Effect of Orthophosphate, Nucleotide Analogues, ADP, and Phosphorylation on the Cytoplasmic Domains of Ca\(^{2+}\)-ATPase from Scallop Sarcoplasmic Reticulum*

The effects of orthophosphate, nucleotide analogues, ADP, and covalent phosphorylation on the tryptic fragmentation patterns of the E\(_1\) and E\(_2\) forms of scallop Ca-ATPase were examined. Sites preferentially cleaved by trypsin in the E\(_1\) form of the Ca-ATPase were detected in the nucleotide (N) and phosphorylation (P) domains, as well as the actuator (A) domain. These sites were occluded in the E\(_3\) (Ca\(^{2+}\)-free) form of the enzyme, consistent with mutual protection of the A, N, and P domains through their association into a clustered structure. Similar protection of cytoplasmic Ca\(^{2+}\)-dependent tryptic cleavage sites was observed when the catalytic binding site for substrate on the E\(_1\) form of scallop Ca-ATPase was occupied by P\(_i\), AMP-PNP, AMP-PCP, or ADP despite the presence of saturating levels of Ca\(^{2+}\). These results suggest that occupation of the catalytic site on E\(_1\) can induce condensation of the cytoplasmic domains to yield a unique structural intermediate that may be related to the form of the enzyme in which the active site is prepared for phosphoryl transfer. The effect of P\(_i\) on the E\(_2\) form of the scallop Ca-ATPase was also investigated, when it was found that formation of E\(_2\)-P led to extreme resistance toward secondary cleavage by trypsin and stabilization of enzymatic activity for long periods of time.

The sarco(endo)plasmic reticulum ATPases (SERCAs)\(^1\) transport Ca\(^{2+}\) against an electrochemical gradient from the cytoplasm into the intracellular membranous compartment of the sarcoplasmic or endoplasmic reticulum and play a major role in Ca\(^{2+}\) homeostasis in both muscle and non-muscle cells (1, 2). In the course of transporting Ca\(^{2+}\), these enzymes pass through a number of relatively well defined intermediate biochemical states that can be isolated and studied. These include forms in which the β-carboxyl group of a specific aspartyl residue (Asp\(^{351}\) in the case of rabbit SERCA1a) is present as an acylyphosphate mixed anhydride. Much of the experimental data has been interpreted in terms of the enzyme being able to exist in two basic forms: the E\(_1\) state, in which the enzyme possesses high affinity binding sites for Ca\(^{2+}\) and can be phosphorylated by ATP but not by P\(_i\), and the E\(_2\) state, which possesses low affinity Ca\(^{2+}\) sites and can be phosphorylated by P\(_i\) but not by ATP (3).

Comparison of the tryptic digestion patterns of rabbit SERCA1a in its Ca\(^{2+}\)-bound and -free states has provided some of the strongest direct evidence supporting such a model where the enzyme can exist in two fundamentally different conformational states (4–6). Recent studies have found that the E\(_1\) (Ca\(^{2+}\)\(_2\)) form of rabbit SERCA1a shows clear structural differences to the enzyme in its vanadate-stabilized Ca\(^{2+}\)-free (E\(_2\)) state (7–10). In the E\(_1\) form, the Actuator (A) domain is isolated from the nucleotide (N) and phosphorylation (P) domains, whereas in the E\(_2\) form all three domains are clustered together.

The Ca-ATPase from the cross-striated part of the adductor muscle of the sea scallop has been the subject of a number of biochemical and structural studies (11, 12). Provided the E\(_2\) form of the scallop Ca-ATPase is stabilized against loss of activity, it adopts a dimeric type of quaternary organization (13) that is absent in the E\(_1\) form of the enzyme (14), and in both the E\(_1\) and E\(_2\) P forms the enzyme is arranged in parallel instead of antiparallel helical strands in the tubular vesicles with only a single asymmetric subunit in each unit cell (p1 lattice) (15).

