pb^{2+} bound to the enzyme stabilizes an E_2-type conformation. In particular, under conditions that promote enzyme phosphorylation, Pb^{2+} ions are able to confine the Na^+,K^+-ATPase into a phosphorylated E_2 state (4).

Acknowledgments
The authors thank prof. H.-J. Apell for providing them with membrane fragments containing the Na^+,K^+-ATPase. The MIUR (Ministero dell'Istruzione, dell'Università e della Ricerca) and the Ente Cassa di Risparmio di Firenze, are gratefully acknowledged for financial support.

1. Grinvald, A., *et al.*, **Biophys. J.** 39(1982), 301-308.
2. Bühler, R., *et al.*, **J. Membr. Biol.** 121(1991), 141-161.
3. Gramigni, E., *et al.*, **Chem. Res. Toxicol.** 22(2009), 1699-1704.
4. Bartolommei, G., *et al.*, **Biophys. J.** doi:10.1016/j.bpj.2010.07.050(2010), in press.

2517-Pos Board B503

The Mechanism of Bacterial Cu^+-ATPases. Distinct Efflux Rates Adapted to Different Function

Daniel Raimunda, Manuel González-Guerrero, Blaise Leeber, José M. Argüello.
Chemistry and Biochemistry Department, Worcester Polytechnic Institute, Worcester, MA, USA

Copper plays important physiological roles as a protein co-factor. In bacteria, cytoplasmic copper homeostasis is controlled by P_{1B-1}-type Cu^+-ATPases. These highly homologous transporters share a common structure and the classical Albers-Post (E1/E2) mechanism. It is accepted that most of these enzymes drive cytoplasmic metal efflux and consequently confer Cu^+ tolerance. Other members of this subfamily appear required for cuproprotein assembly. Early studies using gene deletion, phenotypical characterization and functional complementation, have suggested that these are in fact Cu^+ importers. To explain this phenotypical observation within the mechanistic constrains of the transport cycle of P-type ATPases, we have studied the Cu^+ transport by three proposed Cu^+ importers: *P. aeruginosa* CopA2, *E. hirae* CopA and *Synechocystis* PCC6803 CtaA. These were expressed in an *E. coli* strain lacking its endogenous Cu^+-ATPase (DC194), functional complementation was tested, and $^{64}Cu^+$ transport into everted vesicles measured. These experiments show that all Cu^+- ATPases drive Cu^+ efflux although with quite different kinetics. ATPases involved in cuproprotein assembly have much slower transport rates and high apparent metal affinities. These characteristics explain phenotypes observed upon mutation of the coding genes.

2518-Pos Board B504

Charge Movement in Recombinant Copper ATPase is Investigated on a Solid Supported Membrane

Francesco Tadini-Buoninsegni, Gianluca Bartolommei, Maria Rosa Moncelli, Rajendra Pilankatta, David Lewis, Giuseppe Inesi.

The ATP7B copper ATPase is included in the P1-type ATPase subfamily, which is selective for soft and transition metals. Its function is to deliver copper to nascent metalloproteins and export excessive copper from the cell (1). Biochemical characterization of this enzyme is hindered by its very low native abundance and difficult detection of copper signal within the catalytic time frame. We recently reported high yield heterologous expression of ATP7B in COS-1 cells infected with adenovirus vector, and functional characterization of membrane-bound ATPase obtained with the microsomal fraction of infected cells (2). Here we show that the membrane-bound copper ATPase obtained under the above-mentioned conditions is suited for adsorption on a solid supported membrane and measurements of charge transfer. Using this method, we detected charge movement within a single catalytic cycle upon addition of ATP. We suggest that the observed charge movement is due to displacement of bound copper, and it is related to formation of phosphoenzyme intermediate. ATP dependent charge movements, as well as phosphoenzyme formation, are totally prevented by single mutations of copper binding sites.

This work was supported by the U.S. National Institutes of Health (RO301-69830 from the NHBLI), the Italian Ministry of Education, University and Research (PRIN 2008), the Ente Cassa di Risparmio di Firenze and the Italian Ministry of Foreign Affairs (Joint Mobility Project n.22 for the Exchange of Researchers between Italy and USA, 2008-10).

1. Lutsenko S., N.L. Barnes, M.Y. Bartee, and O.Y. Dmitriev. 2007. Physiol. Rev. 87:1011-1046.
2. Pilankatta R., D. Lewis, C.M. Adams, and G. Inesi. 2009. J. Biol. Chem. 284:21307-21316.

2519-Pos Board B505

Genetic Incorporation of an Unnatural Fluorescent Amino Acid in a Plant H-ATPase Expressed in Yeast

Bassem Gayed, Hyun Soo Lee, Michael G. Palmgren, Joshua R. Berlin.

