Published in final edited form as:

Biochemistry. 2008 November 25; 47(47): 12448–12456. doi:10.1021/bi8014289.

Concerted but Noncooperative Activation of Nucleotide and Actuator Domains of the Ca-ATPase Upon Calcium Binding

Baowei Chen[†], James E. Mahaney[‡], M. Uljana Mayer[†], Diana J. Bigelow[†], and Thomas C. Squier[†]

[†]Pacific Northwest National Laboratory, P.O. Box 999, Richland, WA 99352

[‡]Virginia College of Osteopathic Medicine, Blacksburg, Virginia 24060

Abstract

Calcium-dependent domain movements of the actuator (A) and nucleotide (N) domains of the SERCA2a isoform of the Ca-ATPase were assessed using constructs containing engineered tetracysteine binding motifs, which were expressed in insect High-Five cells and subsequently labeled with the biarsenical fluorophore 4',5'-bis(1,3,2-dithioarsolan-2-yl)fluorescein (FAsH-EDT₂). Maximum catalytic function is retained in microsomes isolated from High-Five cells and labeled with FAsH-EDT₂. Distance measurements using the nucleotide analog TNP-ATP, which acts as a fluorescence resonance energy transfer (FRET) acceptor from FAsH, identify a 2.4 Å increase in the spatial separation between the N- and A-domains induced by high-affinity calcium binding; this structural change is comparable to that observed in crystal structures. No significant distance changes occur across the N-domain between FAsH and TNP-ATP, indicating that calcium activation induces rigid body domain movements rather than intradomain conformational changes. Calcium-dependent decreases in the fluorescence of FAsH bound respectively to either the N- or A-domains indicate coordinated and noncooperative domain movements, where both A- and N-domains display virtually identical calcium dependencies (i.e., $K_d = 4.8 \pm 0.4 \mu\text{M}$). We suggest that occupancy of a single high-affinity calcium binding site induces the rearrangement of the A- and N-domains of the Ca-ATPase to form an intermediate state, which facilitates phosphoenzyme formation from ATP upon occupancy of the second high-affinity calcium site.

P-type ATPases form a family of primary ion transporters that utilize ATP to actively transport a wide range of different ions against a concentration gradient, acting to control many different fundamental biological processes ranging from muscle contraction and nerve conduction to the extrusion of toxic metals from cells (1). Important insights regarding ion transport mechanisms for this family of P-type ATPases have been obtained from the wealth of structural information available for the sarco/endoplasmic reticulum Ca-ATPase (SERCA), including the 25 atomic resolution structures crystallized in nine different enzyme intermediate states (2–5). These crystal structures confirm earlier spectroscopic measurements that established that ATP binds within a nucleotide binding domain that is spatially distant (i.e., $> 50 \text{ Å}$) from calcium ion binding sites located near the bilayer center (6–8), and that large amplitude domain motions couple ATP hydrolysis to ion transport (2, 5, 9–14). Movements of three cytosolic regions, i.e., the nucleotide binding (N), phosphorylation (P), and actuator (A) domains (Figure 1), are modulated by large

Correspondence should be addressed to : Thomas C. Squier, Pacific Northwest National Laboratory, P.O. Box 999, Mail Stop P7-53; Richland, WA 99352; thomas.squier@pnl.gov; Tel: (509)-376-2218; FAX: 509-372-1632.

SUPPORTING INFORMATION AVAILABLE

Spacefilling depictions are shown of known structures of the Ca-ATPase in different enzyme states highlighting solvent accessibilities of proteinase K cleavage sites Leu119 and T242. This material is available free of charge via the Internet at <http://pubs.acs.org>.

rearrangements of transmembrane helices in response to calcium occupancy of high affinity sites. These changes act to re-position the N-domain relative to the P-domain, thus promoting the transfer of the γ -phosphoryl group of ATP to Asp³⁵¹ that forms a phosphoenzyme intermediate within the P-domain (5).

Solution structures using probes bound to the N- and P-domains have been measured, and are consistent with high-resolution x-ray structures (9, 10). Coordinated motions of the actuator (A) domain are proposed to facilitate the association between the N- and P-domains necessary for phosphoenzyme formation and calcium occlusion (5). However, it remains unclear whether the large amplitude motions of the A-domain apparent in high-resolution crystal structures accurately reflect average structures in solution (15, 16). Furthermore, as crystal structures depict the fully activated enzyme bound to two calcium ions, it is uncertain how conformational rearrangements of cytoplasmic domains contribute to intermediate enzyme states associated specifically with cooperative binding of the second calcium ion (5, 17).

To examine how calcium binding promotes the activation of the Ca-ATPase through changes in average domain conformation, and the relevance of reported crystal structures to the average conformation of the enzyme in solution, we have used molecular probes to label sites engineered into separate constructs of SERCA2a on the nucleotide and actuator domains of the Ca-ATPase. These measurements utilize the biarsenical fluorescent probe 4', 5'-bis(1,3,2-dithioarsolan-2-yl)fluorescein (FLAsH), which binds through a unique tetracoordinate linkage to four introduced cysteines engineered in a tagging sequence loop in the A-domain (i.e., M¹-WDCCKACCK-E²) or the N-domain (i.e., M⁵⁷⁵-WDCCPGCKK-H⁵⁷⁶). These constructs permit the selective labeling of sites in the Ca-ATPase expressed in microsomes (18) (Figure 1). The ability to selectively label the engineered tagging sequence using biarsenical probes permits structural changes to be measured in isolated microsomes, and avoids the requirement for the engineering of mutant proteins to contain single reactive thiols that have to be labeled following their purification and prior to their functional reconstitution (19–21).

Using the nucleotide analog TNP-ATP as a fluorescence resonance energy transfer (FRET) acceptor, we find that the spatial separation between the biarsenical probe FLAsH bound to either the A- or N-domains and TNP-ATP are in excellent agreement with prior crystal structures of the Ca-ATPase (7, 8, 22). Further, we detect coordinated and noncooperative changes in the fluorescence signals from FLAsH bound to the A- and N-domains of the Ca-ATPase upon calcium activation, which indicate that conformational rearrangements associated with enzyme activation involve linked domain movements through direct coupling to occupancy of a single high-affinity calcium binding site.

EXPERIMENTAL PROCEDURES

Materials

4',5'-bis(1,3,2-dithioarsolan-2-yl)fluorescein (FLAsH-EDT₂) was synthesized as previously described (23, 24). N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES) and tris(carboxyethyl)phosphine (TCEP) and 2-mercaptoethanol (β -ME) and 1,2-ethanedithiol (EDT) were obtained from Sigma-Aldrich (Milwaukee, WI). All other chemicals were the purest grade commercially available.

