The Mechanism of Ca\(^{2+}\) Transport by Sarco(Endo)plasmic Reticulum Ca\(^{2+}\)-ATPases

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**Function**

Ca\(^{2+}\) pumps, together with Ca\(^{2+}\) release channels, form ubiquitous Ca\(^{2+}\) regulatory systems in muscle and non-muscle cells. The sarcoplasmic reticulum Ca\(^{2+}\)-ATPases (SERCA)\(^{1}\) and the plasma membrane Ca\(^{2+}\)-ATPases have the highest affinity for Ca\(^{2+}\) removal from the cytoplasm and, together, set resting cytosolic Ca\(^{2+}\) concentrations. Three differentially expressed genes encode SERCA proteins (1). SERCA1a and -1b are expressed in fast-twitch skeletal muscle, but loss of SERCA1 function in Brody disease is sufficiently compensated to preserve life (2). SERCA2a is the cardiac/slow-twitch isoform, whereas SERCA2b, with a C-terminal extension, is expressed in smooth muscle and non-muscle tissues. It is almost certainly an essential gene. SERCA3 is expressed in a limited set of non-muscle tissues, including endothelial, epithelial, and lymphocytic cells and platelets, and its knock-out is not lethal (3).

SERCA enzymes are typical of the class of P-type ATPases, which form a phosphoprotein intermediate and undergo conformational changes during the course of ATP hydrolysis (4, 5). Some of the conformational states can be stabilized, either by adjustment of reaction conditions or through mutagenesis, and characterized as intermediates in the overall reaction cycle (Fig. 1A). The phosphorylated intermediate, \(E_{2}P(Ca)_{2}\), can phosphorylate ADP, whereas \(E_{2}P\) can only react with water. The formation of \(E_{2}P\) requires that two high affinity Ca\(^{2+}\) binding sites be occupied. The enzyme is then phosphorylated by ATP and, concomitantly, the two Ca\(^{2+}\) ions are occluded and can no longer exchange with cytoplasmic Ca\(^{2+}\).

The rate-limiting transition to \(E_{1}P\) is accompanied by loss of Ca\(^{2+}\) into the lumen, the affinity having fallen by 3 orders of magnitude. Hydrolysis of \(E_{1}P\) and regeneration of the high affinity Ca\(^{2+}\) binding sites \(E_{1}(Ca)_{2}\) complete the reversible cycle. High luminal Ca\(^{2+}\) drives the formation of \(E_{2}P\) from phosphate (\(P_{i}\)), and its effect on the level of \(E_{2}P\) led Jencks (5, 6) to postulate a second set of Ca\(^{2+}\) binding sites on the luminal surface. Proton countertransport, involving the exchange of one H\(^{+}\) per Ca\(^{2+}\), has been shown during the reaction cycle (7), emphasizing the similarity between Ca\(^{2+}\) and Na\(^{+}\)/K\(^{+}\)-ATPases.

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\(\S\) The abbreviations used are: SERCA, sarcoplasmic reticulum Ca\(^{2+}\)-ATPases; 8-azido-TNP-ATP, 8-azido-2-[(3-O-[(2,4,6-trimethoxyphenyl)amino]-5'-triphosphate; FTIR, Fourier-transform infrared spectroscopy.

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**Structure**

Two-dimensional arrays and helical tubes of SERCA1a were first produced by treatment of native membranes with decavanadate, and in a negative stain, these yielded a three-dimensional structure with a resolution of 25 Å (8). At high Ca\(^{2+}\) concentration, thin plates were obtained, which have given a 6-Å projection map (8, 9). Thapsigargin, a SERCA-specific inhibitor (10) that appears to bind to the M3 transmembrane sequence (11), stabilizes the \(E_{1}\) state of the pump and promotes formation of helical tubes (12). These are also compatible with bound nucleotides, but they are disrupted by low Ca\(^{2+}\)\. In contrast, the thin plates, probably corresponding to \(E_{1}(Ca)_{2}\), are disrupted by thapsigargin and by nucleotides.

