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Topical Review

The Mechanics of Calcium Transport

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Abstract. With the recent atomic models for the sarcoplasmic reticulum Ca^{2+}-ATPase in the Ca^{2+}-bound state, the Ca^{2+}-free, thapsigargin-inhibited state, and the Ca^{2+}-free, vanadate-inhibited state, we are that much closer to understanding and animating the Ca^{2+}-transport cycle. These "snapshots" of the Ca^{2+}-transport cycle reveal an impressive breadth and complexity of conformational change. The cytoplasmic domains undergo rigid-body movements that couple the energy of ATP to the transport of Ca^{2+} across the membrane. Large-scale rearrangements in the transmembrane domain suggest that the Ca^{2+}-binding sites may alternately cease to exist and reform during the transport cycle. Of the three cytoplasmic domains, the actuator (A) domain undergoes the largest movement, namely a 110° rotation normal to the membrane. This domain is linked to transmembrane segments M1–M3, which undergo large rearrangements in the membrane domain. Together, these movements are a main event in Ca^{2+} transport, yet their significance is poorly understood. Nonetheless, inhibition or modulation of Ca^{2+}-ATPase activity appears to target these conformational changes. Thapsigargin is a high-affinity inhibitor that binds to the M3 helix near Phe^{256}, and phospholamban is a modulator of Ca^{2+}-ATPase activity that has been cross-linked to M2 and M4. The purpose of this review is to postulate roles for the A domain and M1–M3 in Ca^{2+} transport and inhibition.

Introduction

Lipid membranes encapsulate all cells and organelles, forming impermeable barriers that limit the free exchange of solutes. These membranes are embedded with a wide variety of proteins responsible for transporting ions and nutrients needed to sustain life. The P-type ATPases represent an important class of such transporters, which have been the subject of increasingly sophisticated scrutiny over the last 50 years. Since their discovery in 1957 (Skou, 1957), numerous kinetic and biochemical studies have provided insight into the mechanism by which these pumps harness energy inherent in the γ-phosphate of ATP and use it to move ions across the membrane (Albers, 1967; Bader, Sex & Post, 1966; Fagan & Saier, 1994). Thanks to recent structures by x-ray crystallography (Toyoshima et al., 2000; Toyoshima & Nomura, 2002) and electron microscopy (Xu et al., 2002; Zhang et al., 1998), the detailed conformational changes underlying this mechanism can now be addressed, thus opening a new era in the structure and function of P-type ATPases.

Historically, the Na^+ pump (Na^+,K^+-ATPase) and Ca^{2+} pump (Ca^{2+}-ATPase) have served as archetypes for P-type ATPases, owing to their abundant supply from enriched native membranes. These membranes have been used for numerous molecular biological, biochemical and biophysical studies and early enzyme preparations proved that this family of ion pumps was particularly amenable to techniques of structural biology (Skriver, Maunsbach & Jorgensen, 1981; Dux & Martonosi, 1983; Rabon et al., 1986). While initial insights into the structural mechanism of transport were obtained by electron microscopy (Xian & Hebert, 1997; Auer, Scarborough & Kuhlbrandt, 1998; Ogawa et al., 1998; Zhang et al., 1998), recent x-ray crystallographic studies have made the final step to atomic resolution.

To date, we have atomic models for three reaction intermediates, (Toyoshima et al., 2000; Toyoshima & Nomura, 2002; Xu et al., 2002), which reveal an unexpectedly complex molecular machine. This machine consists of ten membrane-spanning helices, interrupted by two long loops on the cytoplasmic side

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of the membrane. The main cytoplasmic loop harnesses the energy inherent in the γ-phosphate of ATP and uses it to instigate the movement of ions through the membrane. To do this, the molecule passes through several major reaction intermediates, denoted E_1, E_1P, E_2P, and E_2, which have different conformations and biochemical characteristics. The progression through these intermediates involves rigid-body movements of the three cytoplasmic domains and complex deformations of the membrane-spanning helices. The structures of some of the intermediates are hugely different from one another, and it is fascinating to see how they combine to animate the ion transport cycle.

A Structural Perspective on Membrane Transport

Significant insight into the transport mechanism can be gleaned simply by comparing the atomic models for Ca^{2+}-ATPase in the Ca^{2+}-bound (Toyoshima et al., 2000), Ca^{2+}-free, thapsigargin-inhibited (Toyoshima & Nomura, 2002) and vanadate-inhibited (Xu et al., 2002) states. The heart of the enzyme is the phosphorylation (P) domain with its conserved Asp^{351} that represents a hallmark of the family of P-type ATPases. The P domain has a core Rossmann fold locked against two long helices that are embedded in the membrane domain, namely M4 and M5. The P domain adopts two distinct orientations in the Ca^{2+}-bound and Ca^{2+}-free conformations, in which the P domain, M4, and M5 appear to move in a concerted way. In addition, the nucleotide-binding (N) and actuator (A) domains adopt remarkably distinct orientations in the Ca^{2+}-bound and Ca^{2+}-free configurations. Unlike the P domain, however, these two domains are flexibly connected to the rest of the protein by unstructured loops, suggesting that they are mobile. While the coordination of these movements remains a matter of speculation, the apparent mobility of the cytoplasmic domains has led to a hypothesis that Ca^{2+}-ATPase functions, in part, as a Brownian ratchet (Xu et al., 2002).