Fluorescence labeling provides an important tool to study the structure and function of proteins. Typically, fluorescence labeling involves either chemical modification or protein fusion with fluorescent probes. These techniques, however, have several significant drawbacks. First, the degree to which fluorescent probes interfere with protein folding and function is a concern. Second, many protein domains are difficult to target specifically. In the particular case of integral membrane proteins, few fluorescent probes can be targeted specifically to transmembrane domains without significantly disturbing protein function. Thus, small fluorescent probes that minimally perturb the structure and function of integral membrane proteins are needed. In this study, we genetically incorporated Anap, an environmentally-sensitive fluorescent unnatural amino acid, into specific locations in the Arabidopsis thaliana type 2 H+-ATPase (AHA2) expressed in Saccharomyces cerevisiae. The nonsense stop codon TAG was substituted for Trp codons to incorporate the unnatural amino acid into specific sites within the first two transmembrane alpha helices of AHA2 using an engineered suppressor tRNA that carries the artificial amino acid and is orthogonal to natural amino acids. Culture conditions were varied to maximize expression of a fluorescent 97 kDa protein in yeast that was only observed when Anap was included in the culture media. By taking advantage of the environmentally sensitive properties of this fluorescent unnatural amino acid, the resultant labeled protein can be used to study conformational dynamics during the enzyme cycle that may result from changes in the local environment surrounding the incorporated fluorescent probe. Fluorescence labeling of membrane proteins using this approach should allow detailed studies of local conformational kinetics that will shed new light on structure function relationships in this class of enzymes.

2520-Pos Board B506

PMCA Differential Exposure of Hydrophobic Domains after Calmodulin and Phosphatidic Acid Activation

Juan Pablo F. Rossi, Irene C. Mangialavori, Ana Maria, Villamil Giraldo, Maria F. Pignataro, Mariela S. Ferreira Gomes, Ariel J. Caride.

The exposure of plasma membrane calcium pump to surrounding phospholipids was assessed by measuring the incorporation of the photoactivatable reagent [125I]TID-PC/16 into the membrane regions of this pump. In the absence of activators, Ca2+ increases the incorporation of [125I]TID-PC/16. On the contrary, in the presence of Ca2+ and either calmodulin or phosphatidic acid the incorporation of the labeled phospholipids is decreased. Proteolysis of PMCA with V8 protease results in 3 main fragments: fragment N (TM 1 and 2), fragment M (TM 3 and 4) and fragment C (TM 5 to 10). In the presence of Ca2+, CaM decreased the level of incorporation of [125I]TID-PC/16 to fragments M and C, while phosphatidic acid decreased the incorporation of [125I]TID-PC/16 to fragments N and M, suggesting that the conformational changes induced by calmodulin or phosphatidic acid extend to the transmembrane domains. The result also indicates differences between the active conformations produced by calmodulin and acidic phospholipids. To verify this, we also measured FRET between PMCA labeled with eosin isothiocianate at the ATP binding site and Rho-PE included in PMCA-containing micelles. CaM decreased the efficiency of the energy transfer between these two probes while PA did not. The result indicates that activation by CaM increases the distance between the ATP binding site and the membrane, but acidic phospholipids do not. Moreover, the access of two proteases to their sites of cleavage was different in the presence of calmodulin or phosphatidic acid. The results indicate structural differences between the PMCA conformations induced by these activators.

2521-Pos Board B507

Different Pathways for Association and Dissociation of the Calmodulin Binding Domain of Plasma Membrane Calcium Pump Isoform 4b

John T. Penniston, Ariel J. Caride, **Emanuel E. Strehler**.

The calmodulin (CaM) binding domain of isoform 4b of the Plasma Membrane Ca^{2+} Pump (PMCA4b) is represented by the peptide C28. CaM binds to either PMCA or C28 by a mechanism in which the primary anchor residue of the binding domain (Trp 1093) binds to the C-terminal lobe of the extended CaM molecule, followed by collapse of CaM with the N-terminal lobe binding to the secondary anchor Phe 1110 (N Juranic et al. [2010] J Biol Chem 285:4015-4024). This is a relatively rapid reaction, with a half time of about 1 sec. The dissociation of CaM from PMCA4b or C28 is much slower, with a half time of about 10 min. Fluorescence measurements with C28 and the fluorescent CaM derivative TA-CaM, and molecular dynamics calculations concur in showing that the path of release of the PMCA4b CaM binding domain is quite different from that of binding. We now show that in the presence

of Ca^{2+}, dissociation of CaM from C28 occurs by a pathway in which Trp 1093, although deeply embedded in a pocket in the C-terminal lobe of CaM, leaves first. The dissociation begins by relatively rapid release of Trp 1093, followed by very slow release of Phe 1110, removal of C28 and return of CaM to its conformation in the free state. The intermediate of dissociation with exposed Trp 1093 has a long lifetime (minutes), and is an activated form of PMCA. This mechanism may explain some unique biological properties of PMCA4b.

