# Cooperative Setting for Long-Range Linkage of $Ca^{2+}$ Binding and ATP Synthesis in the $Ca^{2+}$ ATPase

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ABSTRACT High-affinity and cooperative binding of two $Ca^{2+}$ per ATPase (SERCA) occurs within the membrane-bound region of the enzyme. Direct measurements of binding at various $Ca^{2+}$ concentrations demonstrate that site-directed mutations within this region interfere selectively with $Ca^{2+}$ occupancy of either one or both binding sites and with the cooperative character of the binding isotherms. A transition associated with high affinity and cooperative binding of the second $Ca^{2+}$ and the engagement of N796 and E309 are both required to form a phosphoenzyme intermediate with ATP in the forward direction of the cycle and also to form ATP from phosphoenzyme intermediate and ADP in the reverse direction of the cycle. This transition, defined by equilibrium and kinetic characterization of the partial reactions of the enzyme cycle, extends from transmembrane helices to the catalytic site through a long-range linkage and is the mechanistic device for interconversion of binding and phosphorylation potentials.

## INTRODUCTION

Sarcoplasmic reticulum (SR) vesicles (Ebashi and Lippman, 1962; Hasselbach and Makinose, 1961) have provided an advantageous system for characterization of the catalytic and transport cycle (Hasselbach, 1964; Tada et al., 1978; de Meis and Vianna, 1979) and of the protein structure (MacLennan et al., 1985; Toyoshima et al., 1993, 2000) of the $Ca^{2+}$ ATPase. The cycle begins with enzyme activation by cooperative binding of two $Ca^{2+}$ (Inesi et al., 1980), followed by phosphoryl transfer from ATP to a catalytic site residue (Asp351), vectorial translocation of the bound $Ca^{2+}$, and hydrolytic cleavage of the phosphorylated enzyme intermediate. The mechanism whereby $Ca^{2+}$ binding produces catalytic activation and enzyme phosphorylation causes vectorial translocation of $Ca^{2+}$ is a fundamental question of general interest, pertinent to the role of proteins in energy transduction through biochemical reactions. It was originally proposed that phosphorylation and $Ca^{2+}$ translocation may be directly coupled through a symport mechanism (Hasselbach, 1964; Mitchell and Koppenol, 1982), based on spectroscopic evidence of cation binding within the catalytic site (Grisham and Mildvan, 1974). However, it was later found that single mutations of residues within the membrane-bound region of recombinant ATPase (i.e., E309, E771, N796, T799, and D800) produce enzyme inactivation (Clarke et al., 1989). This effect was attributed to interference with $Ca^{2+}$ binding, suggesting that the $Ca^{2+}$-binding domain resides within the membrane-bound region, as later established by structural analysis (Toyoshima et al., 2000). The location of the $Ca^{2+}$ sites is ~50 Å away from the catalytic site in the cytosolic region, and therefore a long-range intramolecular linkage is required for catalytic activation (Bigelow and Inesi, 1992; Inesi et al., 1992). We have now characterized these mutants, with the aim of defining the mechanism of binding and demonstrating unambiguously whether occupancy of the first and/or the second $Ca^{2+}$ site is required not only for catalytic activation in the forward direction of the cycle but also for synthesis of ATP in the reverse direction.

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## MATERIALS AND METHODS

### Materials

Recombinant ATPase protein was obtained in the microsomal fraction of COS-1 cells infected with adenovirus vectors carrying wild-type (WT) or mutated cDNA encoding the chicken fast muscle $Ca^{2+}$ ATPase (SERCA1). The methods used for construction of vectors, cultures, and preparation of microsomes were previously described in detail (Zhang et al., 2000). The total microsomal protein was determined using bicinchoninic acid with the biuret reaction (Pierce, Rockford, IL). In all experiments, the total protein concentration was adjusted to yield comparable SERCA concentration as determined by Western blotting.

### $Ca^{2+}$ binding in the absence of ATP

Microsomal samples were suspended in 2.0 ml of a medium containing 20 mM 3-(*N*-morpholino)propane sulfonic acid (MOPS), pH 7.0, 80 mM KCl, 5mM $MgCl_2$, and variable EGTA, to yield 0.8 mg of protein/ml, and 8 $\mu$l of 2 mM thapsigargin (TG) in dimethyl sulfoxide ($Me_2SO$), or 8 $\mu$l of $Me_2SO$, was also included. After a 10-min incubation in ice, an equal volume of a medium containing 20 mM MOPS, pH 7.0, 80 mM KCl, 5 mM $MgCl_2$, 80 $\mu$M $^{45}CaCl_2$ was added. The total calcium concentration, after mixing, was 52.5 $\mu$M, including added and contaminant (12.5 $\mu$M) calcium. After a 10-min incubation, the suspension was sonicated three times for 10 s each, and 0.750-ml samples (corresponding to 0.3 mg of protein) were vacuum filtered (0.45 $\mu$m; Millipore, Bedford, MA). The filters were then blotted, and the radioactivity was determined by scintillation counting. The free $Ca^{2+}$ concentration was calculated from total calcium and EGTA concentration, according to Fabiato and Fabiato (1978). The difference between binding obtained in the absence and in the presence of TG was considered to be specific binding. The TG-independent binding varied from 95% of total binding at pCa 7.0 to 40% at pCa 5.0.

