

## Effect of Orthophosphate, Nucleotide Analogues, ADP, and Phosphorylation on the Cytoplasmic Domains of Ca<sup>2+</sup>-ATPase from Scallop Sarcoplasmic Reticulum\*

Received for publication, September 10, 2003, and in revised form, November 12, 2003  
Published, JBC Papers in Press, November 25, 2003, DOI 10.1074/jbc.M310085200

Chris Ryan, David L. Stokes‡, Minggu Chen§, Zhimin Zhang, and Peter M. D. Hardwicke¶

From the Department of Biochemistry and Molecular Biology, Southern Illinois University, Carbondale, Illinois 62901

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  $E_2$  (Ca<sup>2+</sup>-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<sup>2+</sup>-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<sub>i</sub>, AMP-PNP, AMP-PCP, or ADP despite the presence of saturating levels of Ca<sup>2+</sup>. 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<sub>i</sub> 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 (SERCA)<sup>1</sup> transport Ca<sup>2+</sup> 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<sup>2+</sup> homeostasis in both muscle and non-muscle cells (1, 2). In the course of transporting Ca<sup>2+</sup>, 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  $\beta$ -carboxyl group of a specific aspartyl residue (Asp<sup>351</sup> in the case of rabbit SERCA1a) is present as an acylphosphate 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<sup>2+</sup> and can be phosphorylated by ATP but not by P<sub>i</sub>, and the  $E_2$  state, which possesses low affinity Ca<sup>2+</sup> sites and can be phosphorylated by P<sub>i</sub> but not by ATP (3).

Comparison of the tryptic digestion patterns of rabbit SERCA1a in its Ca<sup>2+</sup>-bound and -free forms 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<sup>2+</sup>)<sub>2</sub> form of rabbit SERCA1a shows clear structural differences to the enzyme in its vanadate-stabilized Ca<sup>2+</sup>-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  $E_1$ -P and  $E_2$ -P states 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<sup>2+</sup>-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<sup>2+</sup> 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<sup>2+</sup>)<sub>2</sub>.

\* This work was supported by a grant from the Central Research Committee of the School of Medicine, Southern Illinois University (to P. M. D. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Supported by R01 GM56960. Present address: Skirball Institute, NYU Medical Center, 540 First Ave., New York, NY 10016.

§ Present address: Open System Div., Information Builders Inc., Two Penn Plaza, 28th Floor, New York, NY 10121.

¶ To whom correspondence should be addressed. Tel.: 618-453-6469; Fax: 618-453-6440; E-mail: phardwicke@siumed.edu.

<sup>1</sup> The abbreviations used are: SERCA, sarco(endo)plasmic reticulum ATPase; AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride; AMP-PCP, adenosine 5'-( $\beta$ ,  $\gamma$ -methylene) triphosphate; AMP-PNP, 5'-adenylylimidodiphosphate; DOCNa, sodium deoxycholate; FSR, fragmented sarcoplasmic reticulum; MES, 4-morpholineethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; P<sub>i</sub>, orthophosphate; SR, sarcoplasmic reticulum; A, N, and P, actuator, nucleotide, and phosphorylation domains, respectively; TNP-ADP, 2',3'-O-(2,4,6-trinitrocyclohexyldienylidene) adenosine 5'-diphosphate; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone.

## 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-striated 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 rabbit 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 M KCl, 1 mM CaCl<sub>2</sub>, 20 mM MOPSNa, pH 7.0, onto 1.5 M sucrose, 0.1 M KCl, 1 mM CaCl<sub>2</sub>, 20 mM MOPSNa, pH 7.0, and centrifuge the preparation for 3 h at  $1.5 \times 10^5 \times g$ . 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<sup>-1</sup> in 20% v/v ethylene glycol (Pierce), 0.15 M KCl, 50 mM MOPSNa, pH 7.0. The *E*<sub>1</sub> pattern of tryptic fragments was obtained when the free Ca<sup>2+</sup> concentration was above ~10 μM, but typically a total of 1 or 3 mM CaCl<sub>2</sub> was present for digests in the *E*<sub>1</sub> state. For digests of the *E*<sub>2</sub> form, 10 mM EGTA replaced CaCl<sub>2</sub>. Digestion was at room temperature with TPCK-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 CaCl<sub>2</sub> (*E*<sub>1</sub> digests) or 1 mM EGTA (*E*<sub>2</sub> 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 CaCl<sub>2</sub> (*E*<sub>1</sub> digests), or 1 mM EGTA (*E*<sub>2</sub> 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 thioglycolate (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 with a Bio-Rad Trans Blot apparatus. Blots were stained with 0.02% w/v Coomassie Brilliant Blue R.250 in 1 mM HCl, 50% v/v MeOH. Bands of interest were sent to the University of Florida for N-terminal sequencing.