Current modeling is based on a 14-A structure (Fig. 1B) obtained by cryoelectron microscopy of decavanadate tubes (13). A large cytoplasmic head is linked to the membrane by a narrow stalk. The protein within the membrane is divided into two major densities, A and A2, lying beneath the stalk, and two minor densities, B and C, to one side. A recent structure for the Ca\(^{2+}\)-bound complex (14) locates the nucleotide binding site in the groove on the underside of the head (Fig. 1B).

Two main segments of sequence (Fig. 2) form the cytoplasmic head and stalk (Fig. 1B) (15). The segment of about 130 residues following the M1/M2 hairpin, likely to form a 7–8-membered \(\beta\)-strand domain, is linked by long, amphipathic helices, S2 and S3, to M2 and M3. A central segment of about 440 residues forms the main head region. It includes the phosphorylation site at Asp\(^{501}\) and widely separated residues such as Lys\(^{684}\), Lys\(^{15}\), Cys\(^{674}\), Lys\(^{684}\), Lys\(^{303}\) and Asp\(^{507}\), which are affinity labeled by various nucleotide analogues in either Ca\(^{2+}\)- or homologous Na\(^{+}/K\(^{+}\)-ATPases (16). The N and C termini of the central domain form stalk helices S4 and S5, which link M4 to the phosphorylation site and M5 to the hinge region.

The bulk of the central domain is predicted to be a mixture of \(\alpha\)-helices and \(\beta\)-strands, which alternate fairly regularly in the C-terminal half, a characteristic associated with nucleotide binding, but less regularly in the N-terminal half, referred to as the phosphorylation domain. The phosphorylation domain extends to the variable sequence preceding Lys\(^{492}\), which is labeled by 8-azido-TNP-ATP (17), and is likely to be part of the nucleotide binding domain. On the basis of a 20-residue Walker B region and an overall \(\beta\)-\(\alpha\)-\(\beta\) pattern of secondary structure, kinase-related folds in the nucleotide binding region were proposed (18). However, the inclusion of Lys\(^{492}\) implicated an extra antiparallel \(\beta\)-strand in this
domain, which together with immunological evidence for an exposed epitope on the central strand of the β-sheet (19, 20) suggests that this region has a new fold specific to P-type pumps.

The C terminus of the nucleotide binding domain, close to the top of the stalk, is highly conserved and may form a subdomain, which by analogy with some kinases could form a hinge between phosphorylation and nucleotide binding domains. Several sites in this hinge region are labeled by γ-phosphate-linked affinity labels, suggesting that it is close to the phosphorylation site (16).

The sequences of all P-type ion pumps, except for the shorter ones mostly specific for copper or cadmium, show a common pattern of hydrophobicity (21). A 10-transmembrane helix model for SERCA1a and SERCA2a (Fig. 1) was proposed on the basis of analysis of hydrophobicity (15). It has received growing support from well controlled experiments with proteases, antibodies, and sulfhydryl labels, which showed the N and C termini to be cytoplasmic and the M7/M8 loop to be lumenal, and from proteolysis of intact vesicles with trypsin, which led to the isolation of cytoplasmic and luminal fragments corresponding to M1/M2, M3/M4, M5/M6, and M7/M8 (22). Mutated residues are color coded as follows: red, loss of function; yellow, partial loss of function; green, retention of function. Many of the mutations in the sequence between residues 365 and 412 were carried out with SERCA2a.

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Mechanistic investigations consistent with the 10-helix model have been carried out with a variety of probes and reagents, including antibodies, proteases, and sulfhydryl reagents, which indicate that the stalk incorporates the M9 and M10 hairpins and that the cytoplasmic loops M9 and M10 might occupy the C region, whereas M1 might be located in A1. A second approach to helix arrangement involves analysis of conserved (internal) and variable (lipid-exposed) sites in the transmembrane sequences of diverse Ca2+ pumps (21).

Structure/Function Relationships

Mutagenesis

Site-directed mutagenesis has provided key insights into structure/function relationships in SERCA1 and SERCA2 (24, 25). In these experiments, mutated cDNA is expressed transiently in heterologous cell culture, microsomal vesicles are isolated, and overall and partial reactions of ATP-dependent Ca2+ transport are assayed. In Fig. 3, loss of function, reduced function, and unaltered function mutants in SERCA1 or SERCA2 are located relative to predicted structural domains.