### Enzyme phosphorylation with Pi

Enzyme phosphorylation with Pi was obtained by incubating 60 $\mu$g of microsomal protein in 1 ml of a medium containing 50 mM 2-[*N*-morpholino]ethane sulfonic acid (MES), pH 6.2, 10 mM $MgCl_2$, 100 $\mu$M $^{32}P_i$, 500 $\mu$M EGTA, 20% (v/v) $Me_2SO$, and various concentrations of $Ca^{2+}$ as required by the experimental schedule. After 10 min of incubation at 25°C, the reaction was quenched by the addition of 0.5 ml of 3 M perchloric acid (PCA), and 100 $\mu$g of carrier microsomal protein previously quenched in 0.5 ml of 1 M PCA was then added. The quenched samples were cooled in ice, sedimented by low-speed centrifugation, and resuspended in 1 ml of cold 0.125 M PCA. This washing procedure was repeated three times, resuspending twice in PCA and once with $H_2O$. The final sediment was dissolved in 0.2 ml of a medium containing 5% lithium dodecyl sulfate in 50 mM phosphate buffer, pH 6.3. The residual protein concentration was measured, and 60 $\mu$g per sample was placed on 6.5% acrylamide gels and subjected to electrophoresis by the method of Weber and Osborn (1969). The gels were then dried and the radioactivity determined by phosphoimaging.

### ATP synthesis by reversal of the catalytic cycle

Microsomal samples were added to a medium containing 5 mM MES, pH 6.2, 10 mM $MgCl_2$, 0.1 mM $^{32}P_i$, 0.5 mM EGTA, 20% (v/v) $Me_2SO$, and 2 $\mu$M A23187 ($Ca^{2+}$ ionophore), to yield 50–100 $\mu$g of protein/ml. After a 5-min incubation at 25°C, 0.2-ml samples were rapidly mixed with 1.5 ml of ice-cold 50 mM HEPES, pH 8.0, 10 mM $MgCl_2$, 1 mM $CaCl_2$, and 0.1 mM ADP. Such samples were quenched at serial times by the addition of 1.0 ml of ice-cold 3 M PCA. After a brief centrifugation, the supernatant was collected for extraction of $^{32}P_i$ by addition of 1.0 ml of acetone, 0.5 ml of 5% ammonium molybdate in 2.5 N $H_2SO_4$ and 10 $\mu$l of 100 mM $P_i$ (carrier). When the solution was not completely clear, more acetone was added to obtain a clear yellowish solution. The phosphomolybdate complex was extracted by vortexing with 2 ml of isobutanol-benzene (1:1) and discarding the upper phase. Two additional extractions were carried out with 1 ml of acetone, 10 $\mu$l of 100 mM $P_i$, and 2 ml of isobutanol-benzene. A fourth extraction was performed to eliminate any remnant ammonium molybdate, with 1 ml of acetone, 0.2 ml of 100 mM $P_i$, and 2.0 ml of isobutanol-benzene. A final extraction was performed with 1.0 ml of acetone and 2.0 ml of isobutanol-benzene, and an aliquot of the remaining solution was then processed for determination of radioactivity. The correspondence of residual radioactivity to newly synthesized ATP was independently demonstrated by chromatography.

## RESULTS

The functional consequences of E771, T799, D800, E309, or N796 mutations, i.e., interference with $Ca^{2+}$ activation of ATP use and $Ca^{2+}$ inhibition of enzyme phosphorylation by $P_i$, suggest mutational interference with $Ca^{2+}$ binding (Clarke et al., 1989). Direct measurements of $Ca^{2+}$ binding to recombinant enzyme, however, are quite difficult. Nevertheless, preliminary measurements of binding at a single $Ca^{2+}$ concentration suggested that E771Q, T799A, and D800N mutations interfere with binding of both $Ca^{2+}$ required for enzyme activation, whereas mutations of E309 or N796 allow binding of only one $Ca^{2+}$ (Skerjanc et al., 1993; Zhang et al., 2000). We have now obtained direct measurements of $Ca^{2+}$ binding with recombinant ATPase at various $Ca^{2+}$ concentrations. Fitting the WT binding data requires a cooperative binding equation with two interdependent constants (7e+5 and 2e+6 M-1) for two sites exhibiting positive cooperativity, whereas the mutant data can be fitted with an independent binding equation and a single constant (2e+6 M-1). The resulting equilibrium binding isotherms (Fig. 1 *A*) demonstrate that, as opposed to the positive cooperativity of the WT enzyme, the E309Q, N796A, or E309Q/N796A mutants sustain noncooperative binding with a maximal stoichiometric ratio of one $Ca^{2+}$ per ATPase within the pCa 7.0–5.0 concentration range. It is likely that additional, noncooperative binding occurs at higher concentrations that preclude reliable measurements. However, such an additional binding has no specific functional consequences, because no ATP hydrolysis or ATP synthesis is obtained by addition of $Ca^{2+}$ concentrations as high as millimolar (see below).