DNA Constructs and Vectors

The rat cardiac muscle sarcoplasmic reticulum Ca-ATPase (SERCA2a) cDNA used for these studies was the kind gift of Dr. Jonathan Lytton, University of Calgary. This SERCA2a cDNA was subcloned into the pMT2 vector at the *Eco*RI sites, but the cDNA also

contained a single *EcoRI* restriction site (1443-GAATTC-1448) within the open reading frame. To remove the internal *EcoRI* restriction site, a silent mutation (1443-GAGTTC-1448) was made using the QuikChange® II XL Site-Directed Mutagenesis System and XL10-Gold® ultracompetent cells (Agilent Technologies (Stratagene), La Jolla, CA). The correct mutation was confirmed by DNA sequencing and by *EcoRI* restriction analysis of the mutated SERCA2a pMT2 construct. Next, the SERCA2a cDNA was excised from the pMT2 vector using *EcoRI* restriction digestion and sub-cloned into the *EcoRI* site in the pVL1392 baculovirus transfer vector (BD Biosciences Pharmingen Inc., San Diego, CA). The correct orientation of the SERCA2a cDNA was confirmed by DNA sequencing at the junction of the pVL1392 and SERCA2a cDNA and by restriction analysis, taking advantage of the single *Bgl*II restriction site in the pVL1392 multicloning site and the single *Bgl*II restriction site in the SERCA2a cDNA open reading frame. At that time, the decision was made to utilize the Bac-to-Bac® Baculovirus Expression System (Invitrogen Inc., Carlsbad, CA). Therefore the rat SERCA2a cDNA was excised from pVL1392 using the restriction enzymes *Not* I and *Xba* I, then subcloned into the corresponding sites in the pFastBac1™ baculovirus transfer vector multicloning site (Invitrogen). All subsequent mutagenesis work was carried out using this construct.

SERCA2a mutagenesis

Two tetracysteine motif SERCA2a constructs were made. The first contained the amino sequence Trp-Asp-Cys-Cys-Lys-Ala-Cys-Cys-Lys inserted between Met¹ and Glu² in the A-domain of wild-type SERCA2a. The second construct contained the amino acid sequence Trp-Asp-Cys-Cys-Pro-Gly-Cys-Cys-Lys inserted in the N-domain between Met⁵⁷⁵ and His⁵⁷⁶ in the wild-type sequence. The insertion mutants were made using the QuikChange® XL site-Directed Mutagenesis System and XL10-Gold® ultracompetent cells (Stratagene). The presence of the insert was confirmed by cDNA sequencing.

Recombinant Baculovirus Preparation

All baculovirus preparation work was carried out using serum free Sf-21 cells from PharMingen. Recombinant baculoviruses containing either the wild-type SERCA2a cDNA or the tetracysteine SERCA2a construct cDNAs were prepared using the Bac-to-Bac® Baculovirus Expression System (Invitrogen). The presence of the given cDNA in the recombinant bacmid was verified by PCR analysis according to the manufacturer's instructions, except that M13 Forward and M13 Reverse PCR primers from Integrated DNA Technologies were used in place of the corresponding primers recommended by Invitrogen. Passage 1 viral stocks for each construct were generated according to the manufacturer's instructions. The viral stock was plaque-purified, and the resultant virus solution was amplified twice to produce a high-titer stock. Viral titer in the stock solutions was determined using the BacPAK™ Baculovirus Rapid Titer Kit (Clontech Inc., Mountain View, CO). Baculovirus stocks typically had a titer of 1×10^8 pfu/mL or greater and were stored at 4 °C in the dark.

Protein Expression and Characterization

SERCA2a (wild-type and mutant) was expressed in High-Five insect cells (Invitrogen) as reported elsewhere (25). Microsomes were harvested 48 h after baculovirus infection and stored in small aliquots at -80 °C. Protein concentrations were measured (26), using bovine serum albumin (Sigma) as a standard. The amount of SERCA2a in the microsomes was quantified by gel electrophoresis and immunoblotting to correspond to about 1.5% of expressed protein, using methods described previously (25). For the immunoblotting, the anti-SERCA2a monoclonal antibody, 2A7-A1 (Affinity Bioreagents, Golden, CO) was the primary antibody used, and protein A conjugated with horse-radish peroxidase (BioRad Inc., Hercules, CA) was used as the secondary antibody. Immunoblots were developed

colorimetrically using the horse-radish peroxidase assay (BioRad) and analyzed by densitometry. This permitted the measurement expression levels of the two SERCA2a tetracysteine motif mutant constructs relative to wild-type enzyme.

SERCA2a Activity Assays

[Ca²⁺]-dependent rates of ATP hydrolysis were measured using High-Five insect cell microsomes (0.05 mg/mL) in 50 mM MOPS (pH 7.0), 3.0 mM MgCl₂, 100 mM KCl, 1.0 mM EGTA and variable amounts of CaCl₂ to give the desired ionized [Ca²⁺], as previously determined (25), where the formation of inorganic phosphate was measured using a colorimetric malachite green-ammonium molybdate assay (ATPase activity), as previously described (25). To initiate the ATPase reaction, 5 mM MgATP was added to the incubation tube at 37 °C, and inorganic phosphate liberation was measured as a function of time. SERCA2a samples were pre-treated with 20 µg of the calcium ionophore A23187 per mg of total protein to prevent Ca²⁺ build-up within the microsome during the assay. Calcium-dependent activity curves were fit using the Hill equation fitting function.

Labeling with FLAsH-EDT₂

Following incubation of microsomes (8 mg/mL) in 150 mM MOPS (pH 7.0) with 10 mM β-ME and 10 mM TCEP for 1 hour at 25 °C, the tetracysteine motif engineered into either the A- or N-domains of the Ca-ATPase were reacted with FLAsH-EDT₂ (15 µM) for 30 minutes at 25 °C. Samples were diluted 3-fold to stop the reaction into 150 mM MOPS/TRIS (pH 7.0) and 50 µM DMPS, and unbound probe was separated from microsomes by two successive centrifugation steps (100,000 × g for 45 minutes). The resulting pellet was resuspended in 150 mM MOPS/TRIS (pH 7.0). Labeled proteins were imaged using a Kodak IS2000MM imager (Rochester, NY), using interference filters centered at 465 nm for excitation and 535 nm for detection.

Fluorescence measurements

Fluorescence measurements used a Fluoro Max-2 fluorometer (SPEX, Edison, NJ), using excitation and emission slits of 5 nm. In all cases, the sample temperature was 25 °C. Changes in the solvent accessibility of FLAsH were assessed through collisional quenching, where variable amounts of acrylamide was added to FLAsH-labeled SERCA expressed in microsomes (50 µg/mL). Data were analyzed as F_0/F as a function of the titrated acrylamide concentration and according to the Stern-Volmer equation:

$$F_0/F = 1 + K_{sv}[Q] \quad (1)$$

where F_0 and F are the respective fluorescence intensities in the absence and presence of added acrylamide $[Q]$. K_{sv} is the Stern-Volmer quenching constant (27).

