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Localization of E_1-E_2 Conformational Transitions of Sarcoplasmic Reticulum Ca-ATPase by Tryptic Cleavage and Hydrophobic Labeling

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Summary. Tryptic peptides of Ca-ATPase in E_1 and E_2 conformational states (Andersen, J. P., Jørgensen, P. L., *J. Membrane Biol.* **88**:187–198 (1985)) have been isolated by size exclusion high performance liquid chromatography in sodium dodecyl sulfate. This permitted unambiguous localization of a conformational sensitive tryptic split at Arg 198 by N-terminal amino acid sequence analysis. Other splits at Arg 505 and at Arg 819-Lys 825 were insensitive to E_1-E_2 transitions. Tryptic cleavage of Ca-ATPase after phosphorylation by inorganic phosphate showed that this enzyme form has a conformation similar to that of the vanadate-bound E_2 state, both in membranous and in soluble monomeric Ca-ATPase.

Hydrophobic labeling of Ca-ATPase in sarcoplasmic reticulum vesicles with the photoactivable reagent trifluoromethyl-[^{125}I]iodophenyl-diazirine indicated that E_2 and E_2V states are more exposed to the membrane phase than E_1 and E_1P (Ca^{2+}-occluded) states. The preferential hydrophobic labeling in E_2 forms was found to be localized in the A_1 tryptic fragment.

Key Words conformational change · trypsin · size exclusion HPLC · amino-acid sequence · trifluoromethyl-[^{125}I]iodophenyl-diazirine · CrATP · vanadate

Introduction

Physiological relaxation of myofibrils in muscle is induced by ATP-driven active transport of Ca^{2+} across the sarcoplasmic reticulum membrane [13]. Isolation and reconstitution of the Ca^{2+}-pump protein (Ca-ATPase) [20, 29] has permitted structural resolution of the Ca^{2+} transport process at the molecular level. Conformational transitions in Ca-ATPase protein related to energy transduction and Ca^{2+} translocation can be monitored by proteolytic cleavage, both in the purified membrane-bound state and in detergent solubilized monomeric preparations [1, 2]. The exact nature of these structure changes and their extent in terms of the number of amino acid residues involved remain to be identified [27]. An intriguing problem is whether Ca^{2+} translocation involves movement of the cytoplasmic part of the peptide chain into the lipid bilayer [5].

In the present investigation we have located the conformational changes monitored by tryptic cleavage in the primary structure by comparison of N-terminal sequences of tryptic peptides with the complete amino acid sequence [6, 21]. The previous characterization of tryptic cleavage patterns of the phosphorylated intermediates of Ca-ATPase [1] has been completed by examination of the effect of phosphorylation with P_i. In addition we have studied the exposure of Ca-ATPase in the various conformational states to hydrophobic labeling with the photoactivable reagent trifluoromethyl-[^{125}I]iodophenyldiazirine (TID)[1] [7, 17]. Our data indicate that the E_1P-E_2P transition is accompanied by redistribution of protein mass corresponding to part of the A_1 fragment from the cytoplasmic surface to the membrane phase.

Materials and Methods

Sarcoplasmic reticulum vesicles and purified Ca-ATPase membranes were prepared as in [4]. Tryptic digestion was carried out as previously [1] at 0.5 mg Ca-ATPase protein/ml, 25 μg trypsin/ml and varying buffer conditions: 50 mM Tes/Tris (pH 8.0)[1], 30

[1] *Abbreviations*: Tes, N-(tris-[hydroxymethyl]methyl-2-amino)ethane sulfonic acid; EGTA, ethyleneglycol bis (β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; $C_{12}E_8$, octhaethyleneglycol monododecyl ether; HPLC, high performance liquid chromatography; DTT, dithiothreitol; $NaDodSO_4$, sodium dodecyl sulfate; PTH, phenylthiohydantoin; [^{125}I]TID, 3-trifluoromethyl-3-(m[^{125}I]iodophenyl)diazirine; EP, phosphorylated Ca-ATPase; E_1 and E_2, major conformational states of Ca-ATPase; P_i, inorganic phosphate.

mM NaCl, or 20 mM Tes (pH 7.0), 100 mM NaCl, or 100 mM Tes/Tris (pH 7.0) with further additions of $CaCl_2$, $MgCl_2$, EGTA, Tris phosphate, Na_3VO_4, $C_{12}E_8$, dimethylsulfoxide and glycerol as indicated. Tryptic peptides were analyzed by gel electrophoresis in 5-15% gradient slab gels prepared by a modification of the Laemmli procedure [1]. Phosphorylated peptides were also prepared as in ref. [1].