Historically, limited proteolysis has been a useful tool in characterizing reaction intermediates and delineating domain structures (Andersen & Jorgensen, 1985; Jorgensen & Andersen, 1988). In light of the three atomic models for Ca^{2+}-ATPase, results of proteolysis can be interpreted in terms of the configuration of the A domain and connecting loops. Movement of the A domain protects the T2 tryptic site (Arg^{198}) in E_2P and exposes it in all other reaction intermediates (Jorgensen & Andersen, 1988). Similarly, proteolytic cleavage of the M2 and M3 loops connecting the A domain to the transmembrane helices report on the structure of these loops and, presumably, the disposition of the A domain (le Maire et al., 1990; Juul et al., 1995; Danko et al., 2001).

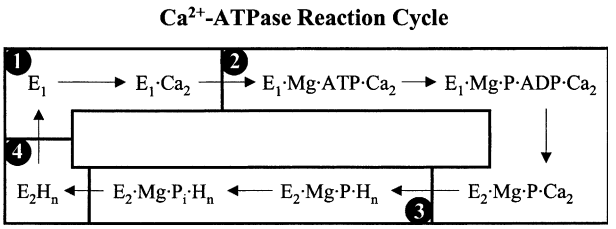

Fig. 1. The Ca^{2+}-ATPase reaction cycle. The four conformations for the actuator (A) domain are numbered and the likely points for the structural transitions are indicated.

These loops are exposed in E_1Ca_2 and E_2 and protected in all other reaction intermediates.

Based on such experiments and the three atomic models, four distinct configurations can be described for the A domain (Fig. 1). Large A-domain rotations take place during the E_2-to-E_1 and E_1P-to-E_2P transitions in the reaction cycle, while smaller A domain movements are suggested for the E_1-to-E_1P and E_2P-to-E_2 transitions. The E_1, Ca^{2+}-bound conformation is the most sensitive to proteases, suggesting an "open" conformation, while the E_2P conformation is the most resistant, suggesting a "closed" conformation. Two further A domain configurations are suggested by differential sensitivity to proteases in the absence and presence of substrates (Danko et al., 2001). Interestingly, the atomic models for the E_1 Ca^{2+}-bound state (Toyoshima et al., 2000) and the E_2 Ca^{2+}-free, inhibited state (Toyoshima & Nomura, 2002) are dramatically different conformations that appear to represent "open" and "closed" states, yet the T2 site remains protease sensitive. However, the atomic model for E_2P (Xu et al., 2002) reveals the "closed" conformation with extensive interaction between the A, N, and P domains (Fig. 2).

In contrast to the rigid-body movements of these cytoplasmic domains, the transmembrane domain undergoes plastic deformations. These deformations are confined to transmembrane helices M1–M6, and are likely driven by interchanging configurations of the ion binding sites that alternately accommodate two Ca^{2+} ions or two protons, possibly as hydronium ions. The direct involvement of M4 and M5 in ion binding and their rigid connection to the P domain provide a plausible explanation for initiating the dramatic changes in P-domain orientation upon Ca^{2+} binding. On the other hand, reorientations of M1–M3 have been speculated to be necessary for H^+ counter-transport (Toyoshima & Nomura, 2002), for movement of the A domain, and for controlling the cytoplasmic Ca^{2+} access channel (Kuhlbrandt, Zeelen & Dietrich, 2002). For these helices, available structures suggest that movements correlate with the disposition of the A domain, with M1 and M2 undergoing larger deformations than M3 (Fig. 2*d*), presumably reflecting their roles in Ca^{2+} transport.