RESULTS

Expression and Activity of the Ca-ATPase

Wild-type rat SERCA2a and mutants that contain tetracysteine tagging sequences inserted into nonconserved regions in the N- or A-domains were expressed in High-Five insect cells. Quantitative immunoblots and a consideration of fluorescent labeling (see below) indicate that the Ca-ATPase represents about 1.5% of the expressed protein in these preparations, and that there is very little endogenous SERCA in these insect microsomes. Consistent with their small size, the introduction of tetracysteine binding motifs engineered in the N-domain (i.e., M⁵⁷⁵-WDCCPGCK-H⁵⁷⁶) or the A-domain (i.e., M¹-WDCKACCK-E²) has a minimal effect on either the abundance of SERCA2a in isolated microsomes or the

migration of SERCA2a on SDS-PAGE, indicating that these tagging sequences do not dramatically affect the overall enzyme conformation to induce, for example, irreversible protein associations. Further, the expressed Ca-ATPase retains full activity irrespective of the introduction of tetracysteine tagging sequences, where maximal rates of ATP hydrolysis (i.e., 30 $\mu\text{mol P}_i/\text{mg min}$) are comparable to SERCA2a activity observed in microsomes isolated from hearts upon correcting for relative differences in SERCA2a abundance (Figure 2) (28).

Targeted Labeling of SERCA using Biarsenical Probes

Microsomes were labeled using the biarsenical probe FAsH-EDT₂ (Figure 1), where the bound ethanedithiol (EDT) is displaced upon association of each arsenic with a pair of vicinal cysteines in the tagging sequence. Prior measurements have established the specificity of FAsH-EDT₂ for the labeling of tagged proteins in complex environments. Subsequently FAsH was used for functional imaging of protein conformational changes, which is facilitated by the small size and tetracoordinate linkage of the probe (18, 23, 24, 29–33).

SERCA2a constructs with engineered tagging sequences in either the A- or N-domains are preferentially labeled by incubation with FAsH-EDT₂ for 30 minutes, each arsenic chelating a pair of vicinal cysteine ligands (Figure 3A). In the absence of competing 2,3-dimercapto-1-propanesulfonic acid (DMPS), labeling is not absolutely specific, as evidenced by the multiple labeled proteins in addition to the Ca-ATPase visible under UV light. Nonspecific labeling is particularly pronounced in control experiments that involve expression of wild-type SERCA2a with no engineered tagging sequence. However, the tetracysteine tagged SERCA2a constructs compete more effectively for added FAsH-EDT₂ than nonspecific sites.

Considerable improvements in labeling specificity are possible if DMPS is added following labeling with FAsH-EDT₂ to displace FAsH bound to lower affinity nonspecific sites. Addition of 50 μM DMPS is sufficient to effectively eliminate nonspecific binding at all sites that do not contain the tetracysteine tagging sequence (Figure 3A). In this respect, it is notable that wild-type SERCA2a which lacks the tetracysteine tagging sequence, is not labeled under these conditions, demonstrating that there is minimal nonspecific labeling of the Ca-ATPase itself (see lane 1 in Figure 3A). Some unbound (i.e., free) FAsH-EDT₂ is apparent near the dye front under these conditions, but can be removed by centrifugation of labeled microsomes (Figure 3B). Thus, we demonstrate the facile ability to selectively label the tagged Ca-ATPase, with background signals associated with nonspecific labeling by FAsH accounting for less than 9% of the total fluorescence signal (Figure 3C).

Coordinated and Noncooperative N- and A-Domain Motions Upon Calcium Binding

An ability to selectively label individual sites on the A- and N-domains of SERCA in native microsomes provides a means, for the first time, to simultaneously assess movements between these key domain elements upon calcium activation of the Ca-ATPase. Calcium-dependent conformational changes were assessed from a consideration of both i) solvent accessibilities to the collisional quencher acrylamide and ii) fluorescence intensity changes for FAsH bound to either the A- or N-domains of the Ca-ATPase. Prior to calcium activation, the solvent accessibilities of FAsH bound to either the A- or N-domains of the Ca-ATPase are very similar (Figure 4). Upon calcium activation, there is a large increase in the solvent exposure of FAsH bound to the A-domain as evidenced by the increased slope. Increases in solvent exposure at this labeling site are consistent with high-resolution structures, where calcium activation results in a large reorientation of the A-domain that repositions the tagging sequence away from the interdomain interface with the N-domain

(i.e., 1iwo.pdb vs 1su4.pdb) (34). These large movements of the A-domain are proposed to allow the N- and P-domains to move into closer proximity to facilitate phosphoenzyme formation (3, 22). In comparison, there are no significant changes in the solvent accessibility of FIAsh bound to the N-domain upon calcium activation, as is expected from the relatively modest calcium-dependent changes in the orientation and structure of the N-domain in available high-resolution structures. Observed changes in the solvent accessibilities of FIAsh bound to the A- or N-domains upon calcium activation are further reflected by the magnitude of the fluorescence intensity, as the fluorescence intensity of FIAsh bound to either the A- or N-domains respectively decreases by 23% and 9% upon calcium activation of the Ca-ATPase (Figure 5A,B).

Using changes in the fluorescence intensity of FIAsh bound to either the A- or N-domains to monitor their calcium-dependent activation, we observe both signals increase with calcium concentrations, but to different extents. However, essentially identical calcium dependencies are observed, which suggest coordinated movements following calcium binding (Figure 5C). Upon fitting both data sets, we find that the macroscopic dissociation constant (K_d) obtained from fitting the data to the Hill equation is $0.48 \pm 0.04 \mu\text{M}$, which is essentially identical to that associated with the calcium-dependent activation of ATP hydrolysis (Figure 2). However, in contrast to observed calcium-dependent increases in rates of ATP hydrolysis ($n_H = 1.6 \pm 0.2$) (Figure 2), we observe that calcium-dependent transitions involving both the A- and N-domains are noncooperative with a Hill coefficient (n_H) of 0.93 ± 0.07 (Figure 5C). These latter results suggest that occupancy of a single high-affinity calcium binding site is sufficient to mediate the large conformational changes apparent in the crystal structure.

Changes in Spatial Arrangement of A- and N-domains upon Calcium Activation

From available high-resolution crystal structures, high levels of ATP in the cell are suggested to modulate the structure of the Ca-ATPase, acting to maintain a compact headpiece organization (22). Movements of the A-domain are proposed to play a central role in mediating concerted movements of transmembrane helices upon calcium binding that promote phosphoenzyme formation from ATP (3). In the case of the A-domain, sensitivities of proteinase K cleavage sites L119 and T242 in stalk regions connecting the A-domain to the transmembrane helices have provided a means to assess ligand-dependent structural changes in solution. These measurements confirm that calcium binding induces an A-domain reorganization that acts to expose T242 for proteolytic cleavage (compare 1iwo.pdb with 1su4.pdb in Figure S1 in Supporting Information) (12, 34, 35). However, in the presence of bound nucleotide there is no direct correspondence between the proteinase K cleavage sensitivities and surface accessibilities in high-resolution crystal structures (e.g., compare 2c88.pdb and 1t5s.pdb in Figure S2 in Supporting Information) (12, 13).