2522-Pos Board B508

Oligomeric Interactions of Sarcolipin and SERCA Detected by FRET Microscopy

Joseph M. Autry, John E. Rubin, Seth L. Robia, David D. Thomas.

We have monitored molecular interactions of sarcolipin (SLN) and the sarcoplasmic reticulum Ca- ATPase (SERCA) by measuring Förster resonance energy transfer (FRET) between fusion proteins labeled with cyan fluorescent protein (donor) and yellow fluorescent protein (acceptor). SLN is a key membrane protein that controls muscle contractility by regulating the calcium transport activity of SERCA, and perhaps by also acting as an ATP-activated anion channel (Becucci et al., Biophys. J. 2007, 2009). Here we used baculovirus expression of fluorescent fusion proteins in insect cells and FRET microscopy to provide novel evidence for (a) independent oligomerization of SLN and (b) regulatory complex formation between SERCA and SLN. FRET assays demonstrated that SLN monomers self-assemble into dimers and higher-order oligomers in the absence of SERCA, but that SLN monomers also bind to SERCA in a 1:1 binary complex when the two proteins are co-expressed. FRET assays further demonstrated that the binding affinity of SLN:SLN homo-oligomers is greater than the binding affinity of SERCA:SLN hetero-dimers, indicating that SLN monomers favor self-association over SERCA binding. Mutating SLN residue isoleucine-17 to alanine (I17A) decreased FRET for SLN self-association and eliminated multimeric assembly of SLN, converting higher-order oligomers into monomers and dimers. The I17A mutation also decreased FRET for SERCA:SLN binding but maintained 1:1 stoichiometry of hetero-dimer formation. Thus, isoleucine-17 is a residue that plays dual roles in (a) determining the distribution of SLN homo-oligomers and (b) stabilizing the formation of SERCA:SLN hetero-dimers. When expressed in bacterial cells, wild-type SLN prevented colony formation but the I17A-SLN mutant did not, indicating that higher-order oligomers of SLN exhibit antibacterial activity, possibly through channel formation. We propose that SLN exists as multiple molecular species in muscle membranes, including SERCA-free (monomer, dimer, oligomer) and SERCA-bound (hetero-dimer).

2523-Pos Board B509

Identification of Phospholemman Residues Critical to Phospholemman Oligomerization and Na Pump Association

Julie Bossuyt, Joseph C. Li, Mounir Khafaga, Linda L. Lee,
Luiza Mamikonian, Sanda Despa, Donald M. Bers.

Phospholemman (PLM or FXYD1) associates with and modulates Na pump (NKA) function in a manner similar to phospholamban (PLB) regulation of SERCA. That is, PLM inhibits NKA by reducing its $[Na]_i$-affinity and PLM phosphorylation relieves this inhibition. We have also shown that PLM forms homo-oligomers (as does PLB) but it is unknown how PLM-PLM interactions occur or what functional role PLM oligomers have. Here we use site-directed mutagenesis and FRET to identify which PLM residues are critical to the PLM-NKA and PLM-PLM association, respectively. We hypothesize that mutations affecting PLM-PLM affinity will enhance PLM-NKA complexes and display stronger NKA inhibition, and vice versa. Based on crystal NKA-FXYD structure, the PLM sites G20, F28 & G31 were selected as potential NKA interaction sites and I26, I29, L30, L33&L36 as putative PLM interaction sites (analogous to leucine zippers thought to mediate PLB pentamerization). Alanine substitution of F28 eliminated PLM-NKA FRET (F_{donor} increase by only $0.2\pm4\%$ vs $18\pm2\%$ for WT). Despite only minor effects on PLM-NKA interaction for G31A (F_{donor} increase by $14\pm1\%$), its PLM-PLM FRET was significantly increased (F_{donor} increase by $55\pm3\%$ vs $37\pm5\%$ for WT). Alanine substitution of I26, L30 & L33 all significantly reduced PLM-PLM FRET (F_{donor} increase by $15\pm2\%$, $13\pm3\%$ and $20\pm3\%$ respectively vs $37\pm5\%$ for WT) and opposing effects on PLM-NKA FRET were observed (e.g. F_{donor} increases by $33\pm6\%$ for L33A vs $18\pm2\%$ for WT). We conclude that PLM homo-oligomers have a structural basis that parallels PLB (Leucine zipper). Moreover our hypothesis that altering the PLM affinity for NKA also changes the interaction of PLM with other PLM molecules (and vice versa) seems to be correct. Our data support the existence of a dynamic equilibrium of PLM oligomers and PLM-NKA that exerts functional regulation of NKA.