High performance gel permeation chromatography was carried out at 20°C in TSK G 3000 SW columns (Toyo Soda), and absorbance in eluted fractions was read at 226 nm. Trypsin and trypsin inhibitor were removed from the digested sample by one passage through the column equilibrated with 0.5 mg $C_{12}E_8$/ml, 0.1 M NaCl, 20 mM Tes (pH 7.0) and 5 mM DTT. Tryptic peptides were then separated by a second passage in the presence of 1% $NaDodSO_4$ and 100 mM NaAcetate (pH 4.5). For detection of phosphorylation from [γ-^{32}P]ATP the first chromatography was omitted.

Amino acid sequences of the separated peptides were determined using an Applied Biosystems Model 470A gas phase Protein Sequencer, essentially as described by Hewick et al. [15]. PTH amino acids were analyzed by reverse-phase HPLC, using a Waters Nova-Pak column and the gradient elution system described in Waters Associates Applications Brief *M3500. A Waters HPLC system including two M510 pumps, a WISP 710B autoinjector, and a M440 dual channel absorbance detector was used. The detector was set to measure the sum of the absorbances at 254 nm (for quantitative measurement of PTH amino acids) and 313 nm (for qualitative detection of breakdown products of PTH-Ser and PTH-Thr). The recovery of PTH amino acids at each cycle was measured with an integrative recorder (Waters M730 Data Module). PTH-Ser and PTH-Thr were usually obtained in low yields and sometimes were not detectable at all; they could always be identified, however, from the appearance of breakdown peaks which absorbed at 313 nm. PTH-Arg and PTH-His were also often recovered in low yields.

Photolabeling with the hydrophobic carbene precursor [^{125}I]TID (Amersham, 10 Ci/mmol) followed the general procedure described by Hoppe et al. [17] for F_1F_0ATP synthase. Following equilibration of sarcoplasmic reticulum membranes (1 mg protein/ml) with [^{125}I]TID (1-2 μCi/ml) for 30 min at 20°C in the dark in 20 mM Tes (pH 7.0), 0.1 M NaCl, the sample was divided into four aliquots, which were further incubated for 4 hr in the dark in presence of either 0.2 mM $CaCl_2$, 5 mM $MgCl_2$, 0.8 mM CrATP (*A*), 0.2 mM $CaCl_2$, 5 mM $MgCl_2$ (*B*), 2.0 mM EGTA, 5 mM $MgCl_2$ (*C*) or 2.0 mM EGTA, 5 mM $MgCl_2$, 1.0 mM Na_3VO_4(50 μM in some experiments) (*D*). Photolysis of samples was carried out in a Perkin Elmer MPF 44A spectrofluorometer equipped with a 250 W Xenon lamp at 360 nm (bandwidth 20 nm) in a 1-cm quartz cuvette for 40 min (3-ml sample) or 15 min (1-ml sample) at 20°C. Time studies showed that these exposure times resulted in maximum incorporation of [^{125}I]TID in membrane components. In order to remove unbound radioactivity, the membranes were washed once in 20 mM Tes (pH 7.0), 0.1 M NaCl, 0.5 mM $CaCl_2$ in the presence of 1% albumin, and once in the same buffer without albumin. Following solubilization in $C_{12}E_8$, the labeled protein was separated from membrane lipids and lipid-bound label (approx. 90% of all bound label) by the previously described ionic exchange chromatography in the presence of $C_{12}E_8$ [3]. The eluate was analyzed for protein content by the Lowry method and for ^{125}I radioactivity by γ-scintillation counting. In some experiments [^{125}I]TID labeling of tryptic peptides was studied. The washed membranes were then digested for 15 min in 20 mM Tes (pH 7.0), 100 mM NaCl, 0.5 mM $CaCl_2$ at the same Ca-ATPase/trypsin concentration ratio as described above. The digested samples were solubilized by $C_{12}E_8$ and delipidated by ionic exchange chromatography in the presence of 5 mM DTT, and were then subjected to HPLC in $NaDodSO_4$.

The monomeric state of $C_{12}E_8$ solubilized Ca-ATPase was tested by large zone HPLC and analytical ultracentrifugation [4].