To ascertain whether the crystal structures reliably assess average structures of the Ca-ATPase in solution, we have measured the spatial relationship between FIAsh bound to the A- and N-domains of the Ca-ATPase and TNP-ATP bound to the N-domain. These distance measurements use FIAsh as a fluorescence resonance energy transfer (FRET) donor and TNP-ATP as a FRET acceptor, where prior measurements have demonstrated that TNP-ATP effectively substitutes for ATP in the nucleotide binding site, but is not hydrolyzed under these experimental conditions (36, 37). The FIAsh labeling sites in the A- and N-domains permit us to assess calcium-dependent changes in both the interdomain spatial separation between A- and N-domains and possible intradomain distance changes across the N-domain.

Upon titration of TNP-ATP, we observe a progressive increase in energy transfer efficiency for FIAsh bound to either the A- or N-domains of the Ca-ATPase, which saturates in the

presence of approximately 40 μM added TNP-ATP (Figure 6). Fitting these binding curves to the Hill equation, we find that the apparent dissociation constants are very similar to earlier measurements ($K_d \approx 10 \mu\text{M}$) (36, 38). Prior to calcium binding (i.e., apo-form of the Ca-ATPase), the maximal energy transfer efficiencies between TNP-ATP and FIAsh bound to either the A- and N-domains are respectively $47 \pm 3\%$ and $69 \pm 5\%$, which correspond to distances of $34.6 \pm 0.7 \text{ \AA}$ and $30 \pm 1 \text{ \AA}$. These latter distances compare favorably with two reported high-resolution structures of the apo-form of the Ca-ATPase with the bound nucleotide AMPPCP (i.e., 2dqs.pdb and 2c88.pdb)(Table 1). In these two high-resolution crystal structures the intermolecular distances between the protein backbone of the tagging insertion sequence in the A-domain and the 3' position of the ribose insertion site for the TNP moiety bound to the N-domain are 34 and 36 $\text{\AA}$. Intramolecular distances between the FIAsh-binding site in the N-domain and the bound nucleotide in the two available structures in the protein data bank (<http://www.rcsb.org/pdb>) are 28 $\text{\AA}$, which agrees favorably with our measured distance of $30 \pm 1 \text{ \AA}$.

Following calcium activation there are no significant changes in the measured energy transfer efficiency between FIAsh bound to the N-domain and TNP-ATP (Figure 6B). This latter result indicates that there are no significant changes in the overall dimension of the N-domain upon calcium activation, in agreement with the high-resolution crystal structures (5). In comparison, we observe that there is a substantial decrease in the energy transfer efficiency between FIAsh bound to the A-domain and TNP-ATP, which decreases from $47 \pm 3\%$ for the apo-form of the enzyme to $39 \pm 3\%$ (Figure 6A). The $8 \pm 4\%$ decrease in FRET is indicative of a $2.4 \pm 1.0 \text{ \AA}$ increase in the spatial separation between the A- and N-domains. This latter result is consistent with the reorientation of the A-domain apparent in the high-resolution crystal structure that acts to enhance the association between N- and P-domains necessary for nucleotide utilization (Figure 7). Together, these results indicate a central role for calcium binding in modulating interdomain interactions in both the absence and presence of bound nucleotide (Figures 5, 6, and 7), and argues against prior suggestions that “calcium binding has comparatively minor effects” in the presence of bound nucleotide (11).

DISCUSSION

We have expressed a fully functional SERCA2a isoform of the Ca-ATPase engineered to contain tetracysteine tagging sequences in either the A- or N-domains (Figure 2). Following isolation of microsomes, the encoded tagging sequence in the Ca-ATPase is labeled specifically using the small biarsenical probe FIAsh (Figures 1 and 4). We detect coordinated but noncooperative local structural changes of both the A- and N-domains upon calcium binding that suggest binding of a single calcium acts to reposition cytosolic domain elements (Figure 5C). There are only minimal changes in either the fluorescence intensity or solvent accessibility of FIAsh bound to the N-domain upon calcium activation, and no changes in the intradomain FRET efficiency between FIAsh bound to the N-domain and TNP-ATP. In contrast, large calcium-dependent changes in the solvent accessibilities and fluorescence intensities of FIAsh in the A-domain are observed (Figures 5 and 6), as well as a $2.4 \text{ \AA}$ increase in the interdomain distance between FIAsh and bound TNP-ATP upon calcium binding (Figure 6, Table 1). These measurements are consistent with the substantial reorientation of the A-domain observed comparing crystal structures of the homologous SERCA1 in the absence (1iwo.pdb) with that in the presence (1su4.pdb) of bound calcium (4) (Figure 7).

Prior measurements have demonstrated that the binding affinity of ATP to the Ca-ATPase is unaffected by calcium activation despite the fact that occupancy of calcium binding sites is necessary for the transfer of the γ -phosphate from bound ATP to Asp³⁵¹ to form a

phosphoenzyme intermediate (12). This observation is consistent with the millimolar ATP concentrations present in the cell, and suggests that calcium binding acts to promote phosphoenzyme formation rather than nucleotide binding. Further, in view of the substantial amount of dynamic disorder associated with cytoplasmic domains of the Ca-ATPase (2, 9, 10, 14, 39), calcium binding may promote the adoption of an infrequent conformer required for phosphoenzyme formation. Insight into the molecular mechanism underlying calcium-dependent structural changes is possible from a consideration of the high-resolution structure, which reveals that the two high-affinity calcium binding sites are located near the center of the bilayer in the Ca-ATPase, and share several common ligands (5, 40). However, it has remained uncertain how the association of either the first or second calcium may differentially modify the conformation of headpiece elements to affect phosphoenzyme formation from ATP (5). Our results, demonstrating a noncooperative movement of the A- and N-domains upon calcium activation, suggest that occupancy of each calcium site induces distinct sets of domain rearrangements. For example, binding of a single calcium may induce large-scale domain movements to create a more compact headpiece organization that enhances transfer of the γ -phosphoryl moiety from ATP upon repositioning Asp³⁵¹ to facilitate its phosphorylation upon occupancy of the second calcium. In this respect, closely juxtaposed headpiece domains would have the potential to enhance transport efficiency, as phosphoenzyme formation and calcium occlusion is facilitated upon occupancy of both high affinity calcium binding sites (41).