**Phosphorylation with P<sub>i</sub>**—For digests in the *E*<sub>2</sub>-P state, DOC-extracted scallop FSR was phosphorylated with P<sub>i</sub> essentially as described previously (15, 21) at room temperature for 15 min before addition of trypsin.

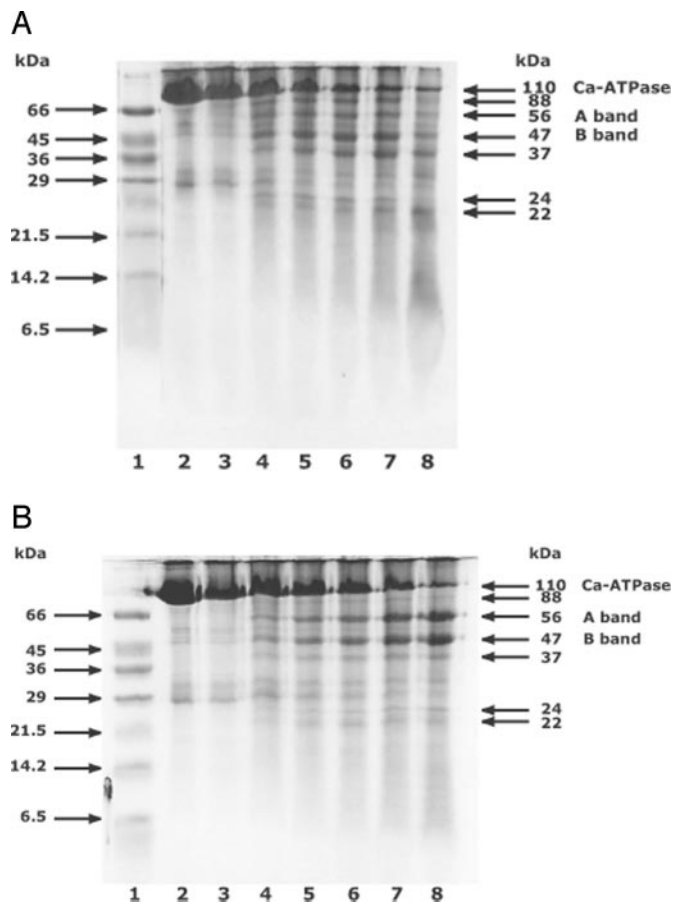
**Detection of E<sub>2</sub>-P Using TNP-ADP Superfluorescence**—This was carried out using an SLM 8000c spectrofluorimeter thermostated at 25 °C. TNP-ADP (Molecular Probes) was added to 35 μg/ml<sup>-1</sup> DOC-extracted scallop FSR suspended in 20% v/v glycerol, 15 mM EGTA-Tris, 15 mM MgCl<sub>2</sub>, 50 mM Mes-Tris, pH 6.0, to a final concentration of 2 μM. Phosphorylation was typically induced by addition of P<sub>i</sub> in the form of H<sub>3</sub>PO<sub>4</sub>-Tris to 8.5 mM. Steady-state fluorescence measurements using 4-nm slits were carried out with excitation at 403 nm, and emission followed at 532 nm.

**Enzyme Assays**—The dependence of the Ca<sup>2+</sup>-activated ATPase activity of the DOC-extracted scallop FSR on ATPMg<sup>2+</sup> concentration was determined using a coupled enzyme assay as previously described (16), except that 5 mM Mg<sup>2+</sup> was present (as MgCl<sub>2</sub>) in excess of the ATPMg<sup>2+</sup> concentration, according to Neet and Green (22). Assays were carried out in a Perkin-Elmer Lambda 40 spectrophotometer in a volume of 1 ml. The cell was thermostated 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



**FIG. 1. Time courses for digestion of the scallop Ca-ATPase in its *E*<sub>1</sub> and *E*<sub>2</sub> states.** Digestions were carried out as described under "Experimental Procedures." Proteolysis was halted by addition of AEBSF to 20 mM and placing the sample on ice. Digested samples were washed free of trypsin. *A*, time course under *E*<sub>1</sub> conditions. *Lane 1*, markers; *lane 2*, undigested FSR control; *lanes 3–8*, membrane-bound fragments from tryptic digestion of 20 μg of scallop FSR at 0, 1, 3, 5, 10, and 30 min, respectively. *B*, time course under *E*<sub>2</sub> conditions. *Lane 1*, markers; *lane 2*, undigested control; *lanes 3–8*, membrane-bound fragments from tryptic digestion of 20 μg of scallop FSR at 0, 1, 3, 5, 10, and 30 min, respectively.