Ca2+ Binding Mutants—Two principles were key to the characterization of two Ca2+ binding sites through mutagenesis. These were: (i) that binding of Ca2+ to the first site (site I), presumably the more distal from the cytoplasm, leads to cooperative binding to the second, presumably more proximal site (site II) (6, 26); and (ii) occupation of both Ca2+ binding sites I and II is required for “forward” phosphorylation from ATP, whereas occupation of site I alone is sufficient to convert P i-reactive E1 conformation to non-reactive E1i, thereby depleting the substrate for “reverse” phosphorylation from P i (25, 27) (Fig. 1).

The initial mutagenic screen identified Glu309 in M4, Glu771 in M5, Asn796, Thr799, and Asn800 in M6, and Glu908 in M8 as potential Ca2+ binding ligands (28). Mutants were Ca2+ transport negative and not phosphorylated by ATP plus Ca2+; for all but N796A, high Ca2+ did not prevent reverse phosphorylation, suggesting that mutation of any of these residues would lead to the loss of at least one Ca2+ binding site. The first measurements of reverse phosphorylation were carried out at pH 6.4, but later measurements, carried out at neutral pH, showed normal Ca2+ inhibition of phosphorylation from Pi, for mutants E309Q as well as for N796A (29, 30). These results are consistent with retention of Ca2+ binding site I, implying that both Glu309 and Asn796 contribute to site I. This conclusion was supported by the direct demonstration that mutant E309Q retains a single Ca2+ binding site and that, at pH 6.4, Ca2+ can gain access to the site (presumably from the luminal side) in detergent-disrupted membranes but not in intact vesicles (31).

By contrast, mutants E771Q, T799A, and E908A showed similar, very low Ca2+ affinity in both forward and reverse phosphorylation assays, implying that site I was disrupted (29, 30). Mutant D800N showed reduced Ca2+ affinity in both assays but to different extents, suggesting that Asp800 was contributing to both sites. In the plasma membrane Ca2+-ATPases, which transport only a single
Minireview: Mechanism of Ca$^{2+}$ Transport by SERCA

[Diagram: Alternative models of the location of the two Ca$^{2+}$ binding sites within transmembrane helices M4, M5, and M6. A. Assignment of ligands to "stacked" Ca$^{2+}$ binding sites (27, 29, 30). B. Different views (schematic, cross-section, and oblique) of a "side-by-side" placement of Ca$^{2+}$ binding sites, consistent with cross-linking results and with a helical structure for M6 (35). C-E. Glu771 in M4; E6, Glu771 in M5; N4, Asn796 in M6; T6. Thr799 in M6. D. Asp800 in M6; E. Glu771 in M8. Ca$^{2+}$ binding ligands are indicated in red, whereas Ca$^{2+}$ is indicated in yellow. In the schematic view of the side-by-side model, the possibility of additional or alternative entry and exit sites is indicated by gray arrows. The oblique view consists of a series of tilted discs representing the seven tiers of the helical nets illustrated in Fig. 2. This view clarifies the proposed geometry of the sites but does not show the proposed right-handed coiling of the helices. Thr799 in M6 is partially obscured.

Ca$^{2+}$, the residues homologous to Glu771, Thr799, and Glu808, all assigned to site I, are replaced by Ala, Met, and Gln, respectively (32).

Mutants E309N, E771Q, N796A, T799A, D800N, as well as G310P and G801V (but not G770A) lost the ability to occlude Ca$^{2+}$ in the presence of CrATP (25, 30, 33). By contrast, E309Q retained full function (mutant E309Q, E771Q, and D800N are non-functional), and the mutant E908A retained Ca$^{2+}$ occlusion and transport with low affinity (25). These observations, together with the fact that Glu808 is the only mutation-sensitive residue in all of M8 (34), suggest, at best, a peripheral role in Ca$^{2+}$ binding and transport for Glu808 and for helix M8.