High-resolution structures indicate that Arg⁷⁶² in TM5 can act as a molecular switch, since this residue has the potential to induce concerted movements of the transmembrane helices that facilitate the formation of high-affinity calcium binding sites and drive movements involving the cytosolic domain elements. Prior to calcium binding, Arg⁷⁶² is proximal to both Asn⁷⁶⁸ (TM5) and Glu⁹⁰⁸ (TM8) within the bilayer interior. Upon calcium binding to site I, which includes ligands Asn⁷⁶⁸ and Glu⁹⁰⁸, Arg⁷⁶² is reoriented by a large-scale rotational movement of TM5 to create a new salt bridge with Asp⁹⁸¹ on TM10 (Figure 8). Thus, charged side-chains within the hydrophobic bilayer remain neutralized. The movement of TM5 and the creation of calcium binding site II promotes the formation of three additional salt bridges located near the aqueous-bilayer interface, that would appear to stabilize the calcium-activated (E1) structure of the enzyme. These salt bridges include Glu⁸⁰-Arg²⁹⁰ and Glu⁹⁰-Lys²⁹⁷, which are respectively located in the loop structures of the luminal interfacial regions connecting TM2/TM3 and TM4/TM5, and Glu²⁴³-Lys⁴⁷ located at the cytoplasmic face of TM3 and TM1 (Figure 8).

Liganding of the second calcium involves Glu³⁰⁹ in TM4, whose conformational rearrangements can directly modify the position of the Asp³⁵¹ side-chain in the P-domain to facilitate the transfer of the γ -phosphoryl group of ATP bound to the N-domain to form the phosphoenzyme intermediate. The importance of site II occupancy in facilitating phosphoenzyme formation is apparent from i) the calcium-dependent cooperativity associated with ATP hydrolysis that mirrors that associated with calcium binding (Figure 2) and ii) from a consideration of the plasma membrane Ca-ATPase (i.e., PMCA), which shares considerable structural homology with SERCA. In particular, a consideration of the sequence of the PMCA indicates a single high-affinity calcium binding site that is homologous to site II in SERCA (42). Moreover, the presence of only one high affinity calcium site is consistent with the larger thermodynamic barrier opposing cation transport across the plasma membrane as compared with that of the sarcoplasmic reticulum. Thus, the calcium-dependent structural transitions and associated rearrangements of TM4 are conformationally coupled to the cytosolic headpiece elements to induce phosphoenzyme formation (43–45).

In conclusion, molecular probes bound specifically to engineered sites on the A- or N-domains of the Ca-ATP reveal coordinated but noncooperative domain movements associated with calcium activation. Large movements of the A-domain are observed, where the average structures of the physiologically important nucleotide bound states are in reasonable agreement with high-resolution crystal structures. Future measurements should measure the kinetics and magnitude of domain motions associated with enzyme activation, and the involvement of specific interfacial sites that stabilize the two major conformers (E1 and E2), taking advantage of the current ability to engineer multiple tags that bind unique small molecule probes (18, 46).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the National Institutes of Health (HL64031) and Genomics:GTL program (45701) within the Office of Biological and Environmental Research at the U. S. Department of Energy. Pacific Northwest National Laboratory is operated for the Department of Energy by Battelle Memorial Institute under contract DE-AC05-76RLO 1830.

Abbreviations used

A-domain	actuator domain
β-ME	beta-mercaptoethanol
DMPS	2,3-dimercapto-1-propanesulfonic acid
EDT	1,2-ethanedithiol
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol bis(β-aminoethylether)-N,N,N',N'-tetraacetic acid
FIAsH-EDT2	4',5'-bis(1,3,2-dithioarsolan-2-yl)fluorescein
FRET	fluorescence resonance energy transfer
β-ME	2-mercaptoethanol
MOPS	3-(N-morpholino)propanesulfonic acid
N-domain	nucleotide binding domain
P-domain	phosphorylation domain
SERCA	sarco/endoplasmic reticulum Ca-ATPase
TCEP	tris(carboxyethyl)phosphine
TM	transmembrane

References

1. Kuhlbrandt W. Biology, structure and mechanism of P-type ATPases. *Nat Rev Mol Cell Biol.* 2004; 5:282–295. [PubMed: 15071553]
2. Moller JV, Olesen C, Jensen AM, Nissen P. The structural basis for coupling of Ca²⁺ transport to ATP hydrolysis by the sarcoplasmic reticulum Ca²⁺-ATPase. *J Bioenerg Biomembr.* 2005; 37:359–364. [PubMed: 16691465]

