

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

Giuseppe Inesi, Zhongsen Zhang, and David Lewis

Department of Biochemistry and Molecular Biology, University of Maryland School of Medicine, Baltimore, Maryland 21201 USA

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.

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, 5 mM MgCl_2 , and variable EGTA, to yield 0.8 mg of protein/ml, and 8 μl of 2 mM thapsigargin (TG) in dimethyl sulfoxide (Me_2SO), or 8 μl 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 μM $^{45}\text{CaCl}_2$ was added. The total calcium concentration, after mixing, was 52.5 μM , including added and contaminant (12.5 μM) 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 μm ; 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.

Submitted May 8, 2002, and accepted for publication June 27, 2002.

Address reprint requests to Dr. Giuseppe Inesi, Department of Biochemistry and Molecular Biology, University of Maryland School of Medicine, 108 N. Greene Street, Baltimore, Maryland 21201. Tel.: 410-706-3220; Fax: 410-706-8297; E-mail: ginesi@umaryland.edu.

© 2002 by the Biophysical Society

0006-3495/02/11/2327/06 \$2.00

Enzyme phosphorylation with Pi

Enzyme phosphorylation with Pi was obtained by incubating 60 μg 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 μM $^{32}\text{P}_i$, 500 μM 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 μg 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 μg 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 phosphorimaging.

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 $^{32}\text{P}_i$, 0.5 mM EGTA, 20% (v/v) Me_2SO , and 2 μM A23187 (Ca^{2+} ionophore), to yield 50–100 μg 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 $^{32}\text{P}_i$ by addition of 1.0 ml of acetone, 0.5 ml of 5% ammonium molybdate in 2.5 N H_2SO_4 and 10 μl 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 μl 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 ($7\text{e}+5$ and $2\text{e}+6$ M $^{-1}$) for

two sites exhibiting positive cooperativity, whereas the mutant data can be fitted with an independent binding equation and a single constant ($2\text{e}+6$ 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

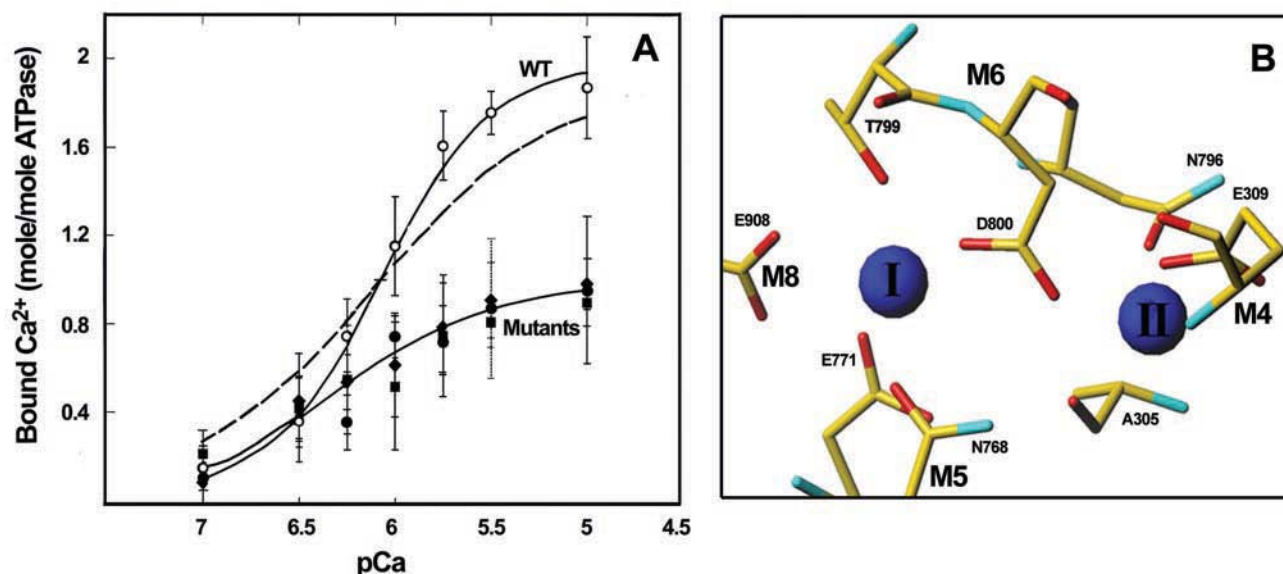


FIGURE 1 Ca^{2+} binding by WT, E309Q, T796A, and E309Q/T796A mutants (A) and molecular graphics representation of the two Ca^{2+} -binding sites in WT ATPase (B). (A) Recombinant ATPase protein was obtained from Cos1 cells infected with adenovirus vectors, and Ca^{2+} binding was measured in the absence of ATP as described by Zhang et al. (2000). \circ , WT; the mutants are E309Q (\blacksquare), N796A (\blacklozenge), and E309Q/N796A (\bullet). 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 ($K_1[\text{Ca}^{2+}] + 2K_1K_2[\text{Ca}^{2+}]^2/1 + K_1[\text{Ca}^{2+}] + K_1K_2[\text{Ca}^{2+}]^2$), whereas those for the E309Q, T796A, and E309Q/T796A mutants could be fitted simply with an independent binding equation ($K[\text{E}_{\text{tot}}][\text{Ca}^{2+}]/1 + K[\text{Ca}^{2+}]$). 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, how-

ever, 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.