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 P<sub>i</sub> (15, 21) and on its specific Ca<sup>2+</sup>-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 tryptic digests where the scallop Ca-ATPase was in the *E*<sub>2</sub> 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 *E*<sub>1</sub> state (10 μM free Ca<sup>2+</sup> or above) gave a pattern in which both the 56- and 47-kDa bands were less intense than in *E*<sub>2</sub> 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 *E*<sub>2</sub> state and then phosphorylated

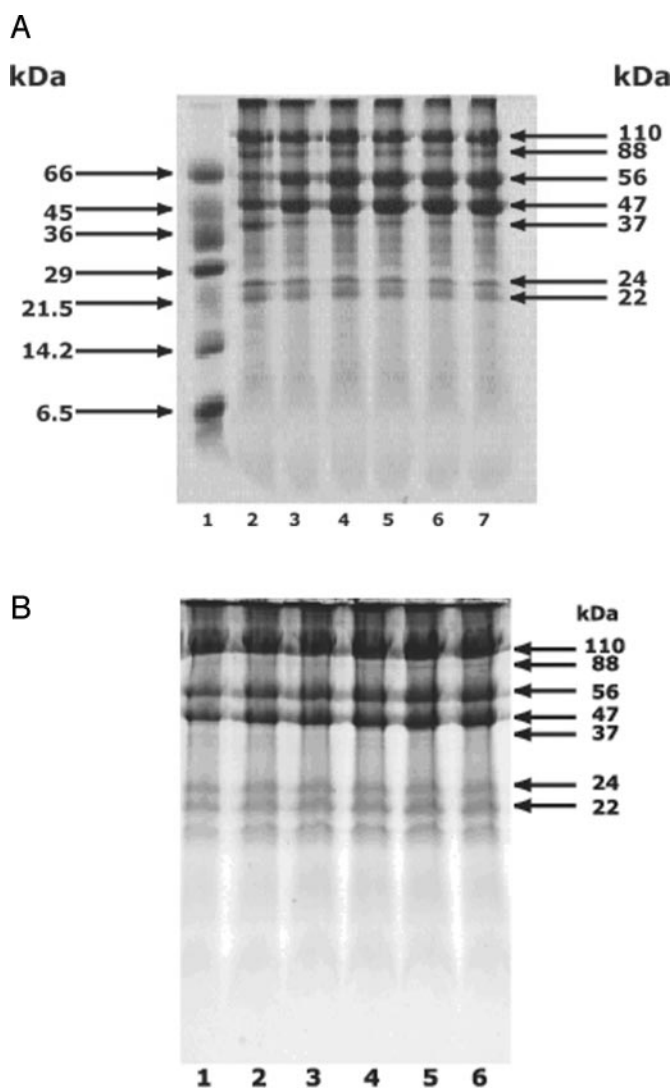
with [ $\gamma$ - $^{32}$ P]ATP showed labeling of the 56-kDa band with  $^{32}$ P as well as the intact ATPase (not shown). Rabbit SERCA1a has a blocked (acetylated) N terminus, as of course does its A tryptic fragment, and the tryptic A fragment representing the N-terminal half of SERCA1a contains Asp $^{351}$ . Thus, the 56-kDa peptide formed in scallop tryptic digests contained Asp $^{350}$  and corresponded as expected to the scallop A tryptic peptide.<sup>2</sup> 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 $^{504}$ -Val $^{505}$  (scallop sequence, Ref. 27) in the N domain and thus represented the scallop B tryptic fragment.

Under otherwise identical conditions, a more rapid overall digestion process occurred in the  $E_1$  compared with the  $E_2$  state. Comparison of time courses obtained when the scallop Ca-ATPase was in its  $E_1$  and  $E_2$  forms (Fig. 1, A and B) showed that in the  $E_1$  state the A band was lost much more rapidly than in  $E_2$ . Although the B fragment was more stable than the A fragment in digests carried out in the presence of Ca $^{2+}$  (when the enzyme was in its  $E_1$  form), it was nevertheless broken down faster than in the presence of EGTA (when the enzyme was in its  $E_2$  form). Therefore, Ca $^{2+}$ -dependent cleavages occurred in both the N- and C-terminal halves of the scallop Ca-ATPase.