In the work reported here, tryptic digests of scallop SERCA detected differences in conformation between the E\(_1\) and E\(_2\) forms of the enzyme associated with the N and P subdomains as well as with the A domain. It was found that some features of the proteolytic stability normally associated with the E\(_2\) state were also observed when ligands ranging from AMP-PNP to simple orthophosphate were bound to the catalytic site of the unphosphorylated E\(_1\) form of the enzyme, despite saturation of the Ca\(^{2+}\)-binding sites. Thus, occupation of the catalytic site on E\(_1\) could induce structural changes that resembled in some respects those produced by emptying of the high affinity Ca\(^{2+}\) sites; however, this modified structural form of the E\(_1\) state of the scallop Ca-ATPase was unique and differed from E\(_2\). The effect of covalent phosphorylation of the scallop Ca-ATPase was also investigated, when the E\(_2\)-P form was found to be exceptionally stable, both in terms of enzymatic activity and resistance to proteolysis. In contrast, the E\(_1\)–P form was highly susceptible to trypsin with a digestion pattern resembling that of the E\(_1\) (Ca\(^{2+}\)\(_2\)).
EXPERIMENTAL PROCEDURES

Deep-sea scallops (Placopecten magellanicus) were obtained from the Marine Biology Laboratory, Woods Hole, MA.

Preparation of Scallop FSR—SR vesicles were made from the cross-stratified part of the adductor muscle as described previously (16, 17). One minor modification to the procedure was the removal of glycogen granules from the final preparation. Glycogen and its associated proteins are very common contaminants of SR vesicle preparations from rat skeletal muscle (18), and the same is true of scallop muscle membranes. In the case of the scallop SR, it was found that a simple and effective method to eliminate much of the glycogen was to layer the purified membrane fraction suspended in 0.32 M sucrose, 0.1 mM KCl, 1 mM CaCl2, 20 mM MOPSNa, pH 7.0, onto 1.5 M sucrose, 0.1 mM CaCl2, 1 mM EGTA, 20 mM MOPSNa, pH 7.0, and centrifuge the preparation for 3 h at 26,000 × g. The glycogen particles collected as a clear button-like pellet at the bottom of the centrifuge tube, whereas the membranes, now largely free of glycogen, banded at the 0.32–1.5 M sucrose interface. Preparation of DOC-extracted scallop FSR was done as described previously (16).

Tryptic Digests—DOC-extracted scallop FSR was suspended at 1 mg ml−1 in 20% v/v ethylene glycol (Fisher), 0.15 mM KCl, 50 mM MOPSNa, pH 7.0. The E1 pattern of tryptic fragments was obtained when the free Ca2+ concentration was above ~10 μM, but typically a total of 1 or 3 mM CaCl2 was present for digests in the E1 state. For digests of the E2 form, 10 mM EGTA-Na replaced CaCl2. Digestion was at room temperature with TPK-treated trypsin (12,000 units/mg, dissolved in 1 mM HCl; Sigma) added in a 1:30 w/w ratio to SR protein (giving 400 units of activity/mg SR protein). Digestions were usually terminated by addition of AEBSF (Calbiochem) to a final concentration of 20 mM, followed by transfer of the sample to ice. The simultaneous addition of HCl to 1 mM with the 20 mM AEBSF proved a very effective way of stopping the digestion. The samples were centrifuged at 16,000 × g for 1/2 h at 4 °C and the supernatant removed. The pellets containing the membrane-bound products were washed to remove trapped protease by resuspension in 0.32 M sucrose, 1 mM AEBSF, 1 mM CaCl2 (E1 digests) or 1 mM EGTA (E2 digests), 25 mM MOPSNa, pH 7.0, followed by recentrifugation. After repeating the washing step, the trypsin-free samples were finally resuspended in 0.32 M sucrose, 1 mM AEBSF, 1 mM CaCl2 (E1 digests), or 1 mM EGTA (E2 digests), 25 mM MOPSNa, pH 7.0, before addition of an equal volume of 2× Laemmli or Tricine sample buffer.

