SUBSTRATE INDUCED CONFORMATIONAL FIT AND HEADPIECE CLOSURE IN THE Ca\textsuperscript{2+} ATPase (SERCA)

Hailun Ma and Giuseppe Inesi*

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

Chikashi Toyoshima

Institute of Molecular and Cellular Biosciences,
University of Tokyo, Bunkyo-ku, Tokyo 113, Japan

*To whom correspondence should be addressed. Tel.: 410-706-3220; Fax: 410-706 8297;
E-mail: ginesi@umaryland.edu.

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SUMMARY

Protection of the Ca\textsuperscript{2+} ATPase (SERCA) from proteinase K digestion has been observed following addition of Ca\textsuperscript{2+}, Mg\textsuperscript{2+} and nucleotide, and interpreted as a substrate dependent conformational change (1). The protected digestion site is located on the loop connecting the A-domain and the M3 transmembrane helix. We studied by mutational analysis the protective effect of AMP-PCP, an ATP analog that is not utilized for enzyme phosphorylation. We found that the nucleotide protective effect is interfered with by single mutations of Arg560 and Glu439 in the N domain, and Lys352, Lys 684, Thr353, Asp703 and Asp707 in the P domain. This is consistent with a transition from the open to the compact configuration of the ATPase headpiece, and approximation of the N and P domains by interactions with the nucleotide adenosine and phosphate moieties, respectively. The A domain - M3 loop is consequently involved. Protection by nucleotide substrate increased following mutations of Asp351 (the residue undergoing phosphorylation by ATP) and neighboring Asn706 to Ala, underlying the importance of side chain specificity in positioning the nucleotide terminal phosphate and limiting the stability of the substrate-enzyme complex. Protection is not observed when AMP-PCP is added in the absence of Ca\textsuperscript{2+} or following mutations (E771Q or N796A) that interfere with Ca\textsuperscript{2+} binding. Therefore, nucleotide binds to the Ca\textsuperscript{2+} activated enzyme in the open headpiece conformation, and the consequent approximation of the N and P domains occurs while the transmembrane domain is still in the Ca\textsuperscript{2+} bound conformation. Mg\textsuperscript{2+} is not required for the protective effect of nucleotide, even though specifically required for the subsequent catalytic reactions.
INTRODUCTION

The sarcoplasmic reticulum (SR\textsuperscript{1}) ATPase is a 110 kDa enzyme that utilizes one mole of ATP for active transport of two calcium ions in exchange for two moles of protons. The catalytic cycle begins with cooperative binding of two calcium ions from the cytosolic medium, followed by ATP utilization and formation of a phosphorylated enzyme intermediate. The bound calcium is then released onto the lumenal side of the SR, and the cycle is completed by hydrolytic cleavage of acylphosphate.

The ATPase molecular structure includes a transmembrane region made of ten clustered helical segments including the Ca\textsuperscript{2+} binding domain, and a cytosolic headpiece including the N (nucleotide binding), P (phosphorylation) and A (actuator) domains. Structural studies have shown that if crystallization is performed in the presence of Ca\textsuperscript{2+} the three headpiece domains are distinctly separate in an open configuration (2). On the other hand, in the absence of Ca\textsuperscript{2+}, those domains gather to form a compact headpiece (3, 4). This suggests that in solution the three domains undergo fluctuations, yielding different enzyme conformations depending on the presence of specific ligands. Taking advantage of a selective ATPase cleavage (5), it was then shown that addition of nucleotides in the presence of Mg\textsuperscript{2+} and Ca\textsuperscript{2+} protects the ATPase from digestion by proteinase K (1). Protection was attributed to a change in the positions of the headpiece domains with respect to the loop connecting the A-domain to the transmembrane helices. These movements are directly related to gate opening and closing in the Ca\textsuperscript{2+} pathway. This is a very important finding, suggesting that the open headpiece of the Ca\textsuperscript{2+} activated enzyme acquires a compact conformation upon substrate binding.