N-terminal sequencing showed that the 88-kDa fragment, which was formed in larger amounts in the  $E_1$  than the  $E_2$  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 $^{582}$ -Phe $^{583}$  peptide bond (scallop sequence, Ref. 27) in the N domain, which will be designated as the T $_3$  site in the scallop enzyme. N-terminal sequencing of the 22 and 24-kDa peptides preferentially formed in the  $E_1$  state showed that formation of both of these peptides involved cleavage of the Lys $^{727}$ -Ser $^{728}$  bond in the C-terminal component of the P domain. The Lys $^{727}$ -Ser $^{728}$  bond will be designated as the T $_4$  tryptic cleavage 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  $\sim 0.1$  mM in tryptic digests of the scallop Ca-ATPase in its  $E_1$  state (with free Ca $^{2+}$  >  $10 \mu$ 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  $E_2$  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



**FIG. 2. Effect of AMP-PNP on tryptic cleavage pattern of the  $E_1$  and  $E_2$  forms of scallop Ca-ATPase.** AMP-PNP (pH 7.0) was added to and mixed with DOC-extracted scallop FSR, and digestion with trypsin was carried out as described under "Experimental Procedures." Samples (36  $\mu$ g) were run in the Laemmli system with a 12.5% separating gel. A, lane 1, markers; lane 2,  $E_1$  digest; lane 3,  $E_1$  digest + 0.1 mM AMP-PNP; lane 4,  $E_1$  digest + 0.5 mM AMP-PNP; lane 5,  $E_1$  digest + 1 mM AMP-PNP; lane 6,  $E_1$  digest + 5 mM AMP-PNP; lane 7,  $E_1$  digest + 10 mM AMP-PNP. B, lane 1,  $E_2$  digest; lane 2,  $E_2$  digest + 0.1 mM AMP-PNP; lane 3,  $E_2$  digest + 0.5 mM AMP-PNP; lane 4,  $E_2$  digest + 1 mM AMP-PNP; lane 5,  $E_2$  digest + 5 mM AMP-PNP; lane 6,  $E_2$  digest + 10 mM AMP-PNP.

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 $^{504}$ ), 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-

<sup>2</sup> Because the scallop Ca-ATPase lacks a residue corresponding to Thr $^{22}$  of rabbit SERCA1a, b (26), over almost all of the sequence (from Gly $^{22}$  until an extra residue is inserted at position 987 (Cys $^{987}$ )) residue position numbers for the scallop ATPase are one less than the corresponding residue number in SERCA1a, b.

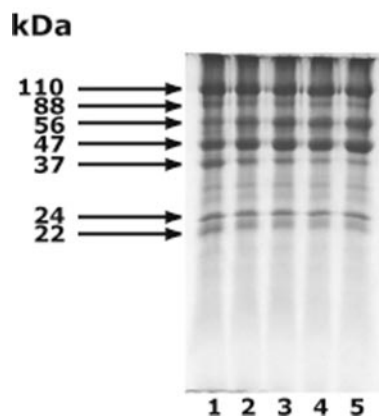


FIG. 3. Effect of  $P_i$  on the tryptic cleavage pattern of the  $E_1$  form of scallop Ca-ATPase. Sodium orthophosphate (pH 7) was added to DOC-extracted scallop SR suspended in the standard  $E_1$  digestion medium, and digestion with trypsin was carried out as described in the legend to Fig. 1 and under "Experimental Procedures." Samples (36  $\mu$ g) were run on a 12.5% Laemmli gel. Lane 1,  $E_1$  digest; lane 2,  $E_1$  digest + 5 mM  $P_i$ ; lane 3,  $E_1$  digest + 10 mM  $P_i$ ; lane 4,  $E_1$  digest + 20 mM  $P_i$ ; lane 5,  $E_1$  digest + 40 mM  $P_i$ .

polyphosphate (5 mM) in  $E_1$  digests produced similar effects to those seen with  $P_i$ .

**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  $K^+$  at pH 7. 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  $E_2$  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 (superfluorescence) 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  $E_2$  form of the scallop Ca-ATPase was suspended in 20% v/v  $Me_2SO$  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  $Me_2SO$  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