Electrophoresis—The discontinuous Tris-glycine and Tris-Tricine systems (19, 20) were used for running SDS-polyacrylamide gels. Sodium thiglysolate (0.1 mM) was present in the sample denaturation medium and cathode buffer.

Electroblotting and N-terminal Sequencing—SDS gels were blotted onto Immobilon-PSQ polyvinylidene difluoride in a medium of 10% v/v MeOH, 10 mM CAPSNa, pH 11, at 4 °C. Blots were stained with 0.02% w/v Coomassie Brilliant Blue R250 in 1 mM HCl, 50% v/v MeOH. Bands of interest were sent to the University of Florida for N-terminal sequencing.

Phosphorylation with Pi—For digestions in the E2, P state, DOC-extracted scallop FSR was phosphorylated with Pi essentially as described previously (15, 21) at room temperature for 15 min before addition of trypsin.

Detection of E2, P Using TNP-ADP Superfluorescence—This was carried out using an SLM 8000c spectrofluorimeter thermostated at 25 °C. TNP-ADP (Molecular Probes) was added to 35 μg/ml-1 DOC-extracted scallop FSR suspended in 20% v/v glycerol, 15 mM EGTA-Tris, 15 mM MgCl2, 50 mM Mes-Tris, pH 6.0, to a final concentration of 2 μM TNP-ADP. Phosphorylation was typically induced by addition of Pi in the form of H2PO4-Tris to 8.5 mM. Steady-state fluorescence measurements using 4-nm slits were carried out with excitation at 463 nm and emission followed at 530 nm.

Enzyme Assays—The dependence of the Ca2+-activated ATPase activity of the DOC-extracted scallop FSR on ATP-Mg2+ concentration was determined using a coupled enzyme assay as previously described (16), except that 5 mM Mg2+ was present (as MgCl2) in excess of the ATP-Mg2+ concentration, according to Neet and Green (22). Assays were carried out in a Varian-Elmer Lambda 40 spectrophotometer in a volume of 1 ml. The cell was thermostatted at 25 °C.

Protein Concentration—The bicinchoninic acid method (23) was used.

RESULTS

General Observations—As previously described, on SDS gels the undigested DOC-extracted scallop FSR fraction showed essentially a single band of ~110 kDa known to contain the polypeptides of the Ca-ATPase (24) and a Na-Ca exchanger (25), the latter probably of sarcolemmal origin. Based on phosphorylation levels of the scallop SR obtained with both ATP and Pi (15, 21) and on its specific Ca2+-activated ATPase activity (13, 16), the Ca-ATPase must represent at least 90% of the 110-kDa material. None of the membrane-bound proteolytic fragments described in this report was derived from the Na-Ca exchanger, although soluble tryptic peptides originating in the exchanger have been identified (25). Traces of 2–3 peptides of 28–32 kDa that arose from contamination of the SR by other elements of the sarcolemma (possibly gap junctions) were sometimes present (15, 25, 26). Gels of trypic digestes where the scallop Ca-ATPase was in the E2 state (+ EGTA) showed two strong bands of apparent molecular mass ~56 and ~47 kDa, together with much weaker bands corresponding to peptides of ~88, ~37, and 22–25 kDa (Fig. 1A). Digestions in the E1 state (10 μM free Ca2+ or above) gave a pattern in which both the 56- and 47-kDa bands were less intense than in E2 digests (Fig. 1B), whereas the 88-, 37-, and 22–25-kDa bands were significantly stronger.