3. Olesen C, Picard M, Winther AM, Gyruup C, Morth JP, Oxvig C, Moller JV, Nissen P. The structural basis of calcium transport by the calcium pump. *Nature*. 2007; 450:1036–1042. [PubMed: 18075584]
4. Toyoshima C. Structural aspects of ion pumping by Ca(2+)-ATPase of sarcoplasmic reticulum. *Arch Biochem Biophys*. 2008; 476:3–11. [PubMed: 18455499]
5. Toyoshima C, Inesi G. Structural basis of ion pumping by Ca²⁺-ATPase of the sarcoplasmic reticulum. *Annu Rev Biochem*. 2004; 73:269–292. [PubMed: 15189143]
6. Bigelow DJ, Inesi G. Contributions of chemical derivatization and spectroscopic studies to the characterization of the Ca²⁺ transport ATPase of sarcoplasmic reticulum. *Biochim Biophys Acta*. 1992; 1113:323–338. [PubMed: 1450205]
7. Sorensen TL, Moller JV, Nissen P. Phosphoryl transfer and calcium ion occlusion in the calcium pump. *Science*. 2004; 304:1672–1675. [PubMed: 15192230]
8. Toyoshima C, Mizutani T. Crystal structure of the calcium pump with a bound ATP analogue. *Nature*. 2004; 430:529–535. [PubMed: 15229613]
9. Huang S, Squier TC. Enhanced rotational dynamics of the phosphorylation domain of the Ca-ATPase upon calcium activation. *Biochemistry*. 1998; 37:18064–18073. [PubMed: 9922175]
10. Chen B, Squier TC, Bigelow DJ. Calcium activation of the Ca-ATPase enhances conformational heterogeneity between nucleotide binding and phosphorylation domains. *Biochemistry*. 2004; 43:4366–4374. [PubMed: 15065881]
11. Mueller B, Zhao M, Negrashov IV, Bennett R, Thomas DD. SERCA structural dynamics induced by ATP and calcium. *Biochemistry*. 2004; 43:12846–12854. [PubMed: 15461457]
12. Inesi G, Lewis D, Ma H, Prasad A, Toyoshima C. Concerted conformational effects of Ca²⁺ and ATP are required for activation of sequential reactions in the Ca²⁺ ATPase (SERCA) catalytic cycle. *Biochemistry*. 2006; 45:13769–13778. [PubMed: 17105196]
13. Ma H, Inesi G, Toyoshima C. Substrate-induced conformational fit and headpiece closure in the Ca²⁺ATPase (SERCA). *J Biol Chem*. 2003; 278:28938–28943. [PubMed: 12750373]
14. Squier TC, Bigelow DJ, Thomas DD. Lipid fluidity directly modulates the overall protein rotational mobility of the Ca-ATPase in sarcoplasmic reticulum. *J Biol Chem*. 1988; 263:9178–9186. [PubMed: 2837480]
15. Winters DL, Autry JM, Svensson B, Thomas DD. Interdomain fluorescence resonance energy transfer in SERCA probed by cyan-fluorescent protein fused to the actuator domain. *Biochemistry*. 2008; 47:4246–4256. [PubMed: 18338856]
16. Picard M, Toyoshima C, Champeil P. The average conformation at micromolar [Ca²⁺] of Ca²⁺-atpase with bound nucleotide differs from that adopted with the transition state analog ADP. AIFx or with AMPPCP under crystallization conditions at millimolar [Ca²⁺]. *J Biol Chem*. 2005; 280:18745–18754. [PubMed: 15757892]
17. Inesi G. Mechanism of calcium transport. *Annu Rev Physiol*. 1985; 47:573–601. [PubMed: 2581506]
18. Chen B, Cao H, Yan P, Mayer MU, Squier TC. Identification of an orthogonal peptide binding motif for biarsenical multiuse affinity probes. *Bioconjug Chem*. 2007; 18:1259–1265. [PubMed: 17569496]
19. Menick DR, Lee JA, Brooker RJ, Wilson TH, Kaback HR. Role of cysteine residues in the lac permease of *Escherichia coli*. *Biochemistry*. 1987; 26:1132–1136. [PubMed: 3552042]
20. van Iwaarden PR, Driessen AJ, Menick DR, Kaback HR, Konings WN. Characterization of purified, reconstituted site-directed cysteine mutants of the lactose permease of *Escherichia coli*. *J Biol Chem*. 1991; 266:15688–15692. [PubMed: 1874727]
21. van Iwaarden PR, Pastore JC, Konings WN, Kaback HR. Construction of a functional lactose permease devoid of cysteine residues. *Biochemistry*. 1991; 30:9595–9600. [PubMed: 1911745]
22. Jensen AM, Sorensen TL, Olesen C, Moller JV, Nissen P. Modulatory and catalytic modes of ATP binding by the calcium pump. *Embo J*. 2006; 25:2305–2314. [PubMed: 16710301]
23. Adams SR, Campbell RE, Gross LA, Martin BR, Walkup GK, Yao Y, Llopis J, Tsien RY. New biarsenical ligands and tetracysteine motifs for protein labeling in vitro and in vivo: synthesis and biological applications. *J Am Chem Soc*. 2002; 124:6063–6076. [PubMed: 12022841]

24. Griffin BA, Adams SR, Tsien RY. Specific covalent labeling of recombinant protein molecules inside live cells. *Science*. 1998; 281:269–272. [PubMed: 9657724]
25. Waggoner JR, Huffman J, Griffith BN, Jones LR, Mahaney JE. Improved expression and characterization of Ca²⁺-ATPase and phospholamban in High-Five cells. *Protein Expr Purif*. 2004; 34:56–67. [PubMed: 14766300]
26. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem*. 1951; 193:265–275. [PubMed: 14907713]
27. Lakowicz, J. Principles of Fluorescence Spectroscopy. 2. Kluwer Academic/Plenum Publishers; New York: 1999.
28. Mahaney JE, Autry JM, Jones LR. Kinetics studies of the cardiac Ca-ATPase expressed in Sf21 cells: new insights on Ca-ATPase regulation by phospholamban. *Biophys J*. 2000; 78:1306–1323. [PubMed: 10692318]
29. Verma S, Xiong Y, Mayer MU, Squier TC. Remodeling of the bacterial RNA polymerase supramolecular complex in response to environmental conditions. *Biochemistry*. 2007; 46:3023–3035. [PubMed: 17319694]
30. Luedtke NW, Dexter RJ, Fried DB, Schepartz A. Surveying polypeptide and protein domain conformation and association with FAsH and ReAsH. *Nat Chem Biol*. 2007; 3:779–784. [PubMed: 17982447]
31. Chen B, Mayer MU, Markillie LM, Stenoien DL, Squier TC. Dynamic motion of helix A in the amino-terminal domain of calmodulin is stabilized upon calcium activation. *Biochemistry*. 2005; 44:905–914. [PubMed: 15654746]
32. Chen B, Mayer MU, Squier TC. Structural uncoupling between opposing domains of oxidized calmodulin underlies the enhanced binding affinity and inhibition of the plasma membrane Ca-ATPase. *Biochemistry*. 2005; 44:4737–4747. [PubMed: 15779900]
33. Giepmans BN, Adams SR, Ellisman MH, Tsien RY. The fluorescent toolbox for assessing protein location and function. *Science*. 2006; 312:217–224. [PubMed: 16614209]
34. Toyoshima C, Nakasako M, Nomura H, Ogawa H. Crystal structure of the calcium pump of sarcoplasmic reticulum at 2.6 Å resolution. *Nature*. 2000; 405:647–655. [PubMed: 10864315]
35. Toyoshima C, Nomura H. Structural changes in the calcium pump accompanying the dissociation of calcium. *Nature*. 2002; 418:605–611. [PubMed: 12167852]
36. Watanabe T, Inesi G. The use of 2',3'-O-(2,4,6-trinitrophenyl) adenosine 5'-triphosphate for studies of nucleotide interaction with sarcoplasmic reticulum vesicles. *J Biol Chem*. 1982; 257:11510–11516. [PubMed: 6214553]
37. Nakamoto RK, Inesi G. Studies of the interactions of 2',3'-O-(2,4,6-trinitrocyclohexyldienylidene)adenosine nucleotides with the sarcoplasmic reticulum (Ca²⁺ + Mg²⁺)-ATPase active site. *J Biol Chem*. 1984; 259:2961–2970. [PubMed: 6142049]
38. Hua S, Ma H, Lewis D, Inesi G, Toyoshima C. Functional role of “N” (nucleotide) and “P” (phosphorylation) domain interactions in the sarcoplasmic reticulum (SERCA) ATPase. *Biochemistry*. 2002; 41:2264–2272. [PubMed: 11841218]
39. Bigelow DJ, Squier TC, Thomas DD. Temperature dependence of rotational dynamics of protein and lipid in sarcoplasmic reticulum membranes. *Biochemistry*. 1986; 25:194–202. [PubMed: 3006752]
40. Inesi G, Kurzmack M, Coan C, Lewis DE. Cooperative calcium binding and ATPase activation in sarcoplasmic reticulum vesicles. *J Biol Chem*. 1980; 255:3025–3031. [PubMed: 6244305]
41. Inesi G, de Meis L. Regulation of steady state filling in sarcoplasmic reticulum. Roles of back-inhibition, leakage, and slippage of the calcium pump. *J Biol Chem*. 1989; 264:5929–5936. [PubMed: 2522442]
42. Di Leva F, Domi T, Fedrizzi L, Lim D, Carafoli E. The plasma membrane Ca(2+) ATPase of animal cells: Structure, function and regulation. *Arch Biochem Biophys*. 2008; 461:1016–1026. [PubMed: 18101016]
43. Clarke DM, Loo TW, Rice WJ, Andersen JP, Vilsen B, MacLennan DH. Functional consequences of alterations to hydrophobic amino acids located in the M4 transmembrane sector of the Ca(2+)-ATPase of sarcoplasmic reticulum. *J Biol Chem*. 1993; 268:18359–18364. [PubMed: 8349711]