2524-Pos Board B510

Highly Specific, Conformationally-Dependent Cross-Linking of Lys27 of PLB to SERCA2a in Cardiac SR Vesicles from Humans

Brandy L. Akin, Larry R. Jones.

Phospholamban (PLB) inhibits SERCA2a, the Ca2+-ATPase of cardiac sarcoplasmic reticulum (SR), by decreasing the apparent Ca2+ affinity of the enzyme. The mechanism of Ca2+ pump inhibition by PLB has been studied by our group using chemical cross-linking agents, which enable PLB-binding interactions with SERCA2a to be measured simultaneously with enzyme inhibition. Previously, cross-linking of canine PLB to SERCA2a was only attainable with Cys-scanning point mutants of PLB. For example, N27C of canine PLB cross-links exclusively to Lys328 of canine SERCA2a with heterobifunctional thiol-to-amine cross-linking agents after co-expression of the two proteins in Sf21 insect cells. Here, we show with SR vesicles prepared from human hearts, that PLB and SERCA2a are cross-linkable using DSG (disuccinimidyl glutarate), a 7.7 Å long, homobifunctional, amine-specific cross-linking agent. Cross-linking of human PLB to SERCA2a takes advantage of the unique Lys residue at position 27 of human PLB, making it susceptible to amine-specific cross-linkers without the need for mutagenesis. This was confirmed by testing SR vesicles prepared from both human and canine ventricles; DSG cross-linked human, but not canine, PLB to SERCA2a. Cross-linking of human PLB to SERCA2a was completely inhibited by either Ca2+ (Ki = 0.50 μM), or the Ca2+ pump inhibitor thapsigargin, but substantially augmented by ATP. This is the first demonstration that PLB binds exclusively to the E2 conformation of SERCA2a in SR vesicles, preferentially the state with bound nucleotide (E2·ATP), and not the state stabilized by thapsigargin (protonated E2). Importantly, similar results were obtained with SR vesicles prepared from both normal and failed human hearts, indicating that PLB-binding interactions with SERCA2a are unchanged in failing myocardium. Studies are in progress to demonstrate with human SR vesicles that Lys27 of PLB cross-links exclusively to Lys328 of SERCA2a.

2525-Pos Board B511

Characterizing the Phospholamban-SERCA Complex by Pulsed EPR Spectroscopy

Zachary M. James, Kurt D. Torgersen, Christine B. Karim,
David D. Thomas.

Muscle contraction and relaxation are controlled through the release and reuptake of Ca^{2+} stored in the sarcoplasmic reticulum (SR). Relaxation is mediated by the SR Ca^{2+} ATPase (SERCA), a pump that drives Ca^{2+} against its concentration gradient while hydrolyzing ATP. Cardiac SERCA is regulated by phospholamban (PLB), a small membrane protein that inhibits the pump except when phosphorylated at Ser16. PLB phosphorylation restores SERCA activity without dissociating the two proteins, instead inducing a structural change within the PLB-SERCA complex. Although a number of studies have investigated the interaction of these proteins, the relationship between phosphorylation, structure, and activity remains unresolved. We have used dipolar electron-electron resonance (DEER) spectroscopy, a technique capable of measuring distances from 2-7nm, to characterize large conformational changes within PLB upon phosphorylation and SERCA binding. Our results show that the transmembrane and cytoplasmic helices of PLB draw closer upon SERCA binding, with subsequent phosphorylation compacting the structure still further. However, relative distances between the cytoplasmic domains of PLB and SERCA remain largely constant before and after phosphorylation, suggesting that the observed structural change occurs in the transmembrane domain of PLB. Ultimately, our goal is to make exhaustive distance measurements within the SERCA-PLB complex in order to understand the structural basis of phosphorylation-mediated inhibition relief.

2526-Pos Board B512

Structural Dynamics of SERCA and Phospholamban by TR-FRET and TIRF

Ji Li, Christine Karim, David D. Thomas.

We used fluorescence to investigate the structure and dynamics of phospholamban (PLB) and its inhibited target, sarcoplasmic reticulum Ca-ATPase (SERCA). Time-resolved fluorescence energy transfer (TR-FRET) was used to study the oligomeric interaction between PLB and SERCA. Previous work on our lab has shown that PLB is primarily pentameric but SERCA binds preferentially to the monomeric form in lipid vesicles. Recent EM studies suggest that the PLB pentamer might also bind to SERCA. We tested this hypothesis by labeling SERCA at C674 with a fluorescent donor (TMRIA) and labeling PLB at K3 with a non-fluorescent acceptor (MGITC), then reconstituting the proteins into lipid vesicles and performing TR-FRET as a function of the fraction of acceptor-labeled PLB (xA), keeping the total PLB/SERCA molar ratio constant at 10. Simulations showed that if a PLB monomer binds to SERCA, the dependence of FRET on xA should be linear, but the binding of a PLB oligomer