Binding by any of the E309Q, N796A, or E309Q/N796A mutants is not significantly different, indicating that the E309 and N796 mutations eliminate completely binding at one site, while allowing stoichiometric occupancy of the other site. On the other hand, we found that the E771Q, T799A, and D800N mutations interfere totally with binding at either site, within the 7.0–5.0 pCa range (Table 1). This indicates that binding of a first $Ca^{2+}$, involving E771, T799, and D800, is required to reposition the N796 and E309 side chains for high-affinity binding of a second $Ca^{2+}$. In the final cooperative complex, the D800 side chain contributes one oxygen to the first $Ca^{2+}$ complex and the other oxygen to the second, as shown by crystallographic analysis (Fig. 1 *B*). A most important feature of this system is that even though the E309Q and N796A mutants retain $Ca^{2+}$ binding at the first site, the ATPase cannot be phosphorylated by ATP, and catalytic activity is totally inhibited (Table 1). Therefore, activation of the catalytic site in the cytosolic region requires a long-range signal that is triggered by cooperative occupancy of the second site. It is noteworthy that the plasma membrane ATPase (Strehler et al., 1990), which handles only one $Ca^{2+}$ per cycle, retains a strong homology corresponding to site II, whose occupancy is strictly required for enzyme activation. On the other hand, divergence in the sequence corresponding to site I (such as M for T799, S for N768, and A for E771) do not allow $Ca^{2+}$ binding but are likely to prime the conformation of site II for high-affinity binding.

Another advantageous feature of the $Ca^{2+}$ pump is its reversal, demonstrated by measurements of ATP synthesis coupled to $Ca^{2+}$ efflux from loaded vesicles (Makinose and Hasselbach, 1971). The partial reactions involved in ATP synthesis can be studied by reacting $P_i$ with the enzyme destabilized by $Ca^{2+}$ dissociation, to yield ADP-insensitive phosphoenzyme (Masuda and de Meis, 1973; Kanazawa and Boyer, 1973). Addition of millimolar $Ca^{2+}$ then renders the phosphoenzyme ADP sensitive, and ATP is obtained if ADP is added with $Ca^{2+}$ (Knowles and Racker, 1975; de Meis and Tume, 1977). It is of interest that ADP-insensitive phosphoenzyme can be obtained by reacting site I and site II mutants with $P_i$ just as well as with recombinant WT ATPase (Table 1). Although the $P_i$ reaction with WT enzyme is readily inhibited by high-affinity $Ca^{2+}$ binding, phosphorylation of site I mutants is