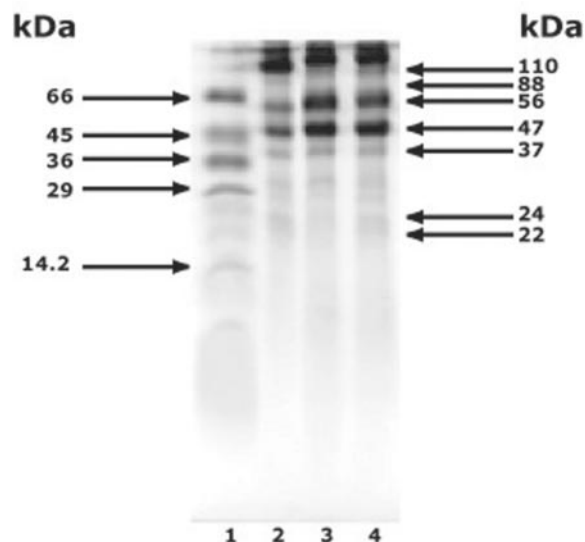


FIG. 4. Effect of phosphorylation of the scallop Ca-ATPase to its  $E_2$ -P state on its tryptic digestion pattern. Samples of DOC-extracted scallop FSR were digested with trypsin as described under "Experimental Procedures" at room temperature for 30 min in a medium promoting phosphorylation of the enzyme with  $P_i$ . A second sample was digested in a medium otherwise identical but lacking  $P_i$ . Samples were electrophoresed in the Tris-glycine system on a 12.5% gel. Lane 1, markers; lane 2, 36  $\mu$ g of the unphosphorylated  $E_2$  form of scallop Ca-ATPase after digestion with trypsin in a medium containing 20% v/v  $Me_2SO$ , 15 mM  $MgCl_2$ , 10 mM EGTA-Tris, 50 mM MOPS-Tris, pH 7.0 but no  $P_i$ ; lane 3, 36  $\mu$ g of the  $E_2$ -P phosphorylated form of scallop muscle Ca-ATPase after digestion with trypsin in 20% v/v  $Me_2SO$ , 15 mM  $MgCl_2$ , 10 mM EGTA-Tris, 50 mM MOPS-Tris, pH 7.0, plus 20 mM  $P_i$ -Tris; lane 4, 36  $\mu$ g of the  $E_2$ -P phosphorylated form of scallop muscle Ca-ATPase after digestion with trypsin in 20% v/v  $Me_2SO$ , 4 mM ADP, 20 mM  $P_i$ -Tris, 15 mM  $MgCl_2$ , 10 mM EGTA-Tris, 50 mM MOPS-Tris, pH 7.0.

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 molecule 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  $T_4$  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 provided 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  $\text{Ca}^{2+}$ ,  $\text{K}^+$ , and  $\text{Na}^+$ , was in complete contrast to the extremely rapid loss of activity that occurs with the membranous unphosphorylated  $E_2$  form of the scallop enzyme under comparable conditions (11, 13). Because inactivation of the unphosphorylated  $\text{Ca}^{2+}$ -free ( $E_2$ ) scallop Ca-ATPase involves an irreversible loss of the  $\text{Ca}^{2+}$ -binding sites, phosphorylation of the enzyme in the absence of  $\text{Ca}^{2+}$  with  $\text{P}_i$  may thus stabilize otherwise labile empty  $\text{Ca}^{2+}$ -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 (+  $\text{Ca}^{2+}$ ) to that described above where the enzyme had been phosphorylated from  $\text{P}_i$  (+ 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  $\text{Mg}^{2+}$  in the presence of  $\text{K}^+$ , *i.e.* under conditions where most (>90%) of the phosphorylated enzyme was in the ADP-sensitive form ( $E_1$ -P) as previously described (15). The pattern of tryptic fragments produced from the  $E_1$ -P preparation of scallop Ca-ATPase as visualized with Coomassie Blue was indistinguishable from that of the unphosphorylated  $E_1$  form of the Ca-ATPase, with significant breakdown of the A and B fragments.

**Dependence of Enzyme Activity on ATPMg<sup>2+</sup> 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  $\text{Ca}^{2+}$ -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.

$$v_o = -K_m(v_o/[\text{ATPMg}]) + V_{\max} \quad (\text{Eq. 1})$$

The graph displayed two limbs, one with a slope corresponding to an apparent Michaelis constant  $K_H$  of 0.29 mM, which extrapolated to give a  $k_{\text{cat}}$  of 368.5  $\text{min}^{-1}$ , and the other to a  $K_m$  of 4.6  $\mu\text{M}$  and a  $k_{\text{cat}}$  of 92.4  $\text{min}^{-1}$ , (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  $\mu\text{M}$  and 0.3–1 mM, respectively, (38) are very similar to the  $K_m$  and  $K_H$  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  $\text{P}_i$ , AMP-PNP, AMP-PCP, and ADP.