Attempts to sequence the 110- and 56-kDa tryptic fragments by the Edman method suggested that they both had blocked N termini. Autoradiography of SDS gels of membranes that had been first proteolyzed in the E2 state and then phosphorylated...
with $[\gamma^{32}\text{P}]\text{ATP}$ showed labeling of the 56-kDa band with $^{32}\text{P}$ as well as the intact ATPase (not shown). Rabbit SERCA1a has a blocked (acetylated) N terminus, as of course does its A tryptic fragment, and the tryptic A fragment representing the N-terminal half of SERCA1a contains Asp$^{351}$. Thus, the 56-kDa peptide formed in scallop tryptic digests contained Asp$^{359}$ and corresponded as expected to the scallop A tryptic peptide.\(^2\) The polypeptide with the apparent size of 47 kDa formed in the same (E\(_2\)) digests was found by Edman N-terminal sequencing of its polyvinylidene difluoride blot to be produced by cleavage at Lys$^{304}$/Val$^{505}$ (scallop sequence, Ref. 27) in the N domain and thus represented the scallop B tryptic fragment.

N-terminal sequencing showed that the 88-kDa fragment, which was formed in larger amounts in the E\(_1\) state, arose by cleavage at Arg$^{197}$-Ala$^{198}$ (scallop sequence, Ref. 27) in the A domain. As with rabbit SERCA1a, this will be designated the T\(_2\) cleavage site. Therefore, for a subpopulation of the scallop Ca-ATPase molecules in the E\(_1\) state, the primary tryptic cleavage site was T\(_2\) rather than T\(_1\). The 37-kDa fragment was relatively stable under E\(_1\) conditions and was often observed to be a very prominent band on SDS gels of tryptic digests made with the E\(_1\) form of the scallop Ca-ATPase (Fig. 1A). N-terminal sequencing showed that this peptide arose by cleavage at the Lys$^{565}$-Phe$^{563}$ peptide bond (scallop sequence, Ref. 27) in the N domain, which will be designated as the T\(_3\) site in the scallop Ca-ATPase.

Effect of AMP-PNP, AMP-PCP, and ADP on the E\(_1\) Form of Scallop SR Ca-ATPase—When AMP-PNP was present at concentrations at or above $\sim 0.1 \text{ mM}$ in tryptic digests of the scallop Ca-ATPase in its E\(_1\) state (with free Ca$^{2+}$ $> 10 \mu\text{M}$), substantial stabilization of both the A and B fragments was observed (Fig. 2, A and B), and there was a significant reduction in the amount of 37-kDa fragment that accumulated. Although AMP-PNP greatly stabilized the A fragment (which contains the T\(_2\) site) and inhibited formation of the 37-kDa peptide by cleavage at T\(_3\) in the B fragment, it had little effect on the accessibility of the T\(_4\) site to trypsin. AMP-PNP did not qualitatively modify the pattern of tryptic peptides formed from the E\(_1\) form of the Ca-ATPase, but both the A and B bands became somewhat stronger as the AMP-PNP concentration was raised to 5 mM (Fig. 2B). This may suggest some additional stabilization through the nucleotide analogue binding to a low affinity site. Addition of AMP-PCP or ADP to tryptic digests of scallop Ca-ATPase gave very similar effects to AMP-PNP over the same (0.1–1 mM) concentration range. In summary, the overall effect of the nucleotide ligands was to make the pattern of proteolytic products formed from the E\(_1\) scallop Ca-ATPase closer to that normally seen in the E\(_2\) state, with strong A and B bands on SDS gels and a weak 37-kDa band.

Effect of Orthophosphate on the Tryptic Cleavage of E\(_1\)—The effect of a range of concentrations of orthophosphate on the tryptic cleavage pattern produced in the presence of Ca$^{2+}$ (enzyme in the E\(_1\) state) is shown in Fig. 3. As the concentration of P\(_i\) was increased to 20 mM, there were significant reductions in the amounts of the 37- and 88-kDa fragments formed, i.e. the cleavages at the T\(_3\) site in the N domain and the T\(_2\) site in the A domain were inhibited. P\(_i\) strongly stabilized the A fragment (Met$^1$-Lys$^{207}$), but as with the nucleotide ligands, there was no significant protective effect on the T\(_4\) site. Thus, the P\(_i\)-bound E\(_1\) form resembled the E\(_2\) form in terms of the stability of the A and B fragments to further proteolysis, despite the presence of saturating concentrations of Ca$^{2+}$. The presence of tri-
polyphosphate (5 mM) in $E_2$ digests produced similar effects to those seen with $E_1$.