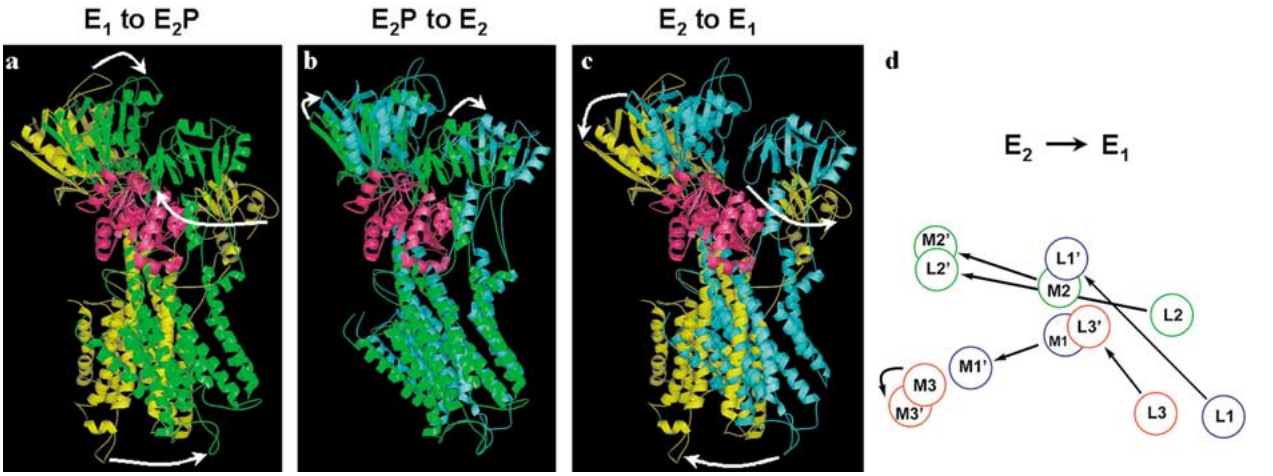

Fig. 2. The atomic models for the E_1, Ca^{2+}-bound form (*yellow*) (Toyoshima et al. 2000), the E_2 P form (*green*) (Xu et al. 2002), and the E_2, Ca^{2+}-free, inhibited form (*blue*) (Toyoshima & Nomura, 2002) of Ca^{2+}-ATPase are shown. The phosphorylation (P) domain is shown in magenta, and all structures have been aligned and are shown relative to the P domain. (*a*) Comparison between E_1 and E_2P; (*b*) Comparison between E_2P and E_2; (*c*) Comparison between E_2 and E_1. Domain movements relative to the P domain are indicated by arrows. (*d*) Relative movements of the loops connecting the A domain and the transmembrane helices. The circles mark the cytoplasmic ends of the transmembrane helices (M1, M2 and M3) and the ends of the loops as they connect to the A domain (L1, L2 and L3), as viewed from the cytoplasmic side of the membrane. The arrows indicate the direction of movement from the E_2 conformation to the E_1Ca_2 conformation. M3 also moves along its axis, and this movement is not shown in the figure.

An Energetic Perspective on Membrane Transport

The Ca^{2+}-ATPase transport cycle relies on mobility of the cytoplasmic domains and the transient formation and stabilization of cytoplasmic domain interfaces. In particular, the structural changes that accompany the transport of Ca^{2+} through the membrane include dramatic association and dissociation of the A, N and P domains. In general, the formation of protein interfaces involves only minor changes in domain structure (Lo Conte, Chothia & Janin, 1999). This is certainly true for Ca^{2+}-ATPase, where dynamic interfaces are formed between mobile, but rigid domains. The extent of these interfaces varies greatly from one reaction intermediate to another, ranging from 740 $Å^2$ in the presence of Ca^{2+} to 4800 $Å^2$ in the absence of Ca^{2+}.

The extensive nature of the cytoplasmic domain interface in some reaction intermediates raises a few energetic considerations. First, the large domain interface may balance the entropic cost of immobilizing the cytoplasmic domains. However, domain association is also an entropy-driven process, where entropy is gained by converting the stronger protein-water interactions to the weaker protein-protein and water-water interactions (Weber, 1993). Second, the size of the interface is related to the conformational changes that bring about association. This is true for Ca^{2+}-ATPase, where the large domain interface accompanies enormous movements of the cytoplasmic domains and deformation of the transmembrane helices. Third, the cytoplasmic domain interface of 4800 $Å^2$ in E_2P must be actively dissociated later in the transport cycle. By way of comparison, the average interface in a protein-protein complex buries 1600 $Å^2$ (Lo Conte et al., 1999). Movement of the P domain or the transmembrane helices must direct the dissociation of the A/N/P domain interface. Thus, while the interface may form by Brownian motion, the size of the interface suggests that a conformational change brings about its dissociation.

Structures of the Reaction Intermediates

E_1Ca_2

We will start with the Ca^{2+}-bound conformation of Ca^{2+}-ATPase, which is characterized by tenuous, flexible connections between the A and N domains and the rest of the protein (Fig. 2*a*). This reaction intermediate was the first one determined by x-ray crystallography (Toyoshima et al., 2000), and has been the subject of extensive studies by electron crystallography (Shi et al., 1995, 1998). The A domain interaction with the P domain buries a meager 740 $Å^2$ of surface area (Fig. 3*a*), and the N domain appears to be held in place by an intermolecular crystal contact. These two domains are obviously very mobile in the E_1 Ca^{2+}-bound state and the minimal contact between the A domain and the rest of the protein explains its protease sensitivity. The T2 tryptic site is fully exposed and the A domain loops are more accessible to V8 and proteinase K proteases (le Maire et al., 1990; Juul et al., 1995; Danko et al., 2001). Although the γ-phosphate of ATP is too far from the conserved Asp^{351} for phosphoryl