Results

We previously showed that E_1-E_2 conformational transitions in Ca-ATPase can be monitored by changes in tryptic cleavage pattern. However, localization of conformation-sensitive tryptic splits was ambiguous due to the presence of a number of secondary cleavage products with similar migration velocity on $NaDodSO_4$ polyacrylamide gels [1]. In order to identify optimum conditions for isolation of tryptic fragments for N-terminal amino acid sequence determination, we have studied tryptic cleavage in various buffer systems. Figure 1, lanes *1–4*, shows that the tryptic cleavage patterns of Ca-ATPase membranes at pH 7.0, 0.1 M NaCl are similar to those observed previously at pH 8.0, 0.03 M NaCl [1]. *I* and *II* indicate the two secondary cleavage products previously designated A_1' and A_1'', respectively. In the presence of Ca^{2+} to saturate high affinity binding sites, fragment *II* is formed at a higher rate than in the absence of Ca^{2+}. By contrast fragment *I* is produced approximately at the same rates with and without Ca^{2+}. A 22-kDa fragment (*III*) appears on the gel simultaneously with *II* and is not observed when *I* is present without *II*.

To distinguish these fragments, their ability to be phosphorylated from [γ-^{32}P]ATP was studied by gel permeation HPLC (TSK G 3000 SW column) in the presence of 1% $NaDodSO_4$ at pH 4.5 after tryptic cleavage for 1 and 20 min in the presence of Ca^{2+}. Elution patterns after phosphorylation and acid denaturation are shown in Fig. 2. Fragment *I* is present only in the initial phase of digestion. After 1 min a heterogeneous peak is observed corresponding to fragments *I*/*II*, whereas a homogenous peak (fragment *II*) is seen after 20 min digestion (*cf.* Fig. 1, lane *3*). ^{32}P radioactivity is associated exclusively with *II*. Therefore only this fragment and not *I* contains the phosphorylated Asp 351.

Fragment *I* can be accumulated by tryptic cleavage of detergent solubilized Ca-ATPase in the presence of vanadate at pH 8.0 and relatively low ionic strength (30 mM NaCl) (Fig. 1, lane *6*). By contrast, vanadate reduces the rate of formation of both *I* and *II* in membrane-bound Ca-ATPase [1] and in the soluble monomeric enzyme at pH 7.0, 0.1 M NaCl (Fig. 1, lane *5*). The cleavage pattern of the soluble enzyme in the presence of Ca^{2+} (E_1 form) is