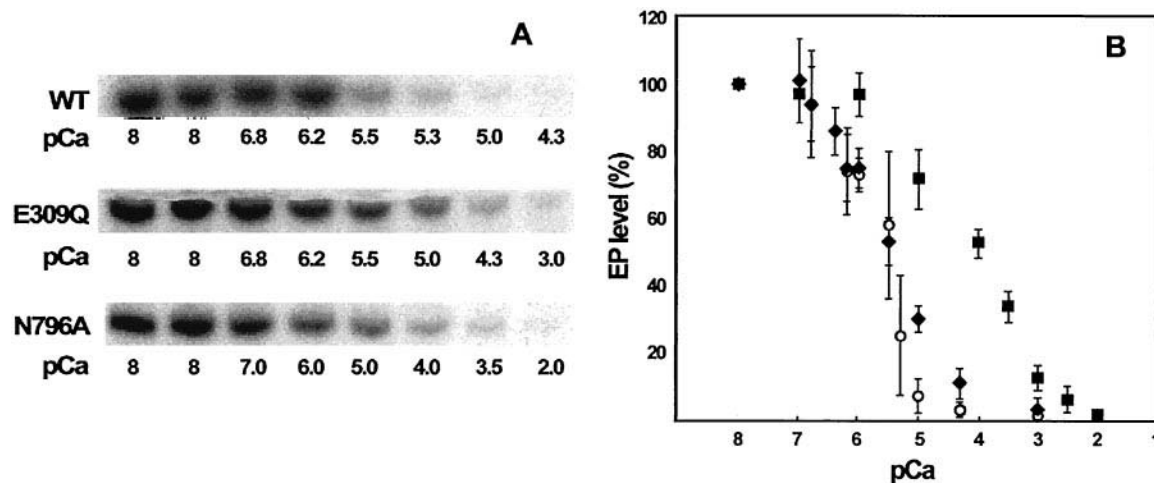


FIGURE 2 Ca^{2+} inhibition of enzyme phosphorylation by P_i . (A) Examples of phosphorylation detected by electrophoretic gels and phosphoimaging; (B) Average levels of phosphoenzyme obtained by equilibrating WT (○), E309Q (■), and T796A (◆) protein with P_i (see Materials and Methods) in the presence 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 hydrolytic 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,