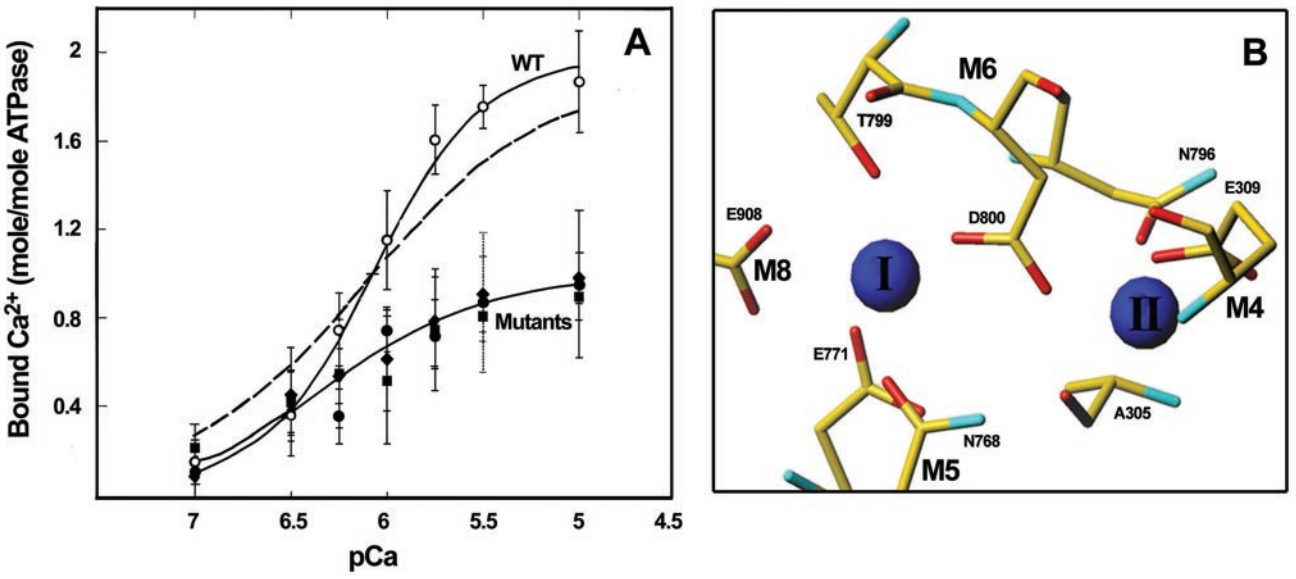


FIGURE 1 $Ca^{2+}$ binding by WT, E309Q, T796A, and E309Q/T796A mutants (*A*) and molecular graphics representation of the two $Ca^{2+}$-binding sites in WT ATPase (*B*). (*A*) Recombinant ATPase protein was obtained from Cos1 cells infected with adenovirus vectors, and $Ca^{2+}$ binding was measured in the absence of ATP as described by Zhang et al. (2000). ○, WT; the mutants are E309Q (■), N796A (◆), and E309Q/N796A (●). Each point is the average of 30–35 samples. The errors bars correspond to standard deviations of the average of all experimental values obtained with the mutants at any $Ca^{2+}$ concentration, which were then used for fitting. The experimental points ($Ca^{2+}$-bound/E) obtained with WT ATPase required fitting with a cooperative two-site equation ($K_1[Ca^{2+}] + 2K_1K_2[Ca^{2+}]^2/1 + K_1[Ca^{2+}] + K_1K_2[Ca^{2+}]^2$), whereas those for the E309Q, T796A, and E309Q/T796A mutants could be fitted simply with an independent binding equation ($K[E_{tot}][Ca^{2+}]/1 + K[Ca^{2+}]$). The dashed line shows the poor fitting of the WT data using an independent binding equation. No significant binding was observed with the E771Q, T799A, or D800N mutants (not shown). (*B*) Representation of the residues involved in $Ca^{2+}$ binding was obtained directly from the crystallographic structure of the SR ATPase with $Ca^{2+}$ bound (Toyoshima et al., 2000) using a silicon graphics (SGI) system with Turbo-FRODO software. M4, M5, M6, and M8 refer to the transmembrane segments originating the binding residues. The section is along the plane of the membrane, viewed from the cytosolic side.

hardly inhibited by millimolar $Ca^{2+}$ (Clarke et al., 1989). On the other hand, inhibition of the $P_i$ reaction with site II mutants is produced by $Ca^{2+}$ within the micromolar concentration range (Andersen and Vilsen, 1992; Vilsen and Andersen, 1992). We show here that the patterns of inhibition are similar to those of $Ca^{2+}$ binding for WT and the T796A mutant (including the difference in cooperativity; compare Figs. 1 *A* and 2). Inhibition of the E309Q mutant phosphorylation, however, requires a significantly higher $Ca^{2+}$ concentration (compare Figs. 1 *A* and 2). It should be pointed out that inhibition of the $P_i$ reaction requires transmission to the catalytic site in addition to $Ca^{2+}$ binding. It is clear that this transmission requires engagement of the M4 helical segments, which normally occurs by participation of E309 in $Ca^{2+}$ binding. It is likely that in the E309Q mutant, alternative oxygen functions participate with lesser affinity.

**TABLE 1 Effects of mutations of site I or site II residues**

| Sample | $Ca^{2+}$ site occupancy | ATPase activity | EP formation (with ATP) | EP formation (with $P_i$) | ATP synthesis |
|---|---|---|---|---|---|
| WT | I and II | 850 ± 52 | 0.61 ± 0.13 | 0.54 ± 0.12 | 100 |
| E309A | I | 0 | 0 | 0.85 ± 0.18 | 0 |
| N796A | I | 0 | 0 | 0.61 ± 0.12 | 0 |
| E309A/N796A | I | 0 | 0 | 0.32 ± 0.10 | 0 |
| E771Q | None | 0 | 0 | 0.84 ± 0.21 | 0 |
| T799A | None | 0 | 0 | 0.41 ± 0.15 | 0 |
| D800N | None | 0 | 0 | 0.60 ± 0.16 | 0 |

$Ca^{2+}$ binding, ATPase activity, phosphoenzyme (EP) formation by ATP in the presence of $Ca^{2+}$, phosphoenzyme formation with $P_i$ in the absence of $Ca^{2+}$, and ATP synthesis by addition of ADP and $Ca^{2+}$ to phosphoenzyme formed with $P_i$ were measured as described previously (Zhang et al., 2000; de Meis and Inesi, 1982). The values refer to nmol/mg of protein/min for ATPase activity, nmol/mg for EP levels, and percentage of EP ($P_i$) used for ATP synthesis. The functional values obtained with mutants were corrected based on ATPase expression as defined by Western blots, with reference to microsomes of cells expressing WT ATPase. As the ATPase content of the microsomal preparation is 0.9–1.0 nmol/mg of protein, the EP obtained with ATP or $P_i$ reflects steady-state or equilibrium levels under these conditions, respectively.