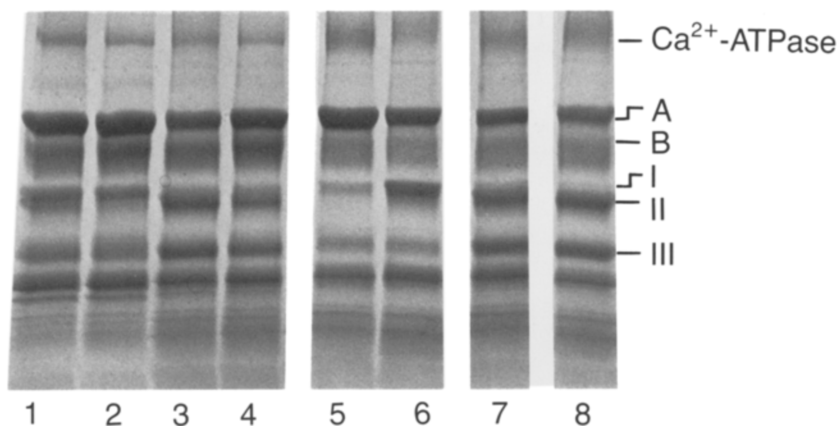

Fig. 1. Tryptic cleavage of Ca-ATPase in different ionic media. Lane *1*: Ca-ATPase membranes in 20 mM Tes (pH 7.0), 0.1 M NaCl, 0.5 mM $CaCl_2$, tryptic digestion for 1 min. Lane *2*: Ca-ATPase membranes in 20 mM Tes (pH 7.0), 0.1 M NaCl, 1.0 mM EGTA, tryptic digestion for 1 min. Lane *3*: as lane *1*, but tryptic digestion for 10 min. Lane *4*: as lane *2*, but tryptic digestion for 10 min. Lane *5*: soluble monomeric Ca-ATPase in 20 mM Tes (pH 7.0), 0.1 M NaCl, 1.0 mM EGTA, 5 mM $MgCl_2$, 0.5 mM Na_3VO_4, 5 mg $C_{12}E_8$/ml, tryptic digestion for 10 min. Lane *6*: soluble monomeric Ca-ATPase in 50 mM Tes/Tirs (pH 8.0), 0.03 M NaCl, 1.0 mM EGTA, 5 mM $MgCl_2$, 0.5 mM Na_3VO_4, 5 mg $C_{12}E_8$/ml, tryptic digestion for 10 min. Lane *7*: soluble monomeric Ca-ATPase in 20 mM Tes (pH 7.0), 0.1 M NaCl, 0.5 mM $CaCl_2$, 5 mg $C_{12}E_8$/ml, tryptic digestion for 10 min. Lane *8*: soluble monomeric Ca-ATPase in 50 mM Tes/Tris (pH 8.0), 0.03 M NaCl, 0.5 mM $CaCl_2$, 5 mg $C_{12}E_8$/ml, tryptic digestion for 10 min

little affected by pH and ionic strength (Fig. 1, lanes *7–8*). For preparation of either fragment *I* or *II* in high yield we used the conditions of Fig. 1, lanes *6* and *8*, respectively. To avoid contamination with trypsin and trypsin inhibitor the digested samples were passed through a HPLC column in the presence of $C_{12}E_8$. After this passage the collected Ca-ATPase peak was treated with 1% $NaDodSO_4$ to dissociate the tryptic fragments and then applied to another HPLC column equilibrated with $NaDodSO_4$. The elution patterns shown in Fig. 3 correspond to soluble Ca-ATPase digested for 30 min in presence of EGTA + Mg^{2+} + vanadate or in the presence of Ca^{2+}. The first peak contains both A and B chains, but in each case the following two peaks represent highly purified fragments (*I, II, III* and *IV*) as evidenced by gel electrophoresis of collected fractions (Fig. 4).

Sequence data obtained for fragments *I*, *II* and *IV* are shown in Table 1. These are in excellent agreement with the data for the complete amino acid sequence deduced by MacLennan and coworkers from the nucleotide sequence of cloned cDNA [6]. Our results indicate the assignment of the tryptic fragments shown in Fig. 5. *II* and *III* are identical to the A_1 and A_2 fragments found previously to be derived from the N-terminal half of the Ca-ATPase polypeptide chain by cleavage at Arg 198 (T_2 site) [6, 22]. Fragment *I* is derived from the C-terminal half (*B*) in accordance with the lack of phosphorylation of *I*. The secondary cleavage of *B* giving rise to fragment I (which we propose designated as B_1)

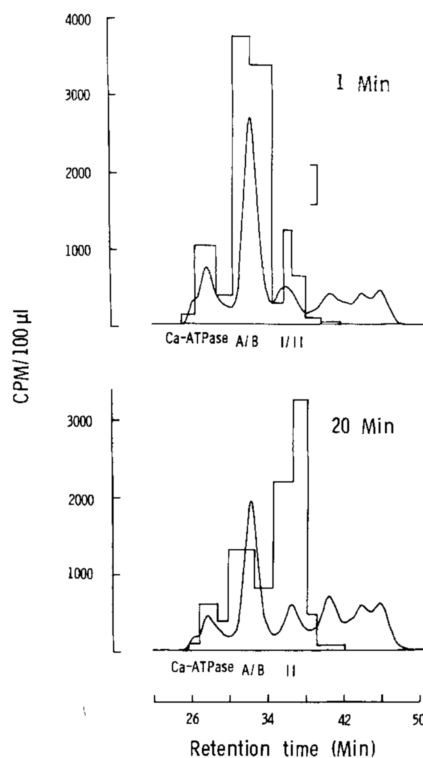

Fig. 2. Phosphorylation of tryptic peptides of Ca-ATPase from [γ-^{32}P]ATP. Ca-ATPase membranes were digested (1 or 20 min) and phosphorylated from [γ-^{32}P]ATP in the presence of 0.5 mM $CaCl_2$, 10 mM $MgCl_2$, 50 mM Tes/Tris (pH 8.0), 30 mM NaCl. After washing of acid denatured protein, 1% $NaDodSO_4$ was added, and 500 µl of the sample were injected into the HPLC column and eluted with 1% $NaDodSO_4$, 0.1 M Na acetate (pH 4.5). The absorbance, as monitored continuously at 226 nm, is shown together with radioactivity in eluted fractions (columns)