\textsuperscript{1} SR, sarcoplasmic reticulum; SERCA, sarco(endo)plasmic reticulum Ca\textsuperscript{2+} ATPase; WT, wild type; MOPS, 3-(N-morpholino) propanesulfonic acid; MES, 2-(N-morpholino) ethanesulfonic acid; PCA, perchloric acid; CDTA, trans-1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid; EGTA, Ethylene glycol-bis(amineoethyl ether)-N,N,N',N'-tetraacetic acid; AMP-PCP, beta,gamma-Methyleneadenosine-triphosphate
We describe here a series of experiments on the interference of various mutations with the protective effect of nucleotide. We demonstrate that occurrence of the nucleotide dependent conformational change requires participation of amino acid residues in both N and P domains, and that nucleotide dependent approximation of the headpiece domains occurs only when both Ca\(^{2+}\) sites are filled.
METHODS

SR vesicles were obtained with the microsomal fraction of rabbit leg muscle homogenate, as described by Eletr and Inesi (6). Recombinant ATPase was obtained by exogenous gene expression in COS-1 cells infected with adenovirus vectors carrying chicken WT or mutant SERCA1 cDNA (7). Adenovirus vector construction, site directed mutations, COS-1 cells culture methods, and preparation of microsomal fractions, were previously described in detail (8). Protein concentration was measured using bicinchoninic acid with the biuret reaction (Pierce). ATPase hydrolytic activity was determined following Pi production by a colorimetric method (9).

Experiments on limited digestion of ATPase with proteinase K, and protection by nucleotides or Ca^{2+}, were performed in media containing 50 mM MOPS, pH 7.0 and 50 mM NaCl, in the presence or in the absence (CDTA or EGTA present) of 5 mM MgCl{sub 2} and/or 0.1 mM CaCl{sub 2}. In experiments with native SR vesicles, the protein concentrations were 0.3 mg SR and 0.01 mg proteinase K per ml. In experiments with recombinant ATPase, the protein concentrations were 1.2 mg microsomal protein, and 0.04 mg proteinase K per ml. The concentration of recombinant ATPase was adjusted to the same level in all experiments, based on ATPase estimates by Western blotting and compensation with empty microsomes. The reaction was quenched at serial times by addition of trichloroacetic acid (TCA) to yield a final 2.5 % concentration. The quenched protein was then solubilized by adding sodium dodecylsulfate (1%), TRIS (0.312 M), pH 6.8, sucrose (3.75%), beta-mercaptoethanol (1.25 mM), and Bromophenol Blue (0.025%). The samples were then subjected to electrophoretic analysis (10) on 12.5 % gels, followed by staining with Coomassie Blue or Western blotting. For this purpose, the monoclonal antibody mAb CaF3-5C3 to the chicken SERCA-1 protein, and
The Ca\textsuperscript{2+} ATPase ATP Site

Goat anti-mouse IgG-horseradish peroxidase conjugated secondary antibodies were used, followed by densitometry of the bands visualized with an enhanced chemiluminescence-linked detection system (Amersham). Amino acid sequencing of peptide fragments eluted from electrophoretic gels was performed at Johns Hopkins University.
RESULTS

Nucleotide and divalent cation protection of ATPase from digestion by proteinase K

Electrophoretic analysis (Fig 1A) shows that limited digestion of ATPase with proteinase K yields a different pattern depending on the absence (EGTA present) or the presence of Ca$^{2+}$. In fact, while 95 and 83 kDa proteolytic products are obtained in the absence of Ca$^{2+}$, the 95 kDa band is not observed in the presence of Ca$^{2+}$. Amino acid sequencing demonstrates that these fragments are produced by cleavage at L119 and T242, respectively. Therefore, the pattern shown in Fig 1 indicates that Ca$^{2+}$ binding protects the L119 site, while leaving the T242 site accessible to proteinase K. On the other hand, in agreement with Danko et al. (1), we found that ATP or AMP-PCP protects the enzyme from proteinase K digestion at the T242 site (Fig 1). Protection by nucleotide occurs in the presence of Ca$^{2+}$, but not in the absence of Ca$^{2+}$, and the overall protection of the ATPase band by nucleotide and Ca$^{2+}$ is much more effective than that of Ca$^{2+}$ alone (Fig 1B). It is of interest that while ADP has still a protective effect, AMPCP (i.e., ADP analog) does not (not shown), suggesting a role of the oxygen atom between the alpha and beta phosphate for substrate stabilization at the catalytic site.