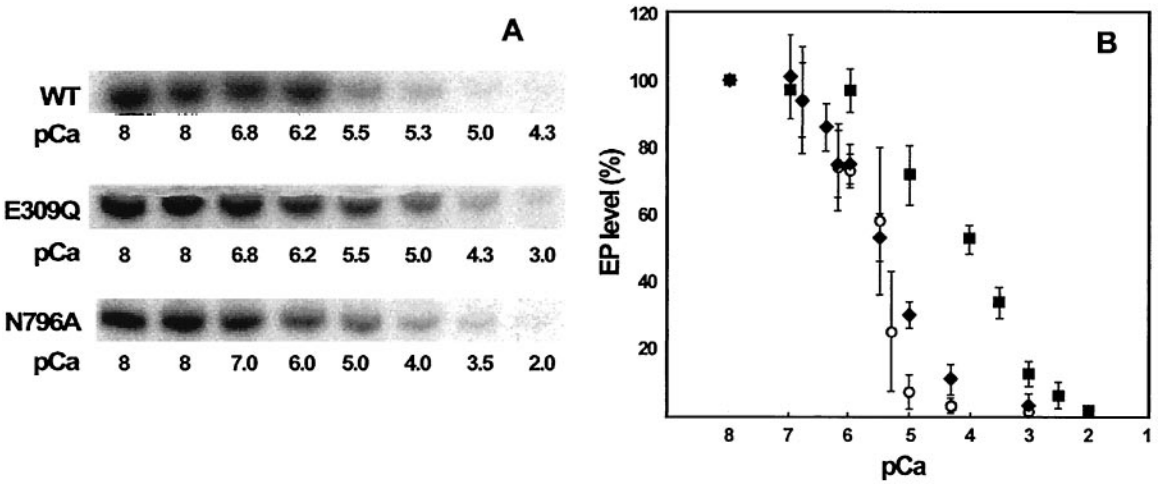


FIGURE 2 $Ca^{2+}$ inhibition of enzyme phosphorylation by $P_i$. (*A*) Examples of phosphorylation detected by electrophoretic gels and phosphoimaging; (*B*) Average levels of phosphoenzyme obtained by equilibrating WT (○), E309Q (■), and T796A (♦) protein with $P_i$ (see Materials and Methods) in the presence of various $Ca^{2+}$ concentrations. (pCa 8 data are repeated to establish reliably the maximal phosphorylation levels. The E771Q, T799A, and D800N exhibited only slight inhibition at pCa 3 (not shown).

A most important finding of our current experiments is that while the WT phosphoenzyme synthesizes ATP with 80% efficiency upon addition of millimolar $Ca^{2+}$ and ADP, no ATP is obtained with either site I or site II mutants, even when millimolar $Ca^{2+}$ is added with ADP (Fig. 3). In fact, the phosphoenzyme formed by reacting the mutant enzyme with $P_i$ remains in its ADP-insensitive form before decaying slowly by hydolytic cleavage (Fig. 3 *A*). Therefore, even though site II mutants can bind $Ca^{2+}$ to inhibit the $P_i$ reaction, they cannot undergo the $Ca^{2+}$ conformational change that is required to form ATP from phosphoenzyme and ADP. It is therefore clear that occupancy of the second $Ca^{2+}$ site in its high-affinity state and the engagement of N796 and E309 are both necessary to confer high phosphorylation potential to the intermediate formed by the $P_i$ reaction.

## DISCUSSION

The sequence of partial reactions comprising the catalytic and transport cycle of the $Ca^{2+}$ ATPase is given in Fig. 4,

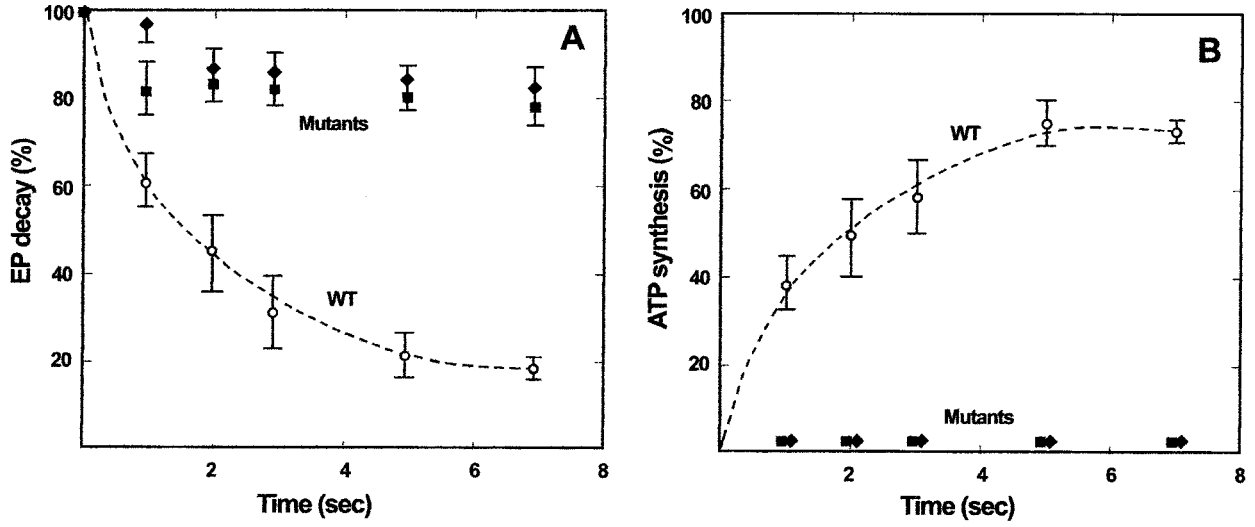


FIGURE 3 Phosphoenzyme decay (*A*) and ATP synthesis (*B*) after addition of $Ca^{2+}$ and ADP to phosphoenzyme formed by reacting the ATPase with $P_i$ in the absence of $Ca^{2+}$. Phosphoenzyme was obtained by reacting WT (○), E309Q (■), and T796A (♦) mutants with $P_i$ in the absence of $Ca^{2+}$ (Table 1). At time 0, 1 mM $Ca^{2+}$ and 0.1 mM ADP were added, and serial samples were taken for measurements of residual phosphoenzyme and newly synthesized ATP Reaction conditions and methods were as described by de Meis and Inesi (1982) (see Materials and Methods). The values are in percentage of the phosphoenzyme level at time 0.