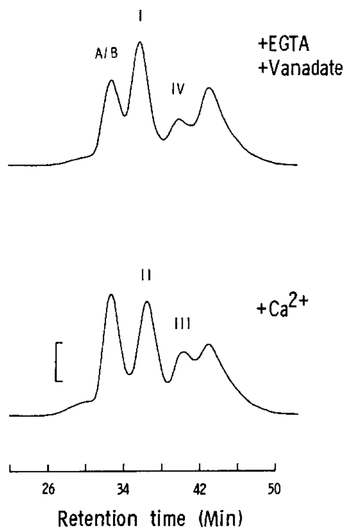

Fig. 3. Isolation of tryptic peptides of Ca-ATPase for N-terminal sequence determination. Ca-ATPase was solubilized by 5 mg $C_{12}E_8$/ml in 50 mM Tes/Tris (pH 8.0), 30 mM NaCl, 10 mM $MgCl_2$ and either 1.0 mM EGTA + 0.5 mM Na_3VO_4 or 0.5 mM $CaCl_2$, and digested for 30 min with trypsin. After addition of trypsin inhibitor the sample was subjected to two consecutive HPLC runs as described in Materials and Methods. The latter, conducted in presence of 1% $NaDodSO_4$, 100 mM Na acetate is shown. Peak fractions were collected and used for sequence determination and electrophoresis (Fig. 4)

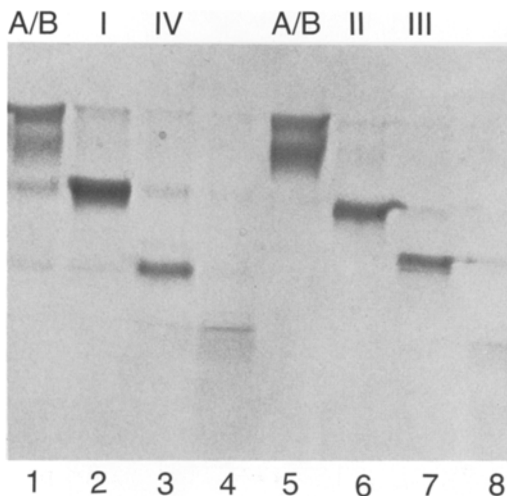

Fig. 4. Electrophoretic analysis of purity of tryptic fragments isolated by HPLC in $NaDodSO_4$. Lanes *1–4*: Peaks from the HPLC shown in the upper half of Fig. 3. Lanes *5–8*: Peaks from the HPLC shown in the lower half of Fig. 3

is localized at either Arg 819, Arg 822, Lys 825, or Arg 836, based on the molecular mass determined by $NaDodSO_4$ polyacrylamide gel electrophoresis. Since Arg 836 belongs to a putative transmembrane segment, the former three bonds are the more likely

Table 1. Sequence data for fragments of Ca-ATPase isolated after tryptic cleavage

I Residue	pmol	II Residue	pmol	IV Residue	pmol
Ala	174	Ala	77	Ser	37
Ala	169	Val	53	Leu	169
Val	199	Asn	58	Pro	80
Gly	133	Gln	74	Ser	n.q.
Asn	100	Asp	52	Val	111
Lys	131	Lys	58	Glu	76
Met	139	Lys	60	Thr	n.q.
Phe	137	Asn	52	Leu	126
Val	92	Met	40	Gly	88
Lys	126	Leu	57	Cys	n.q.
Gly	146	Phe	42	Thr	n.q.
Ala	106	Ser	n.q.	Ser	n.q.
Pro	113	Gly	36	Val	50
Glu	153	Thr	n.q.	Ile	44

Yields are expressed in pmol of PTH amino acids for each cycle determined by HPLC analysis as described in Materials and Methods. n.q., not quantified.

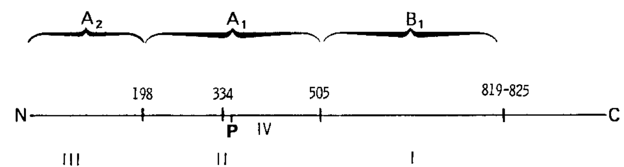

Fig. 5. Assignment of the tryptic fragments based on the sequence data (Table 1). The cleavage site at Arg 198 is protected in E_2 forms. The site in the C-terminal half of the *B* fragment is defined within the limits of Arg 819-Lys 825 (*see* text). The phosphorylated residue (Asp 351) is indicated by *P*

candidates as cleavage sites. Fragment *IV* is seen to be derived from *A* by a split close to the phosphorylated Asp 351. This split is only observed after prolonged incubation with trypsin at alkaline pH and low ionic strength in the presence of detergent, and it may represent partial denaturation of the protein.