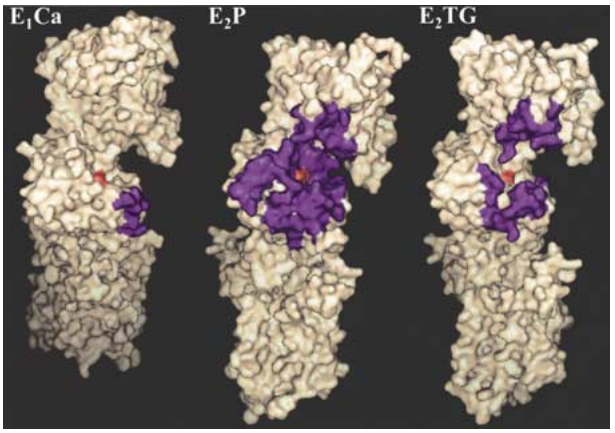

Fig. 3. Buried surface area in the E_1, Ca^{2+}-bound form (Toyoshima et al. 2000), the E_2, Ca^{2+}-free, vanadate-inhibited form (Xu et al. 2002), and the E_2, Ca^{2+}-free, thapsigargin-inhibited form (Toyoshima & Nomura, 2002) of Ca^{2+}-ATPase. The atomic models are shown as molecular surfaces with the actuator (A) domain and connecting loops removed. The buried surface area is shown in *blue* and Asp^{351} is shown in *red*. All structures have been aligned and are shown relative to the P domain. The total buried surface areas are 740 $Å^2$ for E_1Ca_2, 4800 $Å^2$ for E_2P, and 2600 $Å^2$ for E_2. Buried surface areas were calculated using *Surface Racer* (Tsodikov et al., 2002).

transfer to occur in this conformation, a simple rigid-body movement of the N domain is sufficient to close the gap (Xu et al., 2002).

$E_1MgATPCa_2$ AND $E_1MgPADPCa_2$

The reduced protease sensitivity of the enzyme in the presence of nonhydrolyzable ATP analogues and related substrates (Jorgensen & Farley, 1988; Danko et al., 2001) suggests a more closed conformation for the A domain, distinct from E_1Ca_2 (Toyoshima et al., 2000). In particular, the A domain loops and the T2 site become protease resistant, suggesting a change in orientation of the A domain and structuring of the loops connecting M2 and M3. This change is not as extensive as in E_2 (Toyoshima & Nomura, 2002), because the A domain does not restrict closure of the N and P domains and the T2 site retains some protease sensitivity. Thus, this configuration for the A domain must be intermediate between the fully-closed, protease-resistant form, E_2P (Xu et al., 2002), and the fully-open, protease-sensitive form, E_1Ca_2 (Toyoshima et al., 2000). The question remains whether a large A domain movement occurs during the E_1-to-E_1P transition. Compared to the differences between E_2 and E_1Ca_2, it is likely that A-domain movement during the E_1-to-E_1P transition is small, and probably accommodates N- and P-domain closure.

In this scenario, the large A-domain movement would occur during the E_1P-to-E_2P transition. Proteolytic cleavage of the loop connecting the A domain to M3 blocks the reaction cycle at E_1P (Moller et al., 2002), suggesting roles for the M3 loop and A-domain motion in the E_1P-to-E_2P transition. Similarly, deletion of a single amino acid in the M1 loop strongly inhibits the E_1P-to-E_2P transition in the reaction cycle (Daiho et al., 2003). Because the M1 loop is fully extended in the E_2P conformation (Xu et al., 2002), shortening this loop may restrict A-domain movement, thus interfering with its ability to form the necessary interface with the N and P domains.

E_2MgPH_n AND $E_2MgP_iH_n$

An atomic model for E_2P, based on an electron cryomicroscopy structure in the presence of vanadate (Fig. 2*b*) (Xu et al., 2002), predicts an impressive 4800 $Å^2$ of buried surface area between the A, N and P domains (Fig. 3*b*). The partial burial of Arg^{198} at this interface explains its greatly reduced protease sensitivity. Similarly, although the connecting loops are fully extended in this conformation, they may be involved in the new domain interface, possibly explaining their protease resistance. A buried surface area of 4800 $Å^2$ is beyond the range of typical values for macromolecular interactions (Janin, Miller & Chothia, 1988), and it seems surprising that such an interface is transient. The A, N and P domain interface surrounds and contacts the conserved Asp^{351} and its covalently attached $MgPO_4$, which is postulated to interact with the conserved $TGES^{184}$ and $DGVND^{707}$ sequences (Stokes & Green, 2003). This is consistent with Fe-mediated cleavage of the $TGES^{184}$ loop, which is most efficient in E_2P (Patchornik, Goldshleger & Karlish, 2000). Loss of this $MgPO_4$ and the corresponding interactions with $TGES^{184}$ may facilitate dissociation of the interface. In turn, formation of this interface is likely coupled to the ion-transport sites through transmembrane helices M1–M3, thus bringing about the changes in ion affinity and accessibility that represent the critical step in ion transport.