As a preliminary to mutational analysis, we confirmed that identical results are obtained with recombinant ATPase. Since the microsomal preparations derived from COS-1 cells contain several additional proteins, the digestion products of recombinant ATPase were evidenced by Western blotting, which is favored by their reactivity to the same antibody. In fact, since unrelated protein bands are not detected by Western blotting, the results of proteinase K digestion, and the protection by AMP-PCP can be demonstrated quite clearly (Fig 2).
Effects of site directed mutations

Considering that protection from proteinase K digestion is produced by functionally relevant ligands such as Ca^{2+} and nucleotide, we then studied the effects of site directed mutations that may interfere with binding and/or utilization of such ligands for catalytic reactions. A list of mutants produced for these experiments is shown in Table 1. It is also shown in Table 1 that most mutants were expressed at levels nearly as high as those obtained with WT protein. Few mutants, such as D703A and D707A, were expressed at lower levels, perhaps due to defective folding of the nascent peptide. Generally, digestion of mutants by proteinase K proceeded somewhat faster than digestion of WT ATPase, as shown by the half time of disappearance of the main ATPase band (Table 1). This was more pronounced as a consequence of D707A and the K684A mutations, and may be due to some folding destabilization by the mutations.

Mutations of residues involved in Ca^{2+} binding

The specific Ca^{2+} requirement for protection by AMP-PCP was most convincingly demonstrated by the use of the E771Q and N796A mutants. Consistent with inhibition of specific Ca^{2+} binding (11), we found that these mutations interfere also with AMP-PCP protection of ATPase digestion by proteinase K (Fig 3 and Table I). It is of interest that protection by AMP-PCP is totally abolished by a mutation that interferes with binding of only one Ca^{2+} (i.e., N796A), as well as by a mutation that interferes with binding of both Ca^{2+} (i.e., E771Q). Since it is known that enzyme activation requires binding of both Ca^{2+}, it is apparent that protection by nucleotide requires specific enzyme activation by Ca^{2+}.

Mutations of residues in the N domain
We recently found that mutation of R560 to A produces strong inhibition of ATP utilization, but much less inhibition of reverse enzyme phosphorylation with Pi (12). We find now that the R560A mutation interferes completely with AMP-PCP protection of the enzyme from digestion with proteinase K (Fig. 4 and Table I). These combined observations indicate that the R560 side chain is specifically involved in stabilization of nucleotide in the N domain.

Due to their proximity to the nucleotide binding site, we also characterized the effects of E439 and R489 mutations. We found partial catalytic inhibition as a consequence of their mutations, but interference with the AMP-PCP protective effect was observed only in the E439A mutant (Table I).

Considering that the bound nucleotide must reach the P domain to be utilized as a catalytic substrate, and that nucleotide protection from proteinase K may be related to N and P domain approximation, we extended our mutational analysis to the P domain.

Mutations of residues in the P domain

Ca\textsuperscript{2+} dependent utilization of ATP results in phosphorylation of D351 to form a phosphorylated enzyme intermediate. Therefore, mutation of this residue results in complete enzyme inactivation. On the other hand, AMP-PCP protection from proteinase K digestion is retained following mutation of D351 to N (Table I), and is actually much improved by mutation of D351 to A (Fig. 5) or N706 to A (Table I).