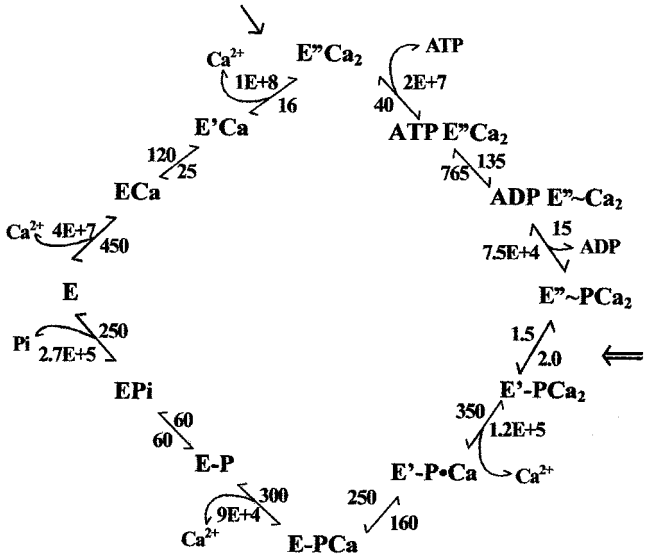


FIGURE 4 The catalytic and transport cycle of the SR ATPase. This sequence of partial reactions, with their microscopic constants, is required to fit the observed ATPase behavior, in equilibrium or kinetic experiments (Inesi et al., 1988). Most partial reactions, and their constants, were determined experimentally. The units are in $s^{-1}$ for first-order reactions, and $s^{-1}$ $M^{-1}$ for second-order reactions. The overall $K_{eq}$ is 4.9e+5, and is the result of ATP terminal phosphate hydrolytic cleavage under standard conditions. E′ and E″ indicate the conformations of the enzyme with one or two calcium ions bound with high affinity. E-P and E~P indicate phosphoenzyme with low or high phosphorylation potential. The constants given here were obtained in studies with rabbit (native) SR ATPase and may not be totally identical to those pertinent to chicken (recombinant) SERCA1.

which is based on the Post-Albers mechanism as written by de Meis and Vianna (1979) for the $Ca^{2+}$ ATPase. Additional reactions, however, and their microscopic constants are included in the sequence, as required to fit the experimentally observed equilibrium and kinetic behavior of the $Ca^{2+}$ ATPase (Inesi et al., 1988). In the scheme, the effect of site II occupancy by $Ca^{2+}$ occurs after the E′Ca + $Ca^{2+}$ → E″ $Ca_2$ reaction, allowing catalytic activation and ATP use to form the phosphorylated enzyme intermediate. A most interesting isomerization of the phosphoenzyme occurs then with the E′-$PCa_2$ ↔ E″~$PCa_2$ reaction, whereby phosphorylation and binding potentials are affected concomitantly, whereas free energy is conserved through conformational change ($K_{eq}$ ≅ 1). The importance of this isomerization, which requires occupancy of both $Ca^{2+}$ sites, is that it leads to $Ca^{2+}$ dissociation in the forward direction of the cycle and ATP synthesis in the reverse direction. The sequential reactions spelled out in Fig. 4 were originally proposed to explain the cooperative character of $Ca^{2+}$ binding and the kinetic behavior (Inesi et al., 1980) of the SR ATPase. Their occurrence and role in the catalytic cycle are here demonstrated unambiguously by mutational analysis. Furthermore, these phenomena can now be explained in structural terms (Fig. 5). In fact, the long-range effect of $Ca^{2+}$ occupancy of site II is due to engagement of E309 and