**Effect of Orthophosphate on the Tryptic Cleavage of $E_2$**—

Thus, $P_i$ profoundly affected the tryptic cleavage of the $E_1$ form of the scallop Ca-ATPase. The effect of $P_i$ on the tryptic cleavage of $E_2$ was then examined. As described above, cleavage at the $T_2$, $T_3$, and $T_4$ sites was inhibited when the scallop Ca-ATPase was in its $E_2$ form under the usual conditions in the presence of 0.13 or 0.15 M K$^+$. The presence of 20 mM $P_i$ did not in any way modify the products of the $E_2$ digest made under these standard conditions, as expected. When the $E_2$ form of the Ca-ATPase was digested under identical conditions but with no K$^+$ present, it was rapidly degraded into small fragments (not shown), as anticipated from previous studies that showed that in the absence of K$^+$, the enzyme adopts a loose and open conformation (21). Orthophosphate (20 mM) had no effect on the very extensive proteolysis of $E_2$ in the standard digestion medium lacking K$^+$ at pH 7.

The $E_2$-$P$ Form of Scallop Muscle SERCA Is Very Stable, with a Tightly Folded Conformation—The studies described above on the effect of $P_i$ on tryptic cleavage of $E_1$ had been carried out under conditions that did not promote formation of $E_2$-$P$. The effect of covalent phosphorylation of $E_2$ was then investigated. Previous studies had shown that membranous scallop Ca-ATPase could be phosphorylated with $P_i$ to yield a form of the enzyme corresponding to the well-studied $E_2$-$P$ form of rabbit SERCA1a (15). Enhancement of steady-state fluorescence (super fluorescence) of TNP-ADP at 532 nm associated with formation of $E_2$-$P$ (28, 29) was used to characterize the affinity of the binding site for $P_i$ on scallop Ca-ATPase involved in formation of $E_2$-$P$ (see “Experimental Procedures”). The intensity increase was half maximal at 3.4 mM added $P_i$, consistent with the expected affinity of the enzyme for $P_i$ (30).

When the $E_2$-$P$ form of the scallop Ca-ATPase was maintained in 20% v/v Me$_2$SO and 15 mM Mg$^{2+}$ in the absence of $P_i$, some limited stabilization of the A and B tryptic fragments was observed (Fig. 4, lane 2). However, when 20 mM $P_i$ was present together with 20% v/v Me$_2$SO and 15 mM Mg$^{2+}$ in the K$^+$-free medium at room temperature to induce formation of the $E_2$-$P$ state, both the A and B fragments became extremely resistant to secondary cleavage by trypsin (Fig. 4, compare lanes 2 and 3). Even after exposure to trypsin for 1/2 h at room temperature, the A and B peptides formed from the $E_2$-$P$ enzyme remained essentially intact. Cleavage was thus effectively restricted to the $T_1$ site with very little secondary proteolysis, whereas after 1/2 h a significant amount of small proteolytic debris had accumulated in the comparable digest of the $E_2$ form. Therefore, although the $T_1$ site remained accessible to trypsin after phosphorylation of the $E_2$ to the $E_2$-$P$ form of scallop SERCA, the rest of the cytoplasmic part of the molecules adopted a conformation that was exceptionally resistant to further attack by trypsin. Because K$^+$ activates hydrolysis of $E_2$-$P$ (31), it was not possible to directly compare the stabilities of $E_2$ and $E_2$-$P$ in the presence of K$^+$. It was noted that the SDS complex of the residual undigested (intact) $E_2$-$P$ form of the Ca-ATPase polypeptide migrated more slowly than the SDS complex of the undigested $E_2$ form (Fig. 4), suggesting that the $E_2$-$P$-SDS complex was more extended than the $E_2$-SDS complex. It is known from studies of rabbit SERCA1a that there is binding site for ADP on $E_2$-$P$ (29). When 4 mM ADP was included in tryptic digests of the $E_2$-$P$ form of scallop Ca-ATPase (Fig. 4, lane 4), the A fragment became more susceptible to tryptic cleavage and traces of the 22–24 kDa doublet appeared in the digest, indicating that the $T_2$ site had become more exposed. Thus, binding of ADP perturbed the structure of $E_2$-$P$.