The behavior of the D351 mutant, in conjunction with the effect of the AMP-PCP pseudo substrate, demonstrates unambiguously that phosphoryl transfer is not required for the protective effect, but simple nucleotide binding is effective. In addition, the greater protection observed with the D351A and N706A mutants indicates that the native side chains of these amino acids play a role in positioning the nucleotide gamma-phosphate and limiting the stability of the
substrate-enzyme complex. In fact, a rather weak substrate-enzyme complex, relative to the subsequent transition state, is required for optimal enzyme kinetics.

It was previously reported that K684, a P domain residue, reacts with adenosine triphosphate pyridoxal in the presence of Ca\(^{2+}\), and this reaction is blocked by ATP (I3). Accordingly, we found that mutation of neighboring K684 to A produces catalytic inactivation as well as interference with the protective effect of AMP-PCP (Table I). It is then apparent that the K684 side chain plays an important and direct role in stabilization of the nucleotide substrate terminal phosphate.

We also found that mutations of K352, T353, D703 and D707 produce total or partial catalytic inactivation, and interfere with the protective effect of AMP-PCP (Table I). All of these residues reside in close proximity of the phosphorylation site (i.e., D351). Analogous mutational analysis of the Na\(^+\),K\(^+\) ATPase (I4) indicates that electrostatic interactions around the phosphorylation site may play an important role in substrate positioning and utilization. We considered that their mutation may alter direct interactions with the nucleotide terminal phosphate or ligation of Mg\(^{2+}\) in conjunction with oxygen atoms of the ATP terminal phosphate. On the other hand, the K352E mutation is not as effective as the K352A mutation. Furthermore, Mg\(^{2+}\) is not required for the nucleotide protective effect (see below).

### Divalent cation specificity and nucleotide concentration dependence

It is shown in Fig 6A that the protective effect of AMP-PCP is obtained to the same extent, even when Mg\(^{2+}\) is omitted (CDTA present), and Ca\(^{2+}\) is present at concentrations (20-100 micromolar) that are much lower than the effective nucleotide concentrations (1 mM).

We also found that, irrespective of the presence or the absence of Mg\(^{2+}\), the protective effect occurs at 0.1-1.0 mM nucleotide concentrations. This range is higher than the 10
micromolar Km observed with ATP as a substrate for ATPase activity. It is of interest that the concentration dependence of the protective effect of AMP-PCP shifts to a lower range when the D351A mutant is used (Fig. 6 B). A high affinity of the D351A mutant for the nucleotide substrate was previously noted by McIntosh et al. (15).
DISCUSSION

Definition of the SR ATPase crystal structure (2, 3) has been a major step in the understanding of this enzyme. With regard to nucleotide binding to the ATPase, studies of crystals soaked with TNP-AMP (2) revealed that the adenosine moiety is located within a flap of the N domain, near the F487, K492 and K515 and R560 residues whose derivatization or mutation interferes with nucleotide binding (12, 16-20). A recent model based on ATP-Fe$^{2+}$ (replacing Mg$^{2+}$) catalyzed oxidation, and on the crystal structure of the ATPase in the E2(TG) state, places the adenosine moiety in a pocket delimited by L492 and K515, with stabilization provided by R560 (21). The ATP-Mg complex is in a folded configuration, with Mg$^{2+}$ stabilized by alpha and beta phosphate oxygen atoms and by the Thr441 side chain (N domain), while the beta-phosphate approximates Thr353 (P domain), and the gamma-phosphate approximates Asn359 and Asp601 (hinge region). However, while this arrangement may result from early collision of the ATP-Mg complex with the ATPase open conformation, the geometry of ATP must then be altered to place its terminal phosphate near D351 (P domain), so that phosphoryl transfer to this residue can take place.

Useful information on this subject was provided by studies on nucleotide induced protection of the ATPase from digestion with proteinase K. The digestion site is at T242, located on the loop connecting the A-domain to the M3 helix. Thus, the susceptibility is expected to be affected by the position of the A-domain. In fact, this is an isolated loop in E1Ca$^{2+}$ (open configuration) but is attached to the P-domain in E2 (TG) (compact configuration), reflecting the different configuration of the cytoplasmic domains. Steric hindrance by the P-domain appears to be the origin of the protection of this site in the E2 (TG) state. However, different degrees of protection from proteolysis are observed in the E2 (TG), Ca E1ATP and E2P states (even though
the headpiece resides in a compact configuration in all these states), reflecting a graded response of the A domain to these ligand induced transitions (1).