The affinity of the catalytic site for AMP-PNP on rabbit SERCA1a ( $K_d \sim 75$ –90  $\mu\text{M}$  (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  $\mu\text{M}$ . 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  $E_1$ -P( $\text{Ca}^{2+}$ )<sub>2</sub> form of rabbit SERCA1a ( $K_d$  values  $\sim 0.73$  mM) (44), it binds more strongly to the unphosphorylated enzyme with reported  $K_d$  values of 12–50  $\mu\text{M}$  (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  $\text{P}_i$  (5–10 mM) (30), whereas the  $K_d$  of the catalytic site for  $\text{P}_i$  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  $\text{P}_i$  of the A and B fragments of the  $E_1$  form of the scallop enzyme was manifested, so that binding of  $\text{P}_i$  to  $E_1$  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  $\text{P}_i$ , 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  $\text{P}_i$  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  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  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<sup>2+</sup> to the  $E_2$  form of the scallop Ca-ATPase leads to  $\sim 4$  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  $\text{Mg}^{2+}$  causes  $\sim 5$  additional thiol groups to become inaccessible to DTNB (21).

All of the ligands that stabilized the A and B fragments in  $\text{Ca}^{2+}$ -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  $\text{Ca}^{2+}$ -free  $E_2$  state, despite  $\text{Ca}^{2+}$  being bound. However, there is no evidence that, for example, AMP-PNP lowers the affinity of the enzyme for  $\text{Ca}^{2+}$ , 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  $\text{Ca}^{2+}$  (48). The  $T_4$  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  $\text{P}_i$  was not identical to  $E_2$  and possessed a unique structure. Hence, although binding of  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  alone is not sufficient to position the nucleotide substrate close enough to Asp<sup>351</sup> for transfer of the  $\gamma$ -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  $E_1$  form of the Ca-ATPase may be related to the closing of the residual distance between Asp<sup>350</sup> (scallop sequence) and the  $\gamma$ -phosphoryl group of ATP.

Recently, it was found that treatment of rabbit SERCA1a with the phosphate transition state analogues  $\text{F}^-$  and orthovanadate caused the enzyme to become extremely resistant to the action of several proteases (50, 51). In the current study, formation of the  $E_2$ -P covalent adduct by addition of  $\text{P}_i$  to the  $E_2$  form of the scallop SR membranes suspended in a  $\text{K}^+$ -free medium prevented an otherwise rapid degradation of the A and B fragments by trypsin. This behavior suggests an unusually condensed conformation for the scallop enzyme in its  $E_2$ -P state; however, because the  $E_2$ -P-SDS complex had a lower mobility than the  $E_2$ -SDS complex, overall the  $E_2$ -P form may not be folded into a more globular shape than  $E_2$ . The unphosphorylated  $E_2$  form of scallop Ca-ATPase loses activity very rapidly and is highly susceptible to proteolysis in the absence of  $\text{K}^+$ , yet formation of the aspartyl phosphate at the active site was able to lock the enzyme into a conformation that was both structurally and functionally exceedingly stable. Thus, the effect of the covalent modification was propagated throughout the cytoplasmic domains of the enzyme.

The resistance of the  $E_2$ -P form of scallop Ca-ATPase toward trypsin and the great stability of its enzyme activity compared with  $E_2$ , both under conditions where  $\text{K}^+$  are absent, are very likely to have a common origin in the unusual state of the active site in  $E_2$ -P. The synergistic binding of  $\text{P}_i$  and  $\text{Mg}^{2+}$  to  $E_2$  is endothermic, but a substantial entropy increase associated with the release of a large number of water molecules from the active site and the hydration shell of  $\text{P}_i$  allows the process to be driven thermodynamically (27, 52). The activity of water at the catalytic site in the  $E_2$ - $\text{Mg}^{2+}$ - $\text{P}_i$  ternary complex is therefore very low; this very non-polar environment promotes spontaneous formation of the acyl phosphate from  $\text{P}_i$  and the  $\beta$ -carboxyl side chain of Asp<sup>351</sup> and then protects it from hydrolysis (3). The very hydrophobic nature of the active site in  $E_2$ -P may underlie its great stability as well as the reported differences in quaternary and tertiary structure between  $E_2$ -P and  $E_2$  (15, 53–55).

Stabilization by  $\text{P}_i$  of  $E_1$  in a form where  $\text{Ca}^{2+}$ -sensitive tryptic cleavage sites on the A and N domains are occluded toward trypsin and the great resistance of  $E_2$ -P toward trypsin are consistent with what is known about the binding site for  $\text{P}_i$ . All three cytoplasmic domains may have to be intimately juxtaposed to form the site in combination with the hinge region. Thus, atomic models suggest that formation of the non-polar cavity in  $E_2$ -P may involve movement of the A domain close to the catalytic site (39). Support for such a structure comes from studies using proteinase K digestion of SERCA1a (56) and chymotryptic digestion of the Na, K-ATPase (57). Further strong evidence that the A domain contributes to the  $\text{P}_i$ -binding site derives from site-directed mutagenesis and chemical modification studies of Gly<sup>233</sup> and Arg<sup>198</sup>, both located in L<sub>23</sub> (58, 59). The close proximity of Glu<sup>485</sup>-Asp<sup>489</sup> to Thr<sup>170</sup>-Leu<sup>172</sup> in