The data of Fig. 1 suggest that complete protection of the T_2 cleavage site in absence of Ca^{2+} requires stabilizing binding of a ligand such as vanadate. In Fig. 6 we have examined the effect of inorganic phosphate, which reacts with E_2 to form E_2P at pH 7.0 in the presence of dimethylsulfoxide or glycerol [23]. Membrane-bound as well as soluble monomeric Ca-ATPase have been treated with trypsin for 15 min with and without phosphate or vanadate. During this relatively long incubation time appreciable amounts of A_1 and A_2 fragments accumulate in the absence of both Ca^{2+} and phosphate/vanadate. It is seen that phosphorylation with P_i provides an almost complete protection of the T_2 site for membrane-bound Ca-ATPase similar

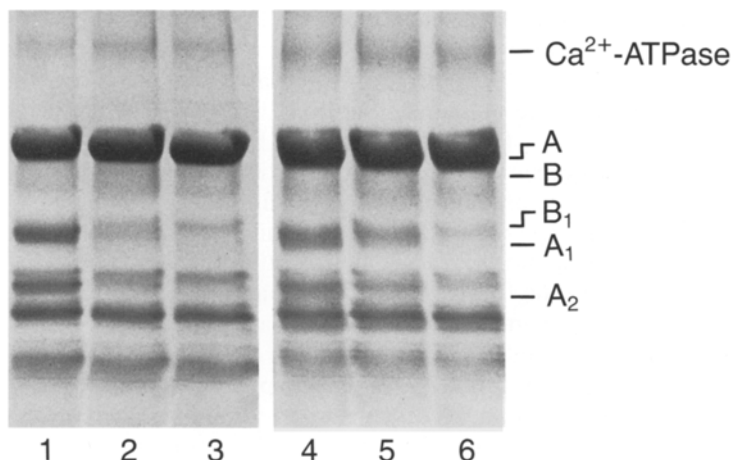

Fig. 6. Effect of phosphorylation with P_i on tryptic cleavage patterns. Lanes *1–3*: Ca-ATPase membranes in presence of 20% dimethylsulfoxide. Lanes *4–6*: soluble Ca-ATPase in presence of 5 mg $C_{12}E_8$/ml and 30% glycerol. In addition the media contained 100 mM Tes/Tris (pH 7.0), 2.0 mM EGTA, 10 mM $MgCl_2$ with 10 mM Tris phosphate (lanes *2* and *5*) or 0.1 mM Na_3VO_4 (lanes *3* and *6*). Tryptic digestion was performed for 15 min

to the protection induced by binding of vanadate. For soluble monomeric Ca-ATPase the unliganded E_2 form is unstable except in the presence of glycerol. Therefore we have used 30% glycerol to promote phosphorylation in this case. Large zone HPLC indicates that inclusion of this component in the medium does not affect the monomeric state of the protein. Phosphorylation with P_i is less complete in glycerol than in dimethylsulfoxide [23]. However, it is evident from Fig. 6 that the T_2 cleavage site on the phosphorylated soluble monomer is protected relative to the site on the unliganded soluble enzyme in agreement with other data showing that soluble monomeric Ca-ATPase undergoes the same conformation transitions as the membrane-bound enzyme during the catalytic cycle [2, 4, 9, 24, 27, 28].

The disappearance of the T_2 cleavage site in E_2P suggests that segments of the peptide chain in the N-terminal half are protected from the aqueous phase in this conformation. In order to see whether there is a redistribution of protein mass into the membrane phase we have labeled Ca-ATPase in sarcoplasmic reticulum membranes with the hydrophobic photoactivable reagent trifluoromethyl[^{125}I]iodophenyldiazirine (TID) under various conditions giving rise to different conformational states. In order to obtain a stable Ca^{2+}-occluded E_1P form the enzyme was phosphorylated for 4 hr with CrATP [28]. The tryptic cleavage pattern of this E_1P form is identical to that observed for E_1 (T_2 site exposed). Alternatively, a stable E_2 form was induced by binding of vanadate. It is evident from Table 2 that E_1 and E_1P forms of the peptide chain are less exposed to the label than E_2 and E_2V. The most significant difference is between E_1P and E_2V with 19% more labeling in E_2V. When 50 μM vanadate was used a