Our mutational analysis indicates that R560 and E439 (N domain), as well as D351, K352, T353, K684, D703, N706 and D707 (P domain) are involved in the nucleotide effect. Participation of N and P residues demonstrates that approximation of the two domains does indeed occur, as required to accommodate the nucleotide by means of adenosine moiety interaction with the N domain, and phosphate interaction with the P domain. The A domain must also reposition as shown by the protection from proteinase K. The Ca$^{2+}$ requirement for nucleotide protection indicates that even though a compact arrangement of the headpiece is favored by nucleotide binding, the transmembrane domain retains bound Ca$^{2+}$. The compact headpiece conformation obtained under these conditions is not identical to that observed in the absence of Ca$^{2+}$ (1), but evidently represents an additional specific state produced by nucleotide binding on the Ca$^{2+}$ activated enzyme. An apparently similar approximation of nucleotide binding (“fingers”) and catalytic (“palm”) domains is known to occur in DNA polymerases (22).

It is of interest that the nucleotide concentration dependence of the protective effect is in the 0.1-1.0 mM range. This range is higher than the 10 µM Km observed with ATP as a substrate for ATPase activity, suggesting that the kinetics of catalytic ATP utilization have a significant influence on the overall ATP concentration dependence of enzyme activity. It should be pointed out that a rather weak substrate-enzyme complex, relative to the stability of the subsequent transition state, is required for effective enzyme kinetics.

An additional finding is the lack of Mg$^{2+}$ requirement for nucleotide protection of ATPase digestion by proteinase K (Fig 6A). It is of interest that in structural snapshots (23) of the phosphoserine phosphatase (PSPase) (its catalytic domain is analogous to the P-domain of
Ca\textsuperscript{2+} ATPase reaction cycle, the initial substrate-enzyme complex does not include Mg\textsuperscript{2+}, while interaction with Mg\textsuperscript{2+} occurs in concomitance with the phosphoryl transfer and hydrolytic reactions. With regard to the Ca\textsuperscript{2+} ATPase, it is likely that a complex with Mg\textsuperscript{2+} is formed initially \((21)\) when ATP is the substrate, due to the cation binding property of the nucleotide. On the other hand, our present experiments suggest that nucleotide binding (and its conformational effect) can be obtained either in the presence or in the absence of Mg\textsuperscript{2+}, although the subsequent phosphoryl transfer and hydrolytic reactions require Mg\textsuperscript{2+}, in analogy to PSPase. This suggestion is consistent with the random mechanism proposed by Reinstein and Jencks \((24)\) for ATP and Mg\textsuperscript{2+} binding to the Ca\textsuperscript{2+} ATPase. In analogy to the PSPase, it is likely that catalytically required Mg\textsuperscript{2+} binding occurs in concomitance with the phosphorylation transition state, including coordination by D703, T353 and gamma-phosphate.

Mutational analysis demonstrates direct roles of R560 (N domain) and K684 in nucleotide stabilization in the N domain and the P domain, respectively. R560 may interact with the nucleotide alpha phosphate. K684, on the other hand, is likely to interact with gamma phosphate, in analogy to stabilization of phosphorylserine by K144 in the PSPase \((23)\). The roles of other residues are more complex. For instance, the absence of nucleotide protection in the D703 mutant could be attributed to a role of this residue in Mg\textsuperscript{2+} stabilization. On the other hand, nucleotide protection is obtained even in the absence of Mg\textsuperscript{2+}. Therefore, D703 must have an additional and important role in structural stabilization by hydrogen bonding with neighboring residues. Similar considerations can be made regarding D707, K352 and T353, whose side chains are likely to establish hydrogen bonding with neighboring residues and/or water molecules \((23)\). Note that the K352A mutation is much more effective than the K352E mutation, indicating that the ability of K352 to establish stabilizing interactions in the open or compact
headpiece conformation are more important than a possible interaction with gamma-phosphate oxygen atoms.