44. Rice WJ, Green NM, MacLennan DH. Site-directed disulfide mapping of helices M4 and M6 in the Ca^{2+} binding domain of SERCA1a, the Ca^{2+} ATPase of fast twitch skeletal muscle sarcoplasmic reticulum. *J Biol Chem.* 1997; 272:31412–31419. [PubMed: 9395473]
45. Skerjanc IS, Toyofuku T, Richardson C, MacLennan DH. Mutation of glutamate 309 to glutamine alters one Ca^{2+} -binding site in the Ca^{2+} -ATPase of sarcoplasmic reticulum expressed in Sf9 cells. *J Biol Chem.* 1993; 268:15944–15950. [PubMed: 8101845]
46. Cao H, Xiong Y, Wang T, Chen B, Squier TC, Mayer MU. A red cy3-based biarsenical fluorescent probe targeted to a complementary binding peptide. *J Am Chem Soc.* 2007; 129:8672–8673. [PubMed: 17585763]
47. Ma H, Lewis D, Xu C, Inesi G, Toyoshima C. Functional and structural roles of critical amino acids within the “N”, “P”, and “A” domains of the Ca^{2+} ATPase (SERCA) headpiece. *Biochemistry.* 2005; 44:8090–8100. [PubMed: 15924428]
48. Toyoshima C, Nomura H, Tsuda T. Lumenal gating mechanism revealed in calcium pump crystal structures with phosphate analogues. *Nature.* 2004; 432:361–368. [PubMed: 15448704]
49. Olesen C, Sorensen TL, Nielsen RC, Moller JV, Nissen P. Dephosphorylation of the calcium pump coupled to counterion occlusion. *Science.* 2004; 306:2251–2255. [PubMed: 15618517]

Figure 1. Locations of Engineered Labeling Motifs Within A- and N-domains of the Ca-ATPase
 The headpiece corresponding to the A-domain (residues 1 to 43 and 124 to 235; orange), N-domain (residues 360 to 600; dark blue), and P-domain (residues 330 to 359 and 601 to 739; green) consists of three discrete structural elements relative to transmembrane helices TM1-TM2 (residues 44 to 123; red), TM3-TM6 (residues 239 to 329 and 740 to 821; cyan), and TM7-TM10 (residues 831 to 994; gray) (*Isu4.pdb*), where domain boundaries are as previously described (7). Positions of FIAsh labeling are shown (CPK shaded side chains), and represent the insertion point corresponding to different constructs with either M¹-WDCKACCK-E² on the A-domain or M⁵⁷⁵-WDCCPGCCK-H⁵⁷⁶ on the N-domain. Arg⁵⁶⁰ represents a reference point located in the nucleotide binding pocket, and acts to stabilize the alpha-phosphate of bound ATP (13). Inset shows structure of 4',5'-bis(1,3,2-dithioarsolan-2-yl)fluorescein (FIAsh:EDT₂).

Figure 2. Catalytic Function of Wild-type and Engineered Constructs of the Ca-ATPase
Rates of ATP hydrolysis were measured for the Ca-ATPase in High-Five insect cell microsomes (50 μg/mL) expressing wild-type rat SERCA2a (●) or engineered constructs with tetracysteine insertion sequences in the A- (◇, ◆) or N-domain (□, ■) of the Ca-ATPase prior to (◇, □) or following (◆, ■) incubation with FIAH:EDT₂. ATPase activity was measured at 37 °C in 50 mM MOPS (pH 7.0), 0.1 M KCl, 3 mM MgCl₂, 1 mM EGTA, and sufficient CaCl₂ to yield the desired ionized calcium concentration, as previously determined (25). The reaction was initiated upon addition of 5 mM MgATP. The dotted line represents the fit to the Hill equation, where $n = 1.6 \pm 0.2$ and $K_d = 0.47 \pm 0.05$ μM free calcium. Maximal ATPase activities were normalized to the abundance of SERCA2a, where quantitative immunoblotting indicates that the Ca-ATPase represents on average about 1.5% of the total protein in isolated microsomes.

Figure 3. Selective Labeling of the Ca-ATPase Expressed in High-Five Microsomes
(Panel A) Fluorescence signals from FIAsh-labeled proteins following SDS-PAGE separation of microsomal proteins (40 µg) expressing wild-type SERCA2a (lane 1) or engineered constructs containing insertion sequences in the A-domain (lane 2) or N-domain (lane 3) assayed immediately after incubation with FIAsh:EDT₂ (5 µM) for 10 minutes at 25 °C in the presence of 5 mM β-ME and 5 mM TCEP and the indicated amount of 2,3-dimercapto-1-propanesulfonic acid (DMPS) to reduce nonspecific binding (18). *(Panel B)* Coomassie blue protein stain (left lanes) or fluorescence (right lanes) imaging following FIAsh labeling, incubation in DMPS (50 µM), and SDS-PAGE separation of High-Five insect cell microsomes (10 µg) expressing engineered SERCA2a, where the insertion sequence is within the A- (WDCCKACCK) or N-domain (WDCCPGCCK) of the Ca-ATPase. *(Panel C)* Fluorescence emission spectra of High-Five insect microsomes (0.1 mg/mL) expressing either wild-type SERCA (dashed line) or the engineered N-domain tag (solid line) following incubation with FIAsh:EDT₂ (10 µM), where $\lambda_{\text{ex}} = 500$ nm. Fluorescence emission spectra of FIAsh-labeled vesicles expressing the A-domain tag were virtually identical to that shown using the N-domain tag.

Figure 4. Solvent Accessibilities of FAsH bound to A-domain or N-domain of the Ca-ATPase
 Stern-Volmer quenching was measured as decreases in the fluorescence intensity (F) relative to the initial value (F_0) for FAsH-labeled microsomes expressing SERCA2a (50 $\mu\text{g/mL}$) in 150 mM MOPS (pH 7.0) and 5 mM MgCl_2 in the presence of either 2 mM EGTA (●) or 0.2 mM CaCl_2 (○) at 25 °C. Slopes of the lines (i.e., the Stern-Volmer quenching constant K_{sv}) for the A-domain are $0.49 \pm 0.03 \text{ M}^{-1}$ (EGTA) and $0.96 \pm 0.05 \text{ M}^{-1}$ (Ca^{2+}); for the N-domain K_{sv} equals $0.56 \pm 0.03 \text{ M}^{-1}$ (EGTA) or $0.58 \pm 0.04 \text{ M}^{-1}$ (Ca^{2+}). $\lambda_{ex} = 500 \text{ nm}$; $\lambda_{em} = 530 \text{ nm}$.