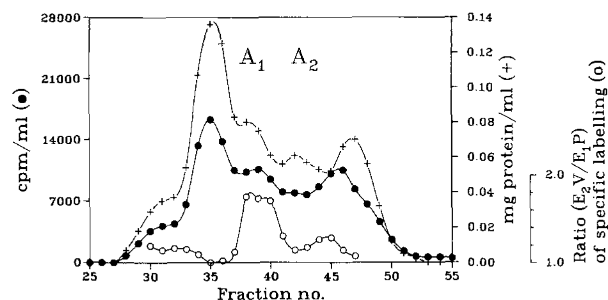

Fig. 7. Effect of E_1-E_2 transition on hydrophobic photolabeling of tryptic peptides of Ca-ATPase. Sarcoplasmic reticulum vesicles were labeled with [^{125}I]TID and further processed as described in Materials and Methods. One ml delipidated and digested protein was injected into the HPLC column. (+), protein concentration; (●), ^{125}I radioactivity in the sample labeled with [^{125}I]TID in the presence of vanadate; (○), ratio between specific labeling (counts per mg protein) in the two samples (E_2V relative to E_1P)

similar result was obtained as with 1.0 mM (121% labeling).

Figure 7 shows the labeling pattern with TID after digestion with trypsin and separation of the Ca-ATPase fragments by HPLC following the procedure in Fig. 2. The protein was labeled either in the E_1P form or in E_2V, and was digested in the presence of Ca^{2+} in both cases. The HPLC elution patterns of the two samples were identical. The one corresponding to labeling in presence of vanadate is shown in Fig. 7 together with the ratio of specific labeling calculated from the combined data of the two experiments. It is seen that the 19% relative increase in specific labeling of the total peptide chain observed for E_2V (Table 2) can be accounted for by a 70% increase in labeling of the A_1 fragment. By contrast the A_2 fragment does not show prefer-

Table 2. Reaction of [^{125}I]TID with various conformational states of Ca-ATPase in sarcoplasmic reticulum vesicles

Ligands present[a]		Predominant conformational state	Relative [^{125}I]labeling[b]
Ca^{2+} + CrATP	(*A*)	E_1P	100%
Ca^{2+}	(*B*)	E_1	104 ± 6 (n = 4)[c]
EGTA	(*C*)	E_2	114 ± 5 (n = 4)
EGTA + vanadate	(*D*)	E_2V	119 ± 4 (n = 6)

Photolabeling of sarcoplasmic reticulum with [^{125}I]TID and delipidation was performed as described in Materials and Methods.
[a] Letters in parentheses refer to the condition detailed in Materials and Methods.
[b] Calculated as percentage of the labeling in condition (*A*) (average of 6 experiments).
[c] Average ± SD.

ential labeling in E_2V. Neither is there any indication of preferential labeling of the *B* fragment in E_2V. This fragment is the major constituent of the peak eluting before A_1. A second peak of preferential labeling is seen after A_2 corresponding to smaller degradation products of A_1. In control experiments with undigested Ca-ATPase we observed no peak of radioactivity corresponding to presence of unbound label in the detergent micelles.

Discussion

The present results indicate that E_1-E_2 and E_1P-E_2P transitions in Ca-ATPase comprise the movement of several amino acid residues in the N-terminal half of the peptide chain from the aqueous phase into the bilayer. These conformational changes form the basis for active transport of Ca^{2+} across the sarcoplasmic reticulum membrane [10]. The tryptic cleavage data and sequence analysis localize the movement to residues close to Arg 198 ("T_2 cleavage site"). This peptide bond is invariably found to be more exposed to trypsin in E_1 states than in E_2 states, in spite of changes in pH and ionic strength, and of inclusion of non-ionic detergent in the medium to solubilize the protein in monomeric form. Tryptic cleavage sites at locations remote from Arg 198 (i.e., the T_1 site at Arg 505 and a bond between Arg 819 and Lys 825) are insensitive to E_1-E_2 transitions.

We have evidence from hydrophobic labeling that residues between Arg 198 and Arg 505 (A_1 fragment) are more exposed to the membrane lipid phase in E_2 states than in E_1 states. The applied label, [^{125}I]TID, has been shown to label intrinsic membrane proteins specifically at faces interacting with the lipid bilayer [7, 17].