the  $E_2$  crystal structure of SERCA1a suggests that the N domain may also be involved in the binding of  $\text{P}_i$ , and there is strong evidence that the C-terminal part of the P domain of SERCA1a is needed for  $\text{P}_i$  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<sup>351</sup>. There is also good evidence that the hinge is directly involved in the binding of  $\text{P}_i$ , because the phosphate transition-state analogue orthovanadate binds close to Thr<sup>353</sup> in h1 (61), and the <sup>601</sup>DPPR motif in h2 provides ligands for the binding of  $\text{P}_i$  (62). Formation of the  $\text{P}_i$ -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  $\text{Ca}^{2+}$  but also by interaction of phosphate groups with the active site. Another example of a major structural reorganization in a protein associated with  $\text{P}_i$  binding/release is the very large conformational change that occurs when  $\text{P}_i$  dissociates from the S-1 head of myosin after hydrolysis of ATP (32).

## REFERENCES

- Lee, A. G. (2001) *Biochem. J.* **356**, 665–683
- McIntosh, D. B. (2000) *Nature Struct. Biol.* **7**, 532–535
- de Meis, L. (1981) *The Sarcoplasmic Reticulum*, pp. 69–101, Wiley, New York
- Andersen, J. P., and Jorgensen, P. L. (1985) *J. Memb. Biol.* **88**, 187–198
- Andersen, J. P., Jorgensen, P. L., and Møller, J. V. (1985) *Proc. Natl. Acad. Sci. (U. S. A.)* **82**, 4573–4577
- Andersen, J. P., Vilsen, B., Collins, J. H., and Jorgensen, P. L. (1986) *J. Memb. Biol.* **93**, 85–92
- Toyoshima, C., Nakasako, M., Nomura, H., and Ogawa, H. (2000) *Nature* **405**, 647–655
- Stokes, D. L., and Green, N. M. (2000) *Biophys. J.* **78**, 1765–1776
- Wu, C., Rice, W. J., He, W., and Stokes, D. L. (2002) *J. Mol. Biol.* **316**, 201–211
- Toyoshima, C., and Nomura, H. (2002) *Nature* **418**, 605–610
- Kalabokis, V. N., Santoro, M. M., and Hardwicke, P. M. D. (1993) *Biochemistry* **32**, 4389–4396
- Castellani, L., Hardwicke, P. M. D., and Vibert, P. (1985) *J. Mol. Biol.* **185**, 579–594
- Kalabokis, V. N., Bozzola, J., Castellani, L., and Hardwicke, P. M. D. (1991) *J. Biol. Chem.* **266**, 22044–22050
- Castellani, L., Hardwicke, P. M. D., and Franzini-Armstrong, C. (1989) *J. Cell Biol.* **108**, 511–520
- Hardwicke, P. M. D., and Bozzola, J. (1989) *J. Musc. Res. Cell Motil.* **10**, 245–253
- Kalabokis, V., and Hardwicke, P. M. D. (1988) *J. Biol. Chem.* **263**, 15184–15185
- Kalabokis, V. N., and Hardwicke, P. M. D. (1993) *Biochim. Biophys. Acta* **1147**, 35–41
- Cuenda, A., Henao, F., Nogues, M., and Gutiérrez-Merino, C. (1994) *Biochim. Biophys. Acta* **1194**, 35–43
- Laemmli, U. K. (1970) *Nature* **227**, 680–685
- Schägger, H., and von Jagow, G. (1987) *Anal. Biochem.* **166**, 368–379
- Hardwicke, P. M. D., and Huvos, P. (1989) *J. Musc. Res. Cell Motil.* **10**, 229–244
- Neet, K. E., and Green, N. M. (1977) *Arch. Biochem. Biophys.* **178**, 588–597
- Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., and Klenk, D. C. (1985) *Anal. Biochem.* **150**, 76–85
- Castellani, L., and Hardwicke, P. M. D. (1983) *J. Cell Biol.* **97**, 557–561
- Chen, M., Zhang, Z., Boateng-Tawiah, M.-A., and Hardwicke, P. M. D. (2000) *J. Biol. Chem.* **275**, 22961–22968
- Hardwicke, P. M. D., Ryan, C., and Kalabokis, V. N. (1999) *Biochim. Biophys. Acta* **1417**, 1–8
- Shi, X., Chen, M., Huvos, P., and Hardwicke, P. M. D. (1998) *Comp. Biochem. Physiol. Part B*, **120**, 359–374
- Dupont, Y., and Pougeois, R. (1983) *FEBS Lett.* **156**, 93–98
- Nakamoto, R. K., and Inesi, G. (1984) *J. Biol. Chem.* **259**, 2961–2970
- Froud, R. J., and Lee, A. G. (1986) *Biochem. J.* **237**, 207–215
- Shigekawa, M., and Wakabayashi, S. (1985) *J. Biol. Chem.* **260**, 11679–11687
- Vale, R. D., and Milligan, R. A. (2000) *Science* **288**, 88–95
- Inesi, G., Goodman, J. J., and Watanabe, S. (1967) *J. Biol. Chem.* **242**, 4637–4643
- The, R., and Hasselbach, W. (1972) *Eur. J. Biochem.* **28**, 357–363
- Dode, L., Van Baelen, K., Wuytack, F., and Dean, W. L. (2001) *J. Biol. Chem.* **276**, 3911–3919
- Hofstee, B. H. J. (1959) *Nature* **184**, 1296–1298
- Taylor, J. S., and Hattian, D. (1979) *J. Biol. Chem.* **254**, 4402–4407
- De Meis, L., and de Mello, M. C. F. (1973) *J. Biol. Chem.* **248**, 3696–3701
- Mignaco, J. A., Lupi, O. H., Santos, F. T., Barrabin, H., and Scofano, H. (1996) *Biochemistry* **35**, 3886–3891
- Stokes, D. L., and Green, N. M. (2003) *Ann. Rev. Biophys. Biomol. Struct.* **32**,