In the course of these studies, it was found that the scallop Ca-ATPase was maintained in the K$^+$-free $E_2$-$P$ phosphorylation medium, it could be kept at room temperature for extended periods of time (>10 days) without loss of activity. Again, as judged by TNP-ADP superfluorescence, both membranous and C$_{12}$E$_8$-solubilized scallop Ca-ATPase phosphorylated with $P_i$ were very stable with little decay of the signal, provided the samples were kept in the dark between measurements to prevent photobleaching. The stability of scallop FSR...
in the $E_2$-P state, formed in the absence of Ca$^{2+}$, K$^+$, and Na$^+$, was in complete contrast to the extremely rapid loss of activity that occurs with the unphosphorylated $E_2$ form of the scallop enzyme under comparable conditions (11, 13). Because inactivation of the unphosphorylated Ca$^{2+}$-free ($E_2$) scallop Ca-ATPase involves an irreversible loss of the Ca$^{2+}$-binding sites, phosphorylation of the enzyme in the absence of Ca$^{2+}$ with P$_i$ may thus stabilize otherwise labile empty Ca$^{2+}$-binding sites.

In the context of the above results, it was of interest to compare the effect of tryptic digestion of the Ca-ATPase phosphorylated from ATP ($E_2$) to that described above where the enzyme had been phosphorylated from P$_i$ ($E_2$-EGTA). Because the enzyme was found to be inactivated by secondary tryptic cleavages within the A and B fragments, phosphorylation was carried out before proteolysis in the absence of Mg$^{2+}$ in the presence of K$^+$, i.e., under conditions where most (>90%) of the phosphorylated enzyme was in the ADP-sensitive form ($E_2$-P) as previously described (15). The pattern of tryptic fragments produced from the $E_2$-P preparation of scallop Ca-ATPase as visualized with Coomassie Blue was indistinguishable from that of the unphosphorylated $E_2$ form of the Ca-ATPase, with significant breakdown of the A and B fragments.

**Dependence of Enzyme Activity on ATPMg$^2+$ Concentration**—Information about the number and type of nucleotide-binding sites on the scallop SERCA was important for interpretation of some of the above results. Many studies of rabbit SERCA1a have suggested the coexistence of catalytic- and regulatory nucleotide-binding sites on that enzyme (33, 34), whereas there is evidence for only one type of site on the SERCA of the cold-resistant wood frog (35). Thus, the dependence of the Ca$^{2+}$-activated ATPase activity of deoxycholate-extracted scallop muscle SERCA vesicles on ATP concentration was examined according to Eadie-Hofstee (36), where the activity ($v_o$) is plotted against the ratio of activity to ATPMg concentration as shown in Equation 1.

$$v_o = - K_i (v_o/\text{ATPMg}) + V_{\text{max}}$$

(Eq. 1)

The graph displayed two limbs, one with a slope corresponding to an apparent Michaelis constant $K_i$ of 0.29 mM, which extrapolated to give a $K_i$ of 368.5 min$^{-1}$, and the other to a $K_m$ of 4.6 $\mu$M and a $K_{cat}$ of 92.4 min$^{-1}$, (assuming a molecular mass of 110 kDa and that the Ca-ATPase constituted 95% of the total protein in the deoxycholate-extracted vesicles used in the experiments.) The central portion of a Hill plot of the data had a slope of $n_H = 0.6$, while the double reciprocal (Lineweaver-Burke) plot was convex upwards (not shown). These results strongly resembled those obtained with rabbit SERCA1a (37, 38), which have been interpreted in terms of a high affinity catalytic nucleotide-binding site and a low affinity regulatory site.