The location of Ca$^{2+}$ ligands on separate helices means that correct helix orientation will be crucial for the formation of high affinity Ca$^{2+}$ binding sites and that reorientation coupled to movements of the cytoplasmic domains could cause occlusion and changes in Ca$^{2+}$ affinity. The packing of these helices is being studied by introduction of pairs of cysteines into selected positions and assay of the expressed products for cross-linking (35). Cross-links observed at different tiers of helices M4 and M6 (A305C/L793C, E309C/N796C, T317CA/A804C) are in relative positions i, i + 4, i + 12, favoring packing of M4 and M6 as a right-handed supercoil at an angle of about 40°. It would normally be difficult to maintain such a large angle over several turns of helix, but the presence of four prolines and three glycines could permit sufficient curvature.

Insights from cross-linking data provide a possible solution to a problem raised by the assignment of Ca$^{2+}$ ligands to two sites in a model with the two sites stacked one above the other (Fig. 3A) (25, 26). Stacking leads to the placement of Asn796 in the more cytoplasmic site (site II), even though it is the most luminal ligand. Andersen (25) suggested that this might be resolved if M6 were not fully helical. However, if M4 and M6 are oriented to optimize cross-links between them, as indicated in Fig. 3B (cross-section), then Glu808, Asn796, and Asp900 would be positioned near the M4/M6 contact, whereas Thr799 would lie to the right of this contact. If M5 is placed so that Glu771 is opposed to Thr799 in M6, then the ligands between M4 and M6 are those assigned to site II (Glu309, Asn796), whereas the ligands between M5 and M6 are those assigned to site I (Glu771, Thr799). Asp800 in M6 would be in a position to contribute to both sites. This new "side-by-side sites" model has the advantage that Asn796, which lies below Asp900 in the helix (Fig. 3B, oblique view), can contribute to site II without distortion of the M5/M6 helices. Positioning of M5 so that Glu808 would be opposed to site I would permit its peripheral contribution to that site.

The side-by-side sites model would mean that the pathway of Ca$^{2+}$ translocation would follow an angular course (Fig. 3B, schematic, large arrows) rather than a direct pathway (Fig. 3A, schematic). In both schemes, occupation of site I by Ca$^{2+}$ initiates cooperative binding to site II, locking in Ca$^{2+}$ at site I, but in the side-by-side sites model Ca$^{2+}$ entry to site II could be through an independent pathway (small arrows). Randomization could follow when links are weakened following occlusion, and exit could be independent or through a single pathway (small or large arrows). There is, however, evidence for interaction during exit, since occupation of the more luminal site (presumably site I) prevents dissociation from the more cytoplasmic site, the inverse of the behavior at the cytoplasmic surface (6).

Scanning mutagenesis revealed that relatively small residues lying within discrete patches on the helix surfaces in the three turns surrounding the five Ca$^{2+}$ ligands in M4, M5, and M6 (Fig. 2), when mutated, block pump activity at a variety of different steps in the reaction cycle (34). By contrast, only a few of the residues in the top and bottom two turns of M4, M5, and M6 are sensitive to mutation and, overall, are much larger, especially in M5. This concentration of small residues in the middle of the membrane could provide a polar cavity with the larger surface residues controlling access to it. In M5 the replacement of the bulky Tyr785, above Glu771 and near the top of M5, by the tiny Glys gives an uncoupled mutant (36) in which Ca$^{2+}$ escapes to the cytoplasm before it can be translocated (Fig. 1). At the bottom of M4, the mutation-sensitive Lys297 (28, 37) has a suitable charge, size, and position to function as a gating residue for release of Ca$^{2+}$ to the lumen.

A double mutant, D813A/D818A, in the M6/M7 loop causes loss of Ca$^{2+}$-ATPase activity and loss of the ability of Ca$^{2+}$ to prevent phosphorylation from P, (38). This loop could form part of the entry portal to Ca$^{2+}$ binding sites I and II or be part of the gate operating during occlusion.