Figure 5.

Calcium-dependent Conformational Sensitivity of A-domain (panel A; $\circ$ in panel C) or N-domain (panel B; $\bullet$ in panel C) constructs of FIAH-labeled SERCA2a (50 $\mu\text{g/mL}$) in 150 mM MOPS/TRIS (pH 7.0), 5 mM MgCl_2 , 0.5 mM EGTA, and sufficient calcium to yield the indicated ionized (free) calcium concentration. Values represent averages and standard errors of the mean from three independent measurements, where $\lambda_{\text{ex}} = 500$ nm; $\lambda_{\text{em}} = 530$ nm. Line in panel C represents nonlinear least squares fit of both data sets to the Hill equation, where the macroscopic equilibrium constant K_d equals 0.48 ± 0.04 μM and the Hill coefficient is 0.93 ± 0.07 . Temperature was 25 $^\circ\text{C}$.

Figure 6. Calcium-Dependent Change in Spatial Separation Between A- and N-domains

Fluorescence resonance energy transfer efficiency (E) was measured using FAsH (donor) bound to either the A-domain or N-domain and TNP-ATP (acceptor), where $E = 1 - F_{da}/F_d$. F_{da} and F_d are the fluorescence intensity of FAsH bound donor prior to and following saturation of the ATP binding site by the nucleotide analog TNP-ATP. Experiments were carried out using FAsH-labeled microsomes expressing SERCA2a (12 μg protein/mL) in 150 mM MOPS/TRIS (pH 7.0) in the presence of either 0.2 mM CaCl_2 (○) or 2 mM EGTA (●). $\lambda_{ex} = 500$ nm; $\lambda_{em} = 530$ nm, and fluorescence intensities were corrected for the inner filter effect (27). Lines represent fits to the Hill equation, where for the A-domain K_d is 13 ± 2 μM and E_{max} is 0.39 ± 0.03 in the presence of calcium (○) and K_d is 11 ± 2 μM and E_{max} is 0.47 ± 0.03 in the presence of EGTA (●). For the N-domain K_d is 11 ± 1 μM and E_{max} is 0.73 ± 0.05 for in the presence of calcium (○) and K_d is 9 ± 1 μM and E_{max} is 0.69 ± 0.05 in the presence of EGTA (●).

Figure 7. Headpiece Domain Reorientation and Calcium Activation

(*Top Panel*): Depiction of selected crystal structures of the Ca-ATPase (top), using domain boundaries defined in the legend to Figure 1. Coordinates were used for the Ca-ATPase (i.e., E) stabilized in six different states, corresponding to i) E, no calcium (1iwo.pdb; shown) (35), ii) E-ATP (2dqs.pdb and 2c88.pdb (shown))(22), iii) 2Ca-E-ATP (1vfp.pdb and 1t5s.pdb (shown))(7, 8), iv) 2Ca-E-P:ADP (2zbd.pdb, 1t5t.pdb (shown), or 3ba6.pdb)(3, 7, 48), v) E-P:ADP (3b9r.pdb or 1wpg.pdb (shown))(3, 48), and vi) E-P (3b9b.pdb and 1xp5.pdb)(3, 49). (*Bottom Panels*): Distance changes (Δr) between sites of FIAH labeling site in the A-domain (i.e., interdomain spatial separation) or N-domain (intradomain spatial separation) relative to the nucleotide binding pocket in N-domain (i.e., NH_1 sidechain of Arg^{560}) that functions to coordinate binding of alpha-phosphate group of ATP (13)), using high-resolution structures of the Ca-ATPase stabilized in different enzymatic states ($\circ$) in comparison to experimental measurements using TNP-ATP as a FRET acceptor ($\bullet$), normalized to an arbitrary reference point.

Figure 8. Calcium Occupancy and Concerted Structural Changes in Transmembrane and Cytosolic Headpiece Associated with Occupancy of Calcium Binding Site One and Enzyme Activation

Amino acids involved in calcium-dependent salt-bridges are highlighted in apo- (2c88.pdb) and calcium-activated (1t5s.pdb) forms of the Ca-ATPase. Arrows in 1t5s.pdb for the calcium-activated form highlight major calcium-dependent structural changes. Side chains associated with calcium binding to site one [involving ligands N⁷⁶⁸ and E⁷⁷¹ in M5 (green), T⁷⁹⁹ and D⁸⁰⁰ in M6 (blue), and E⁹⁰⁸ in M8 (not shown)] induces M5 helix reorientation, bringing R⁷⁶² (M5) into the proximity of D⁹⁸¹ (M10 in red)) to form a stable salt bridge. Binding of the first calcium induces the formation of calcium binding site two (which shares calcium binding site N⁷⁹⁶ and D⁸⁰⁰ on M6) with concomitant reorientation of M4 to stabilize salt bridges i) E80 (M1/M2 loop) with R290 (M3/M4 loop) and ii) E90 in M2 (orange) with K297 in M4 (yellow) to form a helix bundle with the associated formation of the salt bridge between K47 and E243 located near the bilayer surface proximal to M1 (white) and M3 (cyan).

Table 1

Comparison Between Solution and X-ray Structures¹.

Enzyme State	Interdomain A-domain → N-domain Distance		Distance Across N-domain			
	FRET (FlAsH → TNP-ATP)	X-ray Structure		FRET (FlAsH → TNP-ATP)	X-ray Structure	
		(Met ^I → ribose)	(Met ^I → Arg ⁵⁶⁰)		(Met ⁵⁷⁶ → ribose)	(Met ⁵⁷⁶ → Arg ⁵⁶⁰)
E-ATP	34.6 ± 0.7 Å			30 ± 1 Å		
2dqs.pdb		34 Å	35 Å		28 Å	28 Å
2c88.pdb		36 Å	38 Å		28 Å	30 Å
2Ca-E-ATP	37.0 ± 0.7 Å			29 ± 1 Å		
lvfp.pdb		42 Å	42 Å		27 Å	30 Å
1t5s.pdb		42 Å	39 Å		28 Å	29 Å

¹ Solution measurements made using fluorescence resonance energy transfer (FRET) between FlAsH bound to tagging sequences on A- or N-domains and TNP-ATP in comparison to indicated high-resolution X-ray structures (7, 8, 22). Distances represent those between amino-group at insertion position of tag in SERCA1 structures and either the O3' ribose position or the proximal NH1 sidechain of Arg⁵⁶⁰, previously identified as a key interfacial residue that chelates bound ATP (13, 47) and within 4 Å from the O3' ribose.