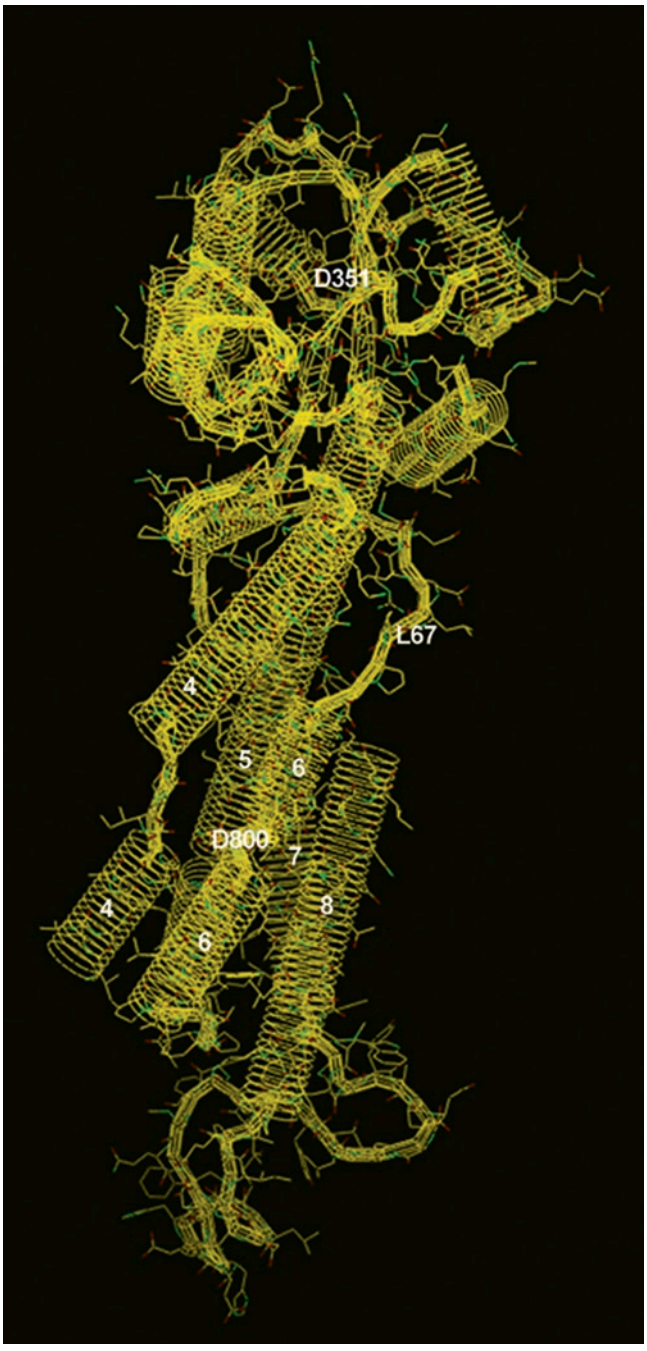


FIGURE 5 Long-range linkage of $Ca^{2+}$ binding and phosphorylation sites. Structural representation of the transmembrane helices involved in $Ca^{2+}$ binding, and its connection to the cytosolic phosphorylation domain, was obtained directly from the crystallographic structure of the SR ATPase with $Ca^{2+}$ bound (Toyoshima et al., 2000). The I298-G360 and R604-S915 sequence segments were selected, using an SGI system with TurboFRODO software. D351 and D800 indicate residues with a central role in phosphorylation and high-affinity $Ca^{2+}$ binding, respectively. The numbers 4–8 refer to transmembrane (M) segments, and L67 refers to the cytosolic loop between M6 and M7. Refer to Fig. 1 *B* for the contribution of M4, -5, -6, and -8 to $Ca^{2+}$ binding. The lower third of the structure corresponds to the membrane-bound region. The M1, M2, M3, M9, and M10 transmembrane segments and A (actuator) and N (nucleotide) cytoplasmic domains are not shown.

displacement of M4 transmembrane segment, which is directly connected to the phosphorylation site (D351) through a highly conserved and mutation-sensitive sequence (Zhang et al., 1995). Cooperative interactions within the two sites results in displacement of additional segments, especially the mutation-sensitive M5 and M6/M7 loop, which are then transmitted to the phosphorylation/catalytic site (Sorensen and Andersen, 2000; Zhang et al., 2001). Additional changes within the enzyme headpiece are expected upon nucleotide binding and use. Our findings define a specific functional role for the $Ca^{2+}$-dependent conformational changes demonstrated by crystallographic studies (Toyoshima et al., 2000; Xu et al., 2002; Toyoshima and Nomura, 2002) and its linkage to the phosphorylation potential.

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## REFERENCES

Andersen, J. P., and B. Vilsen. 1992. Functional consequences of alterations to Glu309, Glu771, and Asp800 in the $Ca^{2+}$-ATPase of sarcoplasmic reticulum. *J. Biol. Chem.* 267:19383–19387.

Bigelow, D., and G. Inesi. 1992. Contributions of chemical derivatization and spectroscopic studies to the characterization of the $Ca^{2+}$ transport ATPase of sarcoplasmic reticulum. *Biochim. Biophys. Acta. Biomembr.* 1113:323–338.

Clarke, D. M., T. W. Loo, G. Inesi, and D. H. MacLennan. 1989. Location of high affinity $Ca^{2+}$-binding sites within the predicted transmembrane domain of the sarcoplasmic reticulum $Ca^{2+}$-ATPase. *Nature.* 339: 476–478.

de Meis, L., and G. Inesi. 1982. ATP synthesis by sarcoplasmic reticulum ATPase following $Ca^{2+}$, pH, temperature, and water activity jumps. *J. Biol. Chem.* 257:1289–1294.

de Meis, L., and R. Tume. 1977. A new mechanism by which a $H^+$ concentration gradient drives the synthesis of ATP: pH jump and ATP synthesis by the $Ca^{2+}$ dependent ATPase of sarcoplasmic reticulum. *Biochemistry.* 16:4455–4463.

de Meis, L., and A. L. Vianna. 1979. Energy interconversion by the $Ca^{2+}$-dependent ATPase of the sarcoplasmic reticulum. *Annu. Rev. Biochem.* 48:275–292.

Ebashi, S., and F. Lippman. 1962. Adenosine triphosphate-linked concentration of calcium ions in a particulate fraction of rabbit muscle. *J. Cell Biol.* 14:389–400.

Fabiato, A., and F. Fabiato. 1978. Effects of pH on the myofilaments and the sarcoplasmic reticulum of skinned cells from cardiac and skeletal muscles. *J. Physiol.* 276:233–255.

Grisham, C. M., and A. S. Mildvan. 1974. Magnetic resonance and kinetic studies of the mechanism of sodium and potassium ion-activated adenosine triphosphatase. *J. Biol. Chem.* 249:3187–3197.

Hasselbach, W. 1964. Relaxing factor and the relaxation of muscle. *Prog. Biophys. Biophys. Chem.* 14:169–222.