E_2 AND E_2H_n

The final intermediate is the E_2, Ca^{2+}-free conformation that also has substantial contact between the A, N, and P domains (Fig. 2*c*), and is stabilized by thapsigargin (Toyoshima & Nomura, 2002). The A domain interaction buries 2600 $Å^2$ of surface area involving both the N and P domains and surrounding Asp^{351} (Fig. 3*c*). A buried surface area of 2600 $Å^2$ is well within the range of typical values for macromolecular interactions (Janin et al., 1988). Again, it seems surprising that such an interface is transient; however, the large interface is likely required to balance the entropic cost of immobilizing the A and N domains. In forming this interface, loss of interactions between bound $MgPO_4$ and $TGES^{184}$ likely allows the A domain to move slightly away. Thus, the

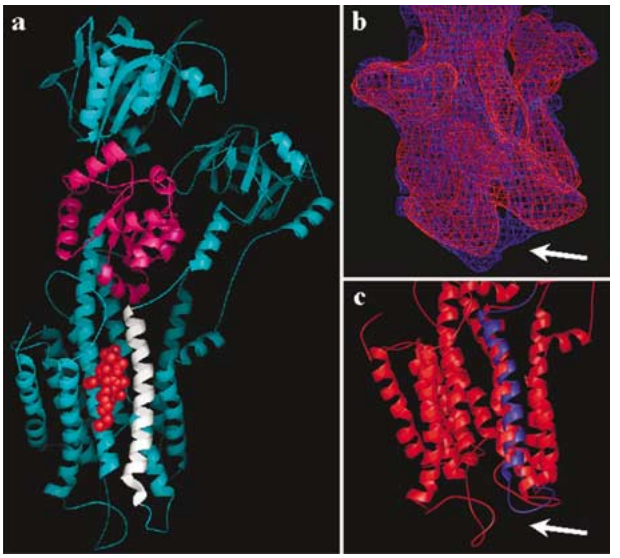

Fig. 4. (*a*) The thapsigargin (TG) binding site on Ca^{2+}-ATPase (Toyoshima & Nomura, 2002). (*b*) The 3-D structure of Ca^{2+}-ATPase in the absence (*red*) and presence (*blue*) of TG, as studied by electron cryomicroscopy (Young et al. 2001a). (*c*) Ribbon diagrams for Ca^{2+}-ATPase in the absence (*red*) and presence (*blue*) of TG, based on the electron cryomicroscopy maps. The atomic coordinates for E_2P (Xu et al., 2002) were remodeled to fit the 3-D map in the presence of TG. The electron cryomicroscopy structures reveal a TG-specific conformational change in M3 and the M3/M4 loop of Ca^{2+}-ATPase. We conclude that the atomic model for the E_2, Ca^{2+}-free, TG-inhibited state of the enzyme (Toyoshima & Nomura, 2002) differs in this region from the physiological E_2 state.

protease sensitivity of E_2 is intermediate between E_2P and E_1Ca_2 (Andersen & Jorgensen, 1985; Danko et al., 2001; Jorgensen & Andersen, 1988).

With respect to ATP, the N domain retains a high affinity and more closely approaches the P domain in the E_2, Ca^{2+}-free conformation, compared to E_1Ca_2 ; however, the γ-phosphate of ATP is still too far from the conserved Asp^{351} for phosphoryl transfer. In the case of E_2, the role of the A domain may be to hold the N and P domains apart, thereby preventing premature activation of the enzyme by ATP prior to Ca^{2+} binding by the membrane domain.

The Reaction Cycle

If the reaction cycle is regulated by Ca^{2+} alone (Toyoshima & Nomura, 2002), then the A domain senses and communicates this regulation to the rest of the enzyme. In the reaction sequence, Ca^{2+} binding directs movement of M4 and M5 and tilting of the P domain. As a consequence, Ca^{2+} binding induces movement of M1–M3 and, by virtue of the connecting loops, movement of the A domain. These movements are designed to break the interface and rotate the A domain out of the way of the N and P domains (Fig. 2*d*). Interestingly, disrupting the M3 loop (Moller et al., 2002) or shortening the M1 loop by one residue (Daiho et al., 2003) does not prevent Ca^{2+}-dependent phosphorylation, suggesting that inclination of the P domain is a critical step.

Prior to forming E_1P, binding of ATP by E_1Ca_2 structures the loops connecting the A domain to the transmembrane helices, and results in rigid-body movements of the N and P domains. Perhaps these movements create a favorable interface for the A domain, resulting in association and re-structuring of its connecting loops. If so, the A domain might serve to stabilize the activated enzyme for phosphoryl transfer from ATP to Asp^{351}. Again, the binding energy of this interface may balance the entropic cost of immobilizing these domains, allowing the energy of ATP to be transferred to the enzyme and expended in subsequent conformational changes.

In the E_1P-to-E_2P transition, the energy from ATP is released, and the A domain appears to be drawn into a more extensive interface with the N and P domains (Fig. 3*b*), perhaps as a result of a thermally driven random search. In addition, structural effects of $MgPO_4$ on the loops of the P domain may instigate conformational changes analogous to those of motor proteins (Fisher et al., 1995; Smith & Rayment, 1995) or the family of PO_4 acceptor proteins (Lee et al., 2001) to produce a surface compatible with tighter binding of the A domain. When bound in this way, the A domain appears to pry the N domain away from the P domain (Fig. 2). This binding also leads to Ca^{2+} release, presumably through elements that link the A and P domains to the transmembrane helices.