Another interesting finding is related to the higher protective effect of nucleotide in the D351A and N706A mutants, as compared with WT D351 and D706 (as well as with the D351N mutant). D351 is in fact the residue undergoing phosphorylation, and N706 (N170 in the PSPase) is a close neighbor that contributes its side chain nitrogen (3, 23) for coordination of the same gamma-phosphate oxygen as K684 (K144 in the PSPase). It is then apparent that, in the WT enzyme, a number of steric and electrostatic constraints guide the gamma phosphate to an optimal position for covalent interaction. On the other hand, even tighter binding may be obtained by removing some of these constraints.

To understand our experimental results, an atomic model was built based on the atomic model of the PSPase with its substrate bound (23). This was possible because of the close similarity between the atomic structures of the catalytic domain of the PSPase and the P-domain of the MgFx complex of the Ca\textsuperscript{2+} ATPase (considered to be an E-P analog; Toyoshima unpublished observations). The model for the Ca\textsuperscript{2+} ATPase was built combining that for the N-domain taken from the E2 (TG) form (PDP ID: 1IWO; (3)), and that for the P-domain from the MgFx complex. ATP in an extended form was placed so that the gamma-phosphate comes exactly to the same position as that of the phosphate in phosphoserine (PDP ID: 1L7P).

The ATP orientation in the model (Fig 7) suggests that Mg\textsuperscript{2+}, coordinated by the beta and gamma phosphates, would not come to a position suitable for coordination by D703. In fact, in the D11N mutant of the PSPase (corresponding to the D351N mutant of the Ca\textsuperscript{2+} ATPase, and used for the substrate bound state) no Mg\textsuperscript{2+} is found, as opposed to the subsequent states of the catalytic cycle in which D167 (D703 in the Ca\textsuperscript{2+} ATPase) participates. This clearly indicates that
the initial step of substrate binding does not require Mg<sup>2+</sup>, consistent with the observation that
Mg<sup>2+</sup> is not required for the nucleotide induced protection from proteinase K digestion of the
Ca<sup>2+</sup> ATPase.

In the D11N mutant of the PSPase, the conformation of N11 is different from other
states. The conformation of N11 is one of the standard rotamers and places the side chain
nitrogen atom close to the phosphate. This conformation is stabilized by hydrogen bonding
between oxygen atom of N11 and main chain amide of D167 (D703 in Ca<sup>2+</sup> ATPase). Thus, this
is likely to be the conformation in the native enzyme when substrates bind initially. Here
phosphates are coordinated by atoms corresponding to K352 (main chain NH), T353 (main chain
NH), T625 (O<gamma>1), G626 (main chain NH), K684 (N<zeta>), N706 (N<delta>2) in the Ca<sup>2+</sup> ATPase. Thus
K684 is the only positively charged residue, likely to be a key attractor for the gamma-
phosphate. Hence the position of the side chain nitrogen atom must be very important. In fact, in
phosphoglucomutase (25, 26), the Ca position of the corresponding residue is different by one
residue, but the position of the nitrogen atom is exactly the same. This position appears to be
controlled by D351 and D707 in the Ca<sup>2+</sup> ATPase when substrates are absent. Thus, mutations of
these residues may strongly affect the affinity for ATP and, consequently, its protective effect.
This may explain the higher protection observed with the D351A, as compared with the D351N
mutant.