**DISCUSSION**

The affinities of the catalytic- and regulatory nucleotide-binding sites on SERCA1a for ATP (2–5 $\mu$M and 0.3–1 mM, respectively, (38) are very similar to the $K_m$ and $K_{cat}$ of scallop Ca-ATPase found here. There is much evidence for two separate binding sites for nucleotide on each Ca-ATPase polypeptide chain (8, 39, 40), with one of the sites functioning as an allosteric regulator site and the other as the active (catalytic) site. Thus, there are two potential nucleotide-binding sites on the scallop Ca-ATPase that have to be taken into consideration with regard to stabilization of the scallop A and B fragments in the $E_1$ form of the Ca-ATPase by P$_i$, AMP-PNP, AMP-PCP, and ADP.

The affinity of the catalytic site for AMP-PNP on rabbit SERCA1a ($K_i = 75–90 \mu$M (36, 41, 42) is in keeping with the concentration of that ligand that stabilized the A and B fragments in tryptic digests of the $E_1$ form of scallop Ca-ATPase. Electron paramagnetic resonance experiments using spin-labeled rabbit SERCA1a (43) suggest that AMP-PCP binds to two sites on rabbit SERCA1a with dissociation constants of 50 and 650 $\mu$M. The lower value fits with stabilization of the A and B fragments of the scallop Ca-ATPase through AMP-PCP binding to the catalytic site. Although metal-free ADP binds only weakly to the phosphorylated $E_2$ form of rabbit SERCA1a ($K_d$ values ~0.73 mM) (44), it binds more strongly to the unphosphorylated enzyme with reported $K_d$ values of 12–50 $\mu$M (45). These are consistent with stabilization of the A and B fragments in digests of the $E_1$ form of scallop Ca-ATPase by ADP being bound to the catalytic site.

In the case of the rabbit enzyme, $E_1$ and $E_2$ have the same affinity for P$_i$ (5–10 mM) (30), whereas the $K_i$ of the catalytic site for P$_i$ on the $E_2$ form of scallop Ca-ATPase was found in the studies reported here to be 3.4 mM on the basis of TNP-ADP fluorescence measurements. These values are in the concentration range where stabilization by P$_i$ of the A and B fragments of the $E_1$ form of the scallop enzyme was manifested, so that binding of P$_i$ to $E_2$ is likely to be mediated through its occupation of some part of the catalytic site. Occupation of only that part of the active site that interacts with the very small orthophosphate ligand was sufficient to cause a very substantial reorganization of the three subdomains (A, N, and P) that comprise most of the cytoplasmic region of the scallop Ca-ATPase. Although the nucleotides were effective at significantly lower concentrations than P$_i$, this probably primarily reflects their higher binding affinities. Thus, binding of the adenosine moiety was not essential for stabilization of the $E_1$ form of scallop Ca-ATPase. Binding of P$_i$, or the polyphosphate moiety of nucleotide to the Ca-ATPase stabilizes a conformation where the cytoplasmic domains of the enzyme lie in close proximity to one another so that tryptic cleavages are inhibited in the A and N domains.

There is already good evidence that binding of nucleotide can modify the conformation of rabbit skeletal muscle and scallop adductor muscle SERCA. In particular, the rate of a conformational change associated with the binding of Ca$^{2+}$ is increased by ATP, and binding of ATPMg changes the conformation of the enzyme to one that is activated for phosphorylation (46). In fact, ADP, AMP-PCP, and AMP-PNP accelerate Ca$^{2+}$-binding in a pH-dependent manner (47), and changes in the amide I and II regions of the IR spectrum show that binding of nucleotide modifies the conformation of rabbit SERCA1a (48). In the case of the scallop Ca-ATPase, addition of 5 mM ATPMg$^{2+}$ to the $E_2$ form of the scallop Ca-ATPase leads to ~4 additional thiol groups becoming less reactive toward the thiol reagent 5,5'-dithiobis(2-nitrobenzoate) (DTNB), whereas addition of 6 mM ATP to the $E_1$ form in the absence of Mg$^{2+}$ causes ~5 additional thiol groups to become inaccessible to DTNB (21).