Preferential labeling of E_2 with [^{125}I]TID was found to be most distinct in the presence of vanadate (19% increase in total labeling *vs.* 14% in the absence of ligand). Similarly the T_2 tryptic cleavage site is fully protected in E_2V and E_2P forms, whereas only partial protection is observed in the mere absence of Ca^{2+}. This finding suggests that E_1 and E_2 forms are in rapid equilibrium in the absence of ligand, exposing the tryptic cleavage site in E_1, and the [^{125}I]TID sites in E_2, and that after reaction with vanadate or P_i the E_2 state is stabilized. Vanadate and P_i also protect the bond at Arg 819-Lys 825, but this effect is very dependent on buffer composition and is not observed at all in unliganded E_2 forms. It may therefore represent a local change in surface charge following ligand binding.

Ca-ATPase is known to form bidimensional membrane crystals after incubation with high concentrations of vanadate [11, 16]. However, crystallization per se does not explain the present observations. Preferential hydrophobic labeling did not require a vanadate concentration in the millimolar range. Furthermore, the T_2 cleavage site is protected by phosphorylation with P_i in the presence of dimethylsulfoxide or detergent plus glycerol, which disrupt membrane crystals [11].

The data in Fig. 6 provides a clearcut demonstration of changes in cleavage pattern related to formation of E_2P in the soluble monomeric enzyme. This adds to the evidence that only a single peptide chain is required to carry out the intermediate reactions involved in Ca^{2+}-transport [2, 4, 9, 24, 27, 28]. Since the soluble monomeric Ca-ATPase is partially delipidated, it can also be concluded that the protection of the T_2 site does not require the presence of a lipid bilayer. The combined information obtained from tryptic cleavage and hydrophobic labeling therefore suggests a model in which part of the moving residues slide between fixed transmembrane helices, whereas another part becomes interposed between these helices and the membrane phospholipid. Such structure changes may increase the hydrophobicity of the phosphorylation site at Asp 351 [10] and at the same time expose binding sites for ions at the luminal surface.

Changes in tryptic cleavage pattern related to E_1-E_2 transitions have previously been demonstrated for the Na,K-ATPase protein [18]. In this enzyme a cleavage site ("bond 3") with a similar location as the T_2 site in Ca-ATPase relative to the transmembrane segments is exposed in E_1 and protected in E_2. In addition an increased labeling with the hydrophobic reagent INA (^{125}I-iodonaphthylazide) has been observed in the E_2 state of the Na, K-pump [19]. Measurements of circular dichroism suggest that E_1-E_2 transitions in Na,K-ATPase are accompanied by large changes in α-helix content

[12]. For Ca-ATPase there is no evidence in favor of a change in secondary structure [8, 25]. Therefore, we consider it likely that the present results reflect mainly redistribution of whole peptide segments between the cytoplasmic surface and the bilayer.

The minimum number of residues involved in the conformational change can be estimated from the observed increase in hydrophobic labeling. If all bound label in E_1 is assumed to be distributed on the ten transmembrane segments proposed by MacLennan et al. [21], the increase in labeling accompanying E_1-E_2 transitions corresponds to a net movement of 38 residues into the bilayer. However, if as much as 40% of Ca-ATPase protein is located in the lipid hydrocarbon phase in the E_1 state, as suggested by combined X-ray and neutron diffraction data [14], the 19% increase in hydrophobic labeling corresponds to movement of as much as 76 residues.

Ultrastructural studies of sarcoplasmic reticulum are in accordance with the present observations in showing a deeper penetration of Ca-ATPase protein into the inner leaflet of the bilayer after stabilization of E_2 with vanadate [16, 26]. Blasie and coworkers on the other hand have concluded that formation of E_1P by phosphorylation from ATP is accompanied by movement of 8% of the peptide mass from the cytoplasmic surface into the lipid phase [6]. According to the present observations the main structural change is related to the E_1P-E_2P transition rather than to the phosphorylation reaction. We have demonstrated that phosphorylation by CrATP, which gives rise to a stable Ca^{2+} occluded E_1P form [28], does not increase the exposure of the peptide to hydrophobic labeling. Nor does it protect tryptic cleavage sites. It is, however, possible that Ca^{2+} occlusion in E_1P results from limited structural changes which escape detection by the present means. More detailed studies of the hydrophobic labeling pattern with assignment of bound radioactivity to individual amino acid residues may provide an important tool in future studies of structure changes in Ca-ATPase.

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