The effect of the N706A mutant is particularly interesting. N706 in the MgFx complex of
the Ca<sup>2+</sup> ATPase, as well as the corresponding N170 in the PSPase, provides a nitrogen atom for
coordination of the same phosphate oxygen as K684. Thus, the strong nucleotide protection
observed in the N706A mutant was contrary to our expectation. Although this Asn residue is not
well conserved in the haloacid dehologenase superfamily, the corresponding residue (N170) of
the PSPase is involved in a large conformation change and appears to regulate the flap (or lid) of the substrate binding site by changing the interaction with F49 in the subdomain. N706 may bear a similar role and might adjust the position of the N- or A-domain. At least in the MgF<sub>x</sub> complex, this residue does interact with the key loop in the A-domain (181TGES). Or, it may have an influence on the position of K684: if mutated to Ala, K684 might take a position that allows stronger interaction with ATP. This may be a likely reason for the strong nucleotide protection observed in the N706 mutant.

Finally, it should be pointed out that in the model (Fig 7) R560 takes a very strategic position. It stabilizes ATP by interacting with the alpha phosphate, and also stabilizes the closed configuration of the N- and P- domains by making a salt bridge with D627, one of the critical residues in the P domain. In this sense, R560 may have a similar role to R56 in the PSPase. Why D627 is important has never been explained. This conformation of D627 is realized by an interaction with K352, which appears to serve also in positioning T353, another critical residue. This may be the reason for the profound effect of the K352A mutation.

In conclusion, our experiments indicate that nucleotide binding occurs by collision with the N domain in the Ca<sup>2+</sup> dependent, open conformation of the enzyme headpiece. A substrate induced conformational fit then takes place, related to stabilization of the headpiece domains in a compact configuration. This allows approximation of the ATP gamma-phosphate to D351 in the P domain, while the membrane bound domain still binds 2 Ca<sup>2+</sup>. The roles of several amino acid residues are demonstrated, in some cases related to direct substrate binding, in other cases related to short and long range interactions of protein structure.
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LEGENDS TO FIGURES

Fig. 1 - Effects of Ca\(^{2+}\) and AMP-PCP on limited digestion of native SR ATPase by Proteinase K. The reaction medium contained 0.3 mg SR protein, 50 mM MOPS, pH 7.0, 50 mM NaCl, 5 mM MgCl\(_2\) and 2 mM EGTA. When indicated, 0.1 mg Proteinase K, 0.1 mM CaCl\(_2\), and/or 1 mM AMP-PCP were added. **A**: Electrophoretic analysis following incubation for 40 minutes at 22 °C. **B**: Time dependence of ATPase band reduction.

Fig. 2 - Comparison of Coomassie staining and Western blotting for electrophoretic analysis of COS-1 microsomes containing recombinant SERCA1, before and after limited digestion with Proteinase K. The reaction medium contained 1.2 mg microsomal protein and 0.04 mg Proteinase K per ml, 50 mM MOPS, pH 7.0, 50 mM NaCl, 5 mM MgCl\(_2\) and 0.1 mM CaCl\(_2\). Incubation for 40 minutes at 22 °C.

Fig 3 - Lack of protection by AMP-PCP in the E771Q mutant. The reaction medium contained 1.2 mg microsomal protein and 0.04 mg Proteinase K per ml, 50 mM MOPS, pH 7.0, 50 mM NaCl, 5 mM MgCl\(_2\) and 0.2 mM CaCl\(_2\). Incubation times as indicated at 22°C. ATPase and fragments visualized by Western blotting.

Fig 4 - Lack of protection by AMP-PCP in the R560 mutant. Reaction conditions as in Fig 3, except for the different mutant.

Fig. 5 - Higher protection by AMP-PCP in the D351A mutant. Reaction conditions as in Fig 3, except for the different mutant.

Fig. 6 - AMP-PCP concentration dependence of the protective effect, in the presence and in the absence of Mg\(^{2+}\): The reaction mixture contained 1.2 mg microsomal protein/ml, 50 mM MOPS, pH 7.0 and 50 mM NaCl. **A**: Lack of Mg\(^{2+}\) requirement: Proteinase K (0.04 mg/ml), CDTA (1 mM), MgCl\(_2\) (1mM), and CaCl\(_2\) (1.1 mM or 0.1 mM) were added as indicated. **B**:...
Comparison of WT and D351A mutant: Proteinase K (0.04 mg/ml) and 0.1 mM CaCl2 were added to all samples; MgCl2 (1 mM) and AMP-PCP were added as indicated. Incubation for 40 minutes at 22°C. The experimental points were fitted with a single site binding equation.