All of the ligands that stabilized the A and B fragments in Ca$^{2+}$-saturated ($E_1$) scallop Ca-ATPase against further digestion by trypsin induced a form of the enzyme with some conformational features in common with the Ca$^{2+}$-free $E_2$ state, despite Ca$^{2+}$ being bound. However, there is no evidence that, for example, AMP-PNP lowers the affinity of the enzyme for Ca$^{2+}$, thereby producing an $E_2$-like state. On the contrary, occupation of the nucleotide-binding site has been reported to increase, not decrease, affinity of the Ca-ATPase for Ca$^{2+}$ (48). The $T_d$ site on $E_1$ was not protected by any of the agents, so that the modified form of $E_1$ induced by binding of substrate analogues or P$_i$, was not identical to $E_2$ and possessed a unique structure. Hence, although binding of Ca$^{2+}$ can profoundly modify interactions among the cytoplasmic subdomains, those
regions of the enzyme still retain some independence from the transmembrane portion of the enzyme in their response to the binding of ligands.

It has been pointed out that the binding of Ca\(^{2+}\) alone is not sufficient to position the nucleotide substrate close enough to Asp\(^{52}\) for transfer of the γ-phosphoryl group (49). The results reported here suggest that the additional reorganization of the catalytic center necessary for this to happen may originate in conformational changes caused by occupation of the active site, an example of the induced fit phenomenon. The occlusion of potentially sensitive sites toward trypsin that occurs when substrate analogues are bound to the catalytic nucleotide-binding site in the E\(_2\) form of the Ca-ATPase may be related to the closing of the residual distance between Asp\(^{352}\) (scallop sequence) and the γ-phosphoryl group of ATP.

Recently, it was found that treatment of rabbit SERCA1a with the phosphate transition-state analogues F\(_{387}\)-P and orthovanadate caused the enzyme to become extremely resistant toward trypsin. Thus, atomic models suggest that formation of the non-polar site derives from site-directed mutagenesis and chemical modification studies of Gly\(^{266}\) and Arg\(^{499}\), both located in L\(_{25}\) (58, 59). The close proximity of Glu\(^{485}\), Asp\(^{486}\) to Thr\(^{170}\), Leu\(^{172}\) in the E\(_2\) crystal structure of SERCA1a suggests that the N domain may also be involved in the binding of P\(_i\), and there is strong evidence that the C-terminal part of the P domain of SERCA1a is needed for P\(_i\) to bind (60). The h1-h2 hinge connecting the N and P domains must participate in the active site in E\(_2\)-P because it contains Asp\(^{52}\). There is also good evidence that the hinge is directly involved in the binding of P\(_i\), because the phosphate transition-state analogue orthovanadate binds the hinge in h1 (61), and the DDRP motif in h2 provides ligands for the binding of P\(_i\) (62). Formation of the P\(_i\)-binding site thus appears to involve all the major structural elements of the cytoplasmic region of the Ca-ATPase. Orthophosphate or the phosphate moieties of nucleotides may therefore act to draw the cytoplasmic domains and the hinge together and so stabilize a compact structure resistant to trypsin.

In conclusion, large-scale structural changes can be initiated in the Ca-ATPase not only by the binding and release of Ca\(^{2+}\), but also by interaction of phosphate groups with the active site. Another example of a major structural reorganization in a protein associated with P\(_i\) binding/release is the very large conformational change that occurs when P\(_i\) dissociates from the S-1 head of myosin after hydrolysis of ATP (32).

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