- 445–468
41. Taylor, J. S. (1981) *J. Biol. Chem.* **256**, 9793–9795
  42. Dupont, Y. (1977) *Eur. J. Biochem.* **72**, 185–190
  43. Mahaney, J. E., Froelich, J., and Thomas, D. D. (1995) *Biochemistry* **34**, 4864–4879
  44. Pickart, C., and Jencks, W. P. (1984) *J. Biol. Chem.* **259**, 1629–1643
  45. Murphy, A. J. (1988) *Biochim. Biophys. Acta* **946**, 57–65
  46. Stahl, N., and Jencks, W. P. (1984) *Biochemistry* **23**, 5389–5392
  47. Mintz, E., Mata, A. M., Forge, V., Passafiume, M., and Guillain, F. (1995) *J. Biol. Chem.* **270**, 27160–27164
  48. von Germar, F., Barth, A., and Mantele, W. (2000) *Biophys. J.* **78**, 1531–1540
  49. MacLennan, D. H., and Green, N. M. (2000) *Nature* **405**, 633–634
  50. Danko, S., Daiho, T., Yamasaki, K., Kamodochi, M., Suzuki, H., and Toyoshima, C. (2001) *FEBS Lett.* **489**, 277–282
  51. Danko, S., Yamasaki, K., Daiho, T., Suzuki, H., and Toyoshima, C. (2001) *FEBS Lett.* **505**, 129–135
  52. Schwarz, F. P., and Inesi, G. (1997) *Biophys. J.* **73**, 2179–2182
  53. Ross, D. C., and McIntosh, D. B. (1987) *J. Biol. Chem.* **262**, 12977–129837
  54. Ross, D. C., Davidson, G. A., and McIntosh, D. B. (1991) *J. Biol. Chem.* **266**, 4613–4621
  55. Myung, J., and Jencks, W. P. (1994) *Biochemistry* **33**, 8775–8785
  56. Møller, J. V., Lenoir, G., Marchand, C., Montigny, C., le Maire, M., Toyoshima, C., Juul, B. S., and Champeil, P. (2002) *J. Biol. Chem.* **277**, 38647–38659
  57. Jorgensen, P. L., Jorgensen, J. R., and Pedersen, P. A. (2001) *J. Bioenerg. Biomembr.* **33**, 367–377
  58. Andersen, J. P., Vilsen, B., Leberer, E., and MacLennan, D. H. (1989) *J. Biol. Chem.* **264**, 21018–21023
  59. Saino, T., Daiho, T., and Kanazawa, T. (1997) *J. Biol. Chem.* **272**, 21142–211450
  60. Shin, J. M., Goldshleger, R., Munson, K., Sachs, G., and Karlish, S. J. D. (2001) *J. Biol. Chem.* **276**, 48440–48450
  61. Hua, S., Inesi, G., and Toyoshima, C. (2000) *J. Biol. Chem.* **275**, 30546–30550
  62. Farley, R. A., Elquza, E., Müller-Ehmsen, J., Kane, D. J., Nagy, A. K., Kasho, V. N., and Faller, L. D. (2001) *Biochemistry* **40**, 6361–6370