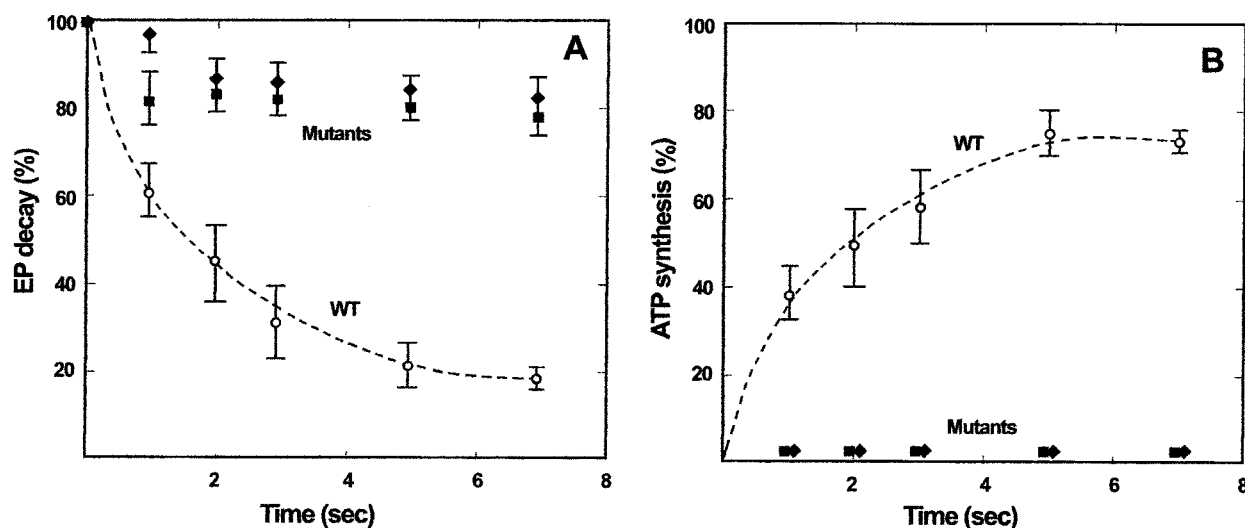
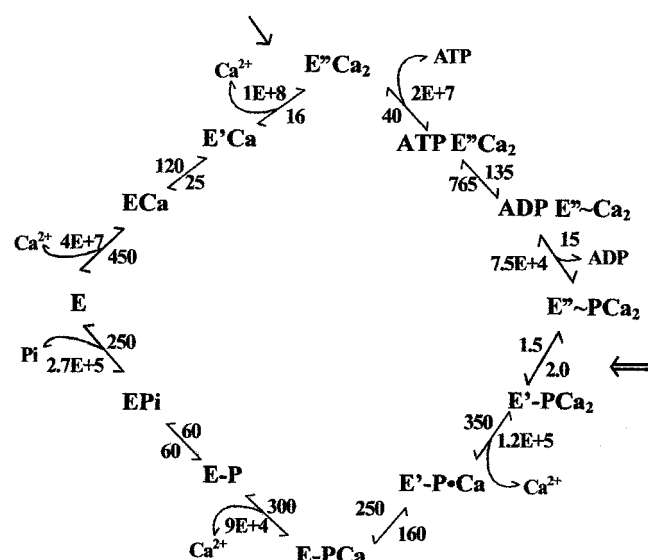
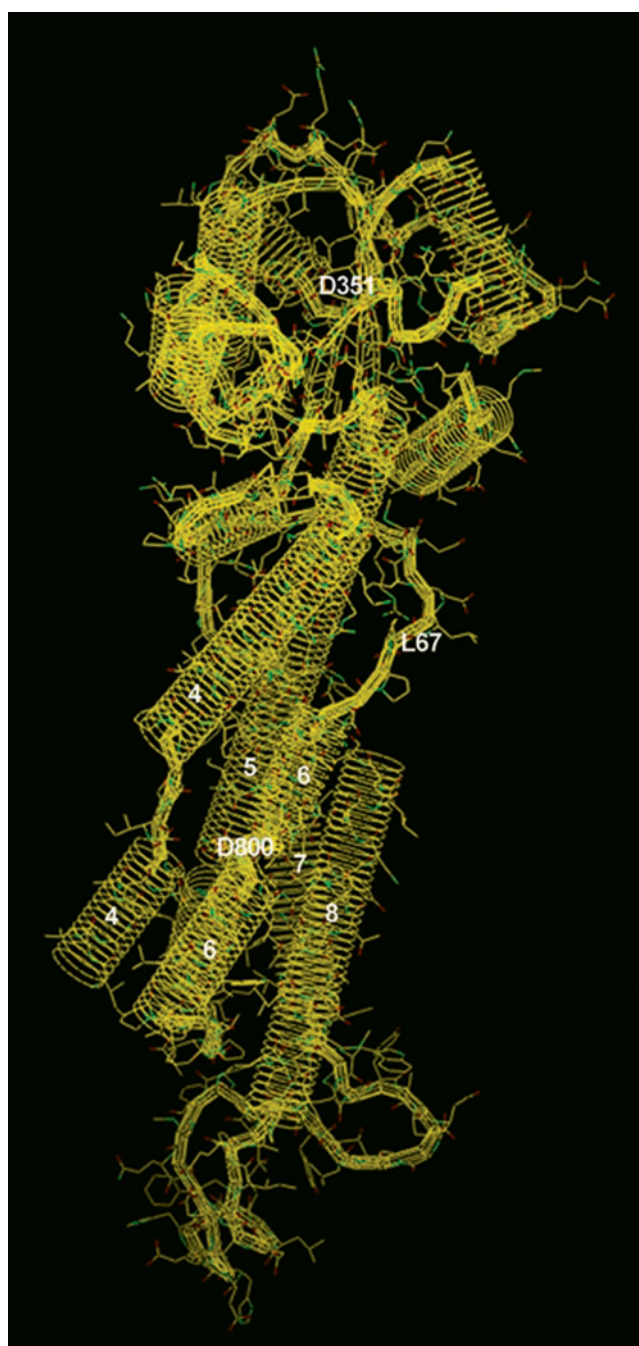


FIGURE 3 Phosphoenzyme decay (A) and ATP synthesis (B) after addition of Ca^{2+} and ADP to phosphoenzyme formed by reacting the ATPase with P_i in the absence of Ca^{2+} . Phosphoenzyme was obtained by reacting WT (○), E309Q (■), and T796A (◆) mutants with P_i in the absence of Ca^{2+} (Table 1). At 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.



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 $\text{E}'\text{Ca} + \text{Ca}^{2+} \rightarrow \text{E}''\text{Ca}_2$ reaction, allowing catalytic activation and ATP use to form the phosphorylated enzyme intermediate. A most interesting isomerization of the phosphoenzyme occurs then with the $\text{E}'\text{-PCa}_2 \leftrightarrow \text{E}''\text{-PCa}_2$ reaction, whereby phosphorylation and binding potentials are affected concomitantly, whereas free energy is conserved through conformational change ($K_{\text{eq}} \cong 1$). 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



Biophysical Journal 83(5) 2327–2332

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.

G. Inesi is grateful to Dr. L. de Meis and Dr. C. Toyoshima for numerous discussions and helpful suggestions. The editorial assistance of Mrs. Kimberly Curry is gratefully acknowledged.

This work was supported by the National Institutes of Health Program Project HL27867 and the Human Frontier Science Program.

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 und ihre Abhängigkeit 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 Å 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.