**Fig. 7 – Structural model of the ATP binding site, spanning the N and P domains.** The model was built by combining the model for the N domain taken from the E2(TG) form (PDB ID, 1IWO; (3)) and that for the P domain taken from the MgFx complex (Toyoshima, unpublished observations) of the Ca$^{2+}$ ATPase. The ATP in extended form was placed so that the gamma-phosphate comes exactly to the same position as in the highly homologous atomic model of the PSPase in the substrate bound form (PDB ID,1L7P; (23)). A: a view along the plane of ribose of ATP. B: a view corresponding to that presented previously (21), approximately orthogonal to the view in A. Side chains of important residues are shown. Their positions match closely those of the corresponding residues in PSPase with the substrate, phosphoserine, bound (23). Green sphere represents Mg$^{2+}$ bound to ATP; purple sphere, Mg$^{2+}$ found in the MgFx complex. The arrow in broken line in A shows the direction of approximation of the N-domain to the P-domain.
| Mutants | Domain | Western Blot (ratio) | ATPase Activity (correction by WB) | T1/2 without AMP-PCP (min) | T1/2 with AMP-PCP (min) | AMP-PCP Effect (% wt) |
|---------|--------|---------------------|-----------------------------------|---------------------------|------------------------|------------------------|
| WT      | -      | 1.00                | 1.00                              | 38.43                     | 82.15                  | 100                    |
| N796A   | Ca$^{2+}$ | 1.00                | 0.06                              | 8.78                      | 9.93                   | 11                     |
| E771Q   | Ca$^{2+}$ | 1.06                | 0.08                              | 17.80                     | 18.59                  | 0                      |
| R560A   | N      | 0.87                | 0.24                              | 21.38                     | 21.91                  | 0                      |
| E439A   | N      | 0.47                | 0.57                              | 20.48                     | 20.10                  | 0                      |
| E439Q   | N      | 0.82                | 0.55                              | 20.35                     | 30.65                  | 45                     |
| R489A   | N      | 0.44                | 0.34                              | 19.03                     | 84.32                  | 300                    |
| R489E   | N      | 0.93                | 0.41                              | 20.48                     | 46.33                  | 120                    |
| D351N   | P      | 0.48                | 0.08                              | 15.12                     | 34.93                  | 115                    |
| D351A   | P      | 0.41                | 0.19                              | 18.77                     | $\infty$               | $\infty$               |
| K352A   | P      | 0.64                | 0.15                              | 14.86                     | 15.89                  | 0                      |
| K352E   | P      | 0.55                | 0.12                              | 8.74                      | 13.64                  | 49                     |
| T353A   | P      | 0.51                | 0.60                              | 22.72                     | 30.26                  | 29                     |
| D703A   | P      | 0.24                | 0.09                              | 22.65                     | 24.44                  | 0.1                    |
| N706A   | P      | 0.63                | 0.13                              | 32.59                     | $\infty$               | $\infty$               |
| D707A   | P      | 0.19                | 0.12                              | 1.28                      | 1.75                   | 32                     |
| K684A   | P      | 1.09                | 0.06                              | 2.24                      | 2.38                   | 0.1                    |

Table 1. Half time for disappearance of ATPase band with and without AMP-PCP
Figure 3

| 0 | 5 | 10 | 20 | 40 | 60 | 80 | 120 min |
|---|---|----|----|----|----|----|---------|
| s AMP-PCP |
| w AMP-PCP |
Substrate induced conformational fit and headpiece closure in the Ca$^{2+}$ ATPase (SERCA)

Hailun Ma, Giuseppe Inesi and Chikashi Toyoshima

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