In the E_2P-to-E_2 transition, the release of $MgPO_4$ may create a steric clash between the $TGES^{184}$ and $DGVND^{707}$ loops. A new set of interactions between these loops pushes the A domain away from the catalytic site and allows the N domain to approach the P domain more closely. Nevertheless, the orientation of the P domain keeps Asp^{351} buried in E_2 and thus unavailable for γ-phosphate transfer from ATP in the absence of Ca^{2+}.

What then is the structural signal for enzyme activation by ATP? Obviously, the reaction cycle is initiated by Ca^{2+} and the signal originates in the membrane domain. In the closed conformation, a putative Ca^{2+} access channel is formed by M1–M3 and is lined at the bottom by Glu^{309} of M4 (Toyoshima & Nomura, 2002). Interaction of the first Ca^{2+} ion with Glu^{309} could be the elusive isomeric transition postulated more than 10 years ago (Cantilina et al., 1993). It is possible that interaction of Ca^{2+} with Glu^{309} initiates structural rearrangements that lead to binding of a second Ca^{2+} ion. This interaction depends on the protonation state of the enzyme, as deprotonation precedes Ca^{2+} binding and could affect the configuration of Glu^{309} (Stokes & Green, 2003). Regardless, initiation of structural rearrangements by M4 will impinge on M1–M3, whose

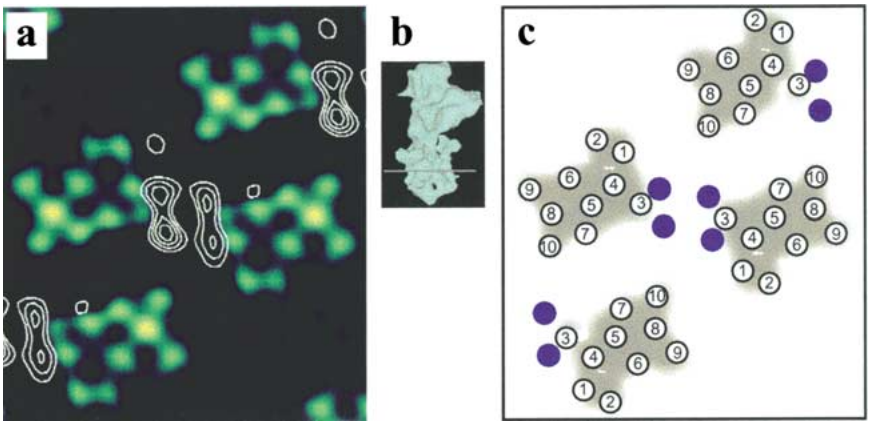

Fig. 5. Electron cryomicroscopy studies of Ca^{2+}-ATPase and phospholamban (PLB) tubular co-crystals (Xu et al., 2002; Young et al., 2001b). (*a*) Cylindrical section through the helical lattice at the middle of the membrane for Ca^{2+}-ATPase. The cylindrical section runs through the center of the membrane and includes four, twofold-related Ca^{2+}-ATPase molecules. The difference map in the presence of PLB is shown as white contour lines with a 2.75 σ cutoff. (*b*) The level of the cylindrical section is shown as a gray bar through the 3-D structure of Ca^{2+}-ATPase. (*c*) Interpretive figure showing the outline of the four, twofold-related Ca^{2+}-ATPase molecules in the helical lattice and the relative positions of membrane helices M1-M10. The blue circles represent the observed difference density peaks.

movements could contribute to the active dissociation of the extensive A/N/P domain interface. Also contributing to this dissociation will be the inclination of the P domain via M4 and M5 lever arms, thus providing access to Asp^{351} by ATP.

A Target for Inhibition

In addition to playing an important role in the reaction cycle, the A domain and its associated transmembrane helices, M1–M3, also appear to be a target for inhibition or regulation via thapsigargin and phospholamban.

Thapsigargin

Thapsigargin (TG) is a sesquiterpene isolated from the plant *Thapsia gargantuan* that has been shown to be a highly specific and avid inhibitor of Ca^{2+}-ATPases from sarcoplasmic reticulum. Inhibition results from locking the pump in an E_2-like state (Sagara & Inesi, 1991), which appears to generate a highly stable configuration of transmembrane helices that effectively resists denaturation (Toyoshima and Nomura, 2002; Stokes & Green, 2003). An atomic model for this conformation revealed TG bound in a groove between M3, M5, and M7 on the cytoplasmic side of the membrane, (Fig. 4*a*; Toyoshima & Nomura, 2002), supporting earlier mutagenesis data (Yu et al., 1998). During the reaction cycle, neither M5 nor M7 move much, although the side chains of Glu^{771} and Asn^{768} rotate slightly toward M4 upon Ca^{2+} binding. In contrast, M3 undergoes significant bending and displacement near the TG binding site. Thus, the mechanism of TG inhibition may be to lock M3 in place, thereby preventing structural rearrangements required for Ca^{2+} binding and communication of this signal to the N and P domains for enzyme activation.