A motif in the mutation-sensitive regions of M4 and M6, (E/ N/D/D/E/E), suggests a sequence duplication (34). A sequence in the same region of M5 (SSNVGE) is related when reversed. However, since there is no symmetry in the ligands contributed by M4, M5, and M6 to the Ca$^{2+}$ binding and transport site, the significance of the similarity in this triad of binding sequences is not clear.

**ATP Binding Mutants**—Mutagenesis of highly conserved sequences in the large cytoplasmic domain between M4 and M5 showed that many mutants did not form phosphoenzyme intermediates from either ATP or P, consistent with, but not proving, their involvement in ATP binding (39, 40). Measurement of ATP binding affinity through competitive inhibition of [γ-32P]-azido-TNP-ATP photolabeling is a promising recent assay (41). With this assay, mutants of Phe487, Arg489, and Lys492 were found to have altered ATP dependence of ATPase activity in low, intermediate, and high ATP concentration ranges, showing their involvement in both catalytic and regulatory ATP binding. Mutants in Phe487 also failed to show CrATP-dependent Ca$^{2+}$ occlusion.

**Conformation Change Mutants**—The crucial labile intermediate E,P(Ca), normally loses its ADP sensitivity and its Ca$^{2+}$ within a few hundred milliseconds, but the intermediate can be stabilized. A number of site-directed mutations in all the major domains block this step (24, 25), showing that the conformational effects accompanying E,P → E,P involve all of these domains. Mutants that affect hydrolysis of E,P have so far been found only in M4, M5, and M6, implying that a limited, long range interaction between cytoplasmic and transmembrane sites controls this step.

**Chemical and Physical Probes of Conformational Changes**

The wide separation of phosphorylation sites and Ca$^{2+}$ sites within the ATPase molecule implies long distance transmission of...
conformational effects, whereas the rigid domains and long helices provide a plausible medium for their transmission. Although the nature and extent of conformational changes cannot be defined without a detailed structure, a variety of chemical and physical methods have been employed to detect conformational changes and, in some cases, to follow their kinetics. Cross-linking of the hinge domain to the N terminus of the nucleotide binding domain blocks $E_P \rightarrow E_P$ (42), providing evidence for essential domain movements. Analysis of x-ray diffraction following the photolysis of caged ATP (43) or comparisons of structural features of the enzyme crystalized in two different conformations (44) provide evidence for large global changes consistent with domain movements. Large domain movements, however, are not reflected in changes in CD (45), in intrinsic fluorescence, or in excitation energy transfer between bound dyes (46). FTIR difference spectra in which absorption bands can be assigned to specific bond types (8, 47, 48) show that changes in the membrane. FTIR difference spectra in which absorption bands can be assigned to specific bond types (8, 47, 48) show that small amino acids and blocked from exit by the juxtaposition of relatively large residues at both ends of the transmembrane helices. Further conformational changes open the gate allowing exit of the two Ca$^{2+}$ ions to the lumen. This conformation also activates dephosphorylation and returns the pump to the high Ca$^{2+}$ affinity form, completing the cycle. (Copyright 1997, Alice Y. Chen.)

**Mechanism of Ca$^{2+}$ Transport**

A basic mechanism for a P-type pump, illustrated in Fig. 4, takes into account the characteristics of the transport process and the structure of the pump (24). The Ca$^{2+}$ binding and translocation sites are in a cavity between M4, M5, and M6, where they are formed by the precise juxtaposition of Ca$^{2+}$ binding residues located in these three helices. Access to the cavity is controlled by interactions between the larger residues near the cytoplasmic ends of the helices. The phosphorylation-induced domain movements that close off cytoplasmic access to the cavity will initiate occlusion. If such movements involved M4, M5, or M6, they might also be expected to disrupt the precise placement of the ligands required to form high affinity sites, so that, after occlusion, the two Ca$^{2+}$ ions would be less firmly bound and capable of exchanging their positions, consistent with kinetic observations (49). Further long range, phosphorylation-induced domain movements will open the exit gate, permitting release of weakly bound Ca$^{2+}$ to the lumen. Later conformational changes will result in dephosphorylation of $E_P$ and reformation of $E_Ca$, completing the Ca$^{2+}$ transport cycle.

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