Hasselbach, W., and M. Makinose. 1961. Die Calciumpumpe der "Erschlaffungsgrana" des Muskels and ihre Abhangigkeit von der ATP-spaltung. *Biochem. Z.* 333:518–528.

Inesi, G., M. Kurzmack, C. Coan, and D. Lewis. 1980. Cooperative calcium binding and ATPase activation in sarcoplasmic reticulum vesicles. *J. Biol. Chem.* 255:3025–3031.

Inesi, G., M. Kurzmack, and D. Lewis. 1988. Kinetic and equilibrium characterization of an energy- transducing enzyme and its partial reactions. *Methods Enzymol.* 157:154–190.

Inesi, G., D. Lewis, D. Nikic, A. Hussain, and M. E. Kirtley. 1992. Long-range intramolecular linked functions in the calcium transport ATPase. *Adv. Enzymol. Relat. Areas Mol. Biol.* 65:185–215.

Kanazawa, T., and P. D. Boyer. 1973. Occurrence and characteristics of a rapid exchange of phosphate oxygens catalyzed by sarcoplasmic reticulum. *J. Biol. Chem.* 248:3163–3172.

Knowles, A. F., and E. Racker. 1975. Formation of adenosine triphosphate from $P_i$ and adenosine diphosphate by purified $Ca^{2+}$-adenosine triphosphatase. *J. Biol. Chem.* 250:1949–1951.

MacLennan, D. H., C. J. Brandl, B. Korczak, and N. M. Green. 1985. Amino-acid sequence of a $Ca^{2+}+Mg^{2+}$ dependent ATPase from rabbit muscle sarcoplasmic reticulum, deduced from its complementary DNA sequence. *Nature.* 316:696–700.

Makinose, M., and W. Hasselbach. 1971. ATP synthesis by the reverse of the sarcoplasmic calcium pump. *FEBS Lett.* 12:271–272.

Masuda, H., and L. de Meis. 1973. Phosphorylation of the sarcoplasmic reticulum membrane by orthophosphate: inhibition by calcium ions. *Biochemistry.* 12:4581–4585.

Mitchell, P., and W. H. Koppenol. 1982. Chemiosmotic ATPase mechanisms. *Annu. NY Acad. Sci.* 402:584–601.

Skerjanc, I. S., T. Toyofuku, C. Richardson, and D. H. MacLennan. 1993. 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.* 268:15944–15950.

Sorensen, T. L., and J. P. Andersen. 2000. Importance of stalk segment S5 for intramolecular communication in the sarcoplasmic reticulum $Ca^{2+}$-ATPase. *J. Biol. Chem.* 275:28954–28961.

Strehler, E. E., P. James, R. Fischer, R. Heim, T. Vorherr, A. G. Filoteo, J. T. Penniston, and E. Carafoli. 1990. Peptide sequence analysis and molecular cloning reveal two calcium pump isoforms in the human erythrocyte membrane. *J. Biol. Chem.* 265:2835–2842.

Tada, M., T. Yamamoto, and Y. Tonomura. 1978. Molecular mechanism of active calcium transport by sarcoplasmic reticulum. *Physiol. Rev.* 58: 1–79.

Toyoshima, C., M. Nakasako, H. Nomura, and H. Ogawa. 2000. Crystal structure of the calcium pump of sarcoplasmic reticulum at 2.6 A resolution. *Nature.* 405:647–655.

Toyoshima, C., and H. Nomura. 2002. Structural changes in the calcium pump accompanying calcium dissociation. *Nature.* 481:605–611.

Toyoshima, C., H. Sasabe, and D. L. Stokes. 1993. Three-dimensional cryo-electron microscopy of the calcium ion pump in the sarcoplasmic reticulum membrane. *Nature.* 362:469–471.

Vilsen, B., and J. P. Andersen. 1992. Mutational analysis of the role of Glu309 in the sarcoplasmic reticulum $Ca^{2+}$-ATPase of frog skeletal muscle. *FEBS Lett.* 306:247–250.

Weber, K., and M. Osborn. 1969. The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. *J. Biol. Chem.* 244:4406–4417.

Xu, C., W. J. Rice, W. He, and D. L. Stokes. 2002. A structural model for the catalytic cycle of $Ca^{2+}$-ATPase. *J. Mol. Biol.* 316:201–211.

Zhang, Z., D. Lewis, C. Strock, G. Inesi, M. Nakasako, H. Nomura, and C. Toyoshima. 2000. Detailed characterization of the cooperative mechanism of $Ca^{2+}$ binding and catalytic activation in the $Ca^{2+}$ transport (SERCA) ATPase. *Biochemistry.* 39:8758–8767.

Zhang, Z., D. Lewis, C. Sumbilla, G. Inesi, and C. Toyoshima. 2001. The role of the M6–M7 loop (L67) in stabilization of the phosphorylation and $Ca^{2+}$ binding domains of the sarcoplasmic reticulum $Ca^{2+}$-ATPase (SERCA). *J. Biol. Chem.* 276:15232–15239.

Zhang, Z., C. Sumbilla, D. Lewis, S. Summers, M. G. Klein, and G. Inesi. 1995. Mutational analysis of the peptide segment linking phosphorylation and $Ca^{2+}$-binding domains in the sarcoplasmic reticulum $Ca^{2+}$-ATPase. *J. Biol. Chem.* 270:1–8.