Early attempts to identify the site of TG binding utilized electron cryomicroscopy of Ca^{2+}-ATPase tubular crystals in the absence and presence of TG (Young et al., 2001a). A statistically significant difference density was observed in the lumenal domain of Ca^{2+}-ATPase between the M3/M4 and M7/M8 loops (Fig. 4*b*). The simplest explanation for this result was a TG binding site involving these lumenal loops. In light of the x-ray structure (Toyoshima & Nomura, 2002), however, a more likely explanation is that this difference density reflects a conformational change of M3 and the M3/M4 loop (Fig. 4*c*). More specifically, this change appears to result from a small downward movement of the M3 helix and re-structuring of the M3/M4 loop (H.S. Young, *unpublished obervations*). This suggests that the atomic model for the E_2, Ca^{2+}-free, TG-inhibited state of the enzyme (Toyoshima & Nomura, 2002) differs in this region from the physiological E_2 state.

Phospholamban

Phospholamban (PLB) is a small regulatory protein from cardiac sarcoplasmic reticulum that interacts with Ca^{2+}-ATPase to modulate cardiac contractility in response to β-adrenergic stimuli. It is thought that PLB binds directly to Ca^{2+}-ATPase with a 1:1 stoichiometry to inhibit Ca^{2+} transport. Phosphorylation of PLB relieves this inhibition, thereby increasing Ca^{2+} transients as well as relaxation rates. Although the PLB binding site on Ca^{2+}-ATPase remains a matter for debate (Young, Jones & Stokes,

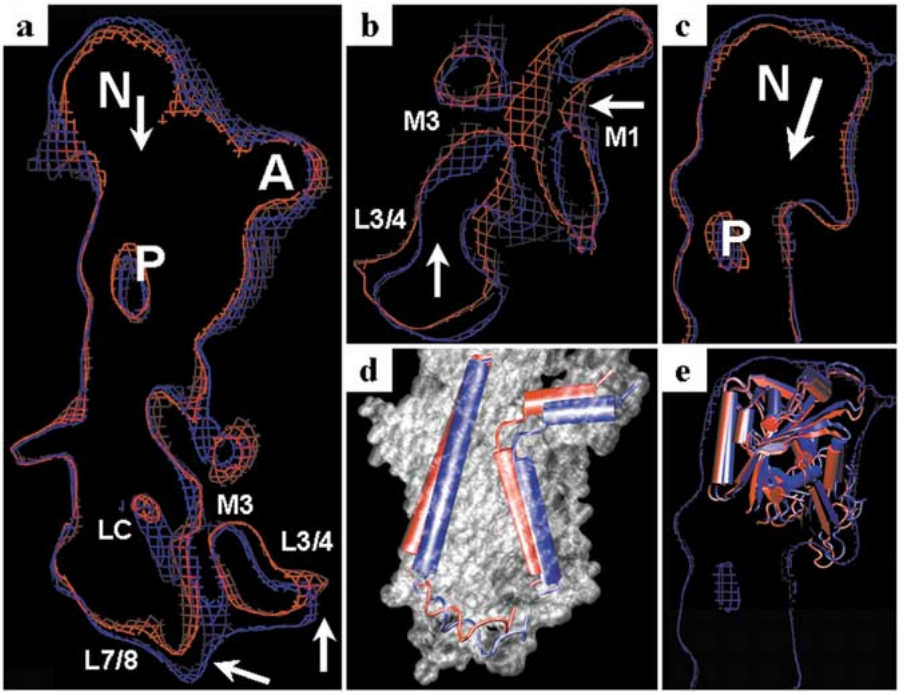

Fig. 6. Conformational changes in co-crystals of Ca^{2+}-ATPase and PLB. (*a*) Comparison of the 3-D structure of Ca^{2+}-ATPase in the absence (*blue*) and presence (*red*) of PLB. A section is shown through the molecules, illustrating the observed conformational changes. M3 and the M3/M4 loop (*L3/4*) are shifted upwards. The M7/M8 loop (*L7/8*) forms a more compact lumenal structure and the lumenal channel (*LC*) is greatly restricted. The A and P domains precisely superimpose, while the N domain rotates downward, (*b*) A section through the membrane showing the lateral movement of M1 and the upward movement of M3 and L3/4. (*c*) A section through the cytoplasmic domain showing the rotation of the N domain. (*d*) A model for the movements of M1, M3 and L3/4 in the absence (*blue*) and presence (*red*) of PLB. The Ca^{2+}-ATPase structure is shown as a transparent white surface. (*e*) A model for the rotation of the N domain in the absence (*blue*) and presence (*red*) of PLB.

2001b; Hutter et al., 2002; Toyoshima et al., 2003), biochemical cross-linking studies point to a membrane binding site involving M2 and M4 (Jones, Cornea & Chen, 2002; Chen et al., 2003; Toyoshima et al., 2003). In particular, the chemical cross-linking studies have led to models for the inhibitory complex with PLB positioned in a groove formed by M2, M4, M6, and M9 (Chen et al., 2003; Toyoshima et al., 2003). Earlier mutagenesis and co-immunoprecipitation studies (Asahi et al., 1999) focused on interaction sites along M6 and the M6/M7 loop, which now appear to be implausible. Also, direct interaction of the $KDDK^{400}$ loop in the N domain of Ca^{2+}-ATPase with the N-terminus of PLB was suggested by early cross-linking studies (James et al., 1989) and mutagenesis (Toyofuku et al., 1994). Although this has represented an important constraint in models (Young et al., 2001b; Hutter et al., 2002; Toyoshima et al., 2003), failure to reproduce this result with the more efficient hetero- and homo-bifunctional reagents (Chen et al., 2003) casts doubt on the veracity of this constraint.

With regard to the transport cycle, both M2 and M4 undergo dramatic rearrangements in the E_2-to-E_1 transition, and the movement of M4 is essential for forming the Ca^{2+} binding sites. It is possible that PLB binding serves to rigidify M2 and M4, thus preventing these rearrangements, similar to the proposed effects of TG. Interestingly, TG has been shown to prevent cross-linking between PLB and Ca^{2+}-ATPase (Jones et al., 2002; Chen et al., 2003) and, conversely, PLB has been shown to reduce the sensitivity of the cardiac Ca^{2+}-ATPase to TG (Mahaney et al., 1999). These effects are not due to physical overlap of the PLB and TG binding sites, because mutation of Phe^{256} disrupts TG binding without affecting PLB cross-linking (Chen et al., 2003). Nevertheless, the sites appear to be allosterically coupled, which likely explains the conformational changes in M1–M4 seen by electron microscopy upon PLB binding (*see* below).

The location of the PLB binding site has also been studied by electron cryomicroscopy of tubular co-crystals of PLB and Ca^{2+}-ATPase (Young et al. 2001b). Based on this work, we proposed a structural model for the interaction between PLB's cytoplasmic domain and Ca^{2+}-ATPase, which is inconsistent with most cross-linking results. Given the availability of higher-resolution structures of Ca^{2+}-ATPase in the E_2 and E_2P conformations (Toyoshima & Nomura, 2002; Xu et al., 2002), we have re-examined the Ca^{2+}-ATPase structure in the presence and absence of

PLB. Specifically, comparison of the 3-D structures of Ca^{2+}-ATPase in the absence (Xu et al., 2002) and presence of PLB (Young et al., 2001b) confirm difference densities, which we previously attributed to the cytoplasmic domain of PLB, and reveal new transmembrane difference densities (Fig. 5) (H.S. Young, *unpublished observations*).

The difference densities can be partly explained in terms of conformational changes in Ca^{2+}-ATPase upon PLB binding (Fig. 6). The Ca^{2+}-ATPase transmembrane domain in the presence of PLB more closely resembles the E_2, Ca^{2+}-free, inhibited state (Toyoshima & Nomura, 2002) than the E_2P state in the presence of vanadate (Xu et al., 2002). In the presence of PLB, M3 and M4 and their connecting loop shift upwards, which partly explains the transmembrane difference densities. As a result, the lumenal channel (Zhang et al., 1998) is partially occluded. Assigning a binding site for the transmembrane helix of PLB has been more problematic, due to both the moderate resolution and the disorder and/or partial occupancy of PLB in the helical lattice. Nonetheless, the location of our difference densities together with the cross-linking data (Jones et al., 2002; Toyoshima et al., 2003) imply that PLB may stabilize some or all of the N-terminal transmembrane helices of Ca^{2+}-ATPase (M1–M4) as part of its inhibitory mechanism. Such stabilization will undoubtedly limit activation by Ca^{2+} and communication by the A domain, and may explain the coupling between the TG binding site on M3 and the PLB binding site on M2 and M4 (Jones et al., 2002; Chen et al., 2003; Toyoshima et al., 2003).

Concluding Remarks

In the Ca^{2+}-ATPase transport cycle, the A domain forms at least four distinct interfaces with the N and P domains, at first controlling phosphate transfer from ATP and later controlling the stability of the phosphoenzyme. Two structural elements couple ATP hydrolysis to Ca^{2+} transport by coordinating the respective sites in the cytoplasmic and transmembrane domains. First, M4 and M5 provide rigid lever arms between the Ca^{2+} binding sites and the P domain. Second, the A domain and M1–M3 appear to be responsive to one another, forming a link between the Ca^{2+}-binding sites and the P domain. The interface between the A and P domains is the most responsive in the reaction cycle, and we conclude that this interface is a communicative step. Both thapsigargin and phospholamban appear to interfere with this coordination by preventing mobility of M1–M4. Challenges for the future include a structure for E_1P and a structure for the inhibitory complex of phospholamban and Ca^{2+}-ATPase.

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