Reversible Inactivation of the Sarcoplasmic Reticulum Ca^{2+}-ATPase Coupled to Rearrangement of Cytoplasmic Protein Domains As Revealed by Changes in Trypsinization Pattern†

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Received May 24, 1994; Revised Manuscript Received August 11, 1994®

ABSTRACT: Repetitive homogenization of skeletal muscle sarcoplasmic reticulum (SR) membranes in the presence of chelating agents at low ionic strength leads to the loss of the Ca-ATPase activity. This inactive state of the enzyme is coupled to an extensive rearrangement of the cytosolic domains as visualized by a completely different trypsinization pattern of the enzyme. In addition to the primary cleavage site (Arg 505), a novel trypsinization site (Arg 334), just N-terminal of the phosphorylation domain and localized on the primary tryptic fragment A, becomes exposed. Cleavage at the latter site yields a soluble fragment of M_r 20 117 and the membrane-bound N-terminal one-third of the ATPase of M_r 35 279. Two additional trypsinization sites C-terminal of the nucleotide binding domain become exposed in the inactive Ca^{2+}-ATPase conformation. Rapid cleavage at these sites yields two soluble fragments of about 15 and 10 kDa. All together, the three soluble fragments comprise most of the large cytosolic loop of the Ca^{2+}-ATPase. The inactivation and the change in trypsinization pattern can be reversed by rehomogenization of the extracted membranes in the presence of divalent cations. The results suggest the presence of an occluded site for divalent cations which can be depleted or refilled during application of sheer forces. Occupation of this site is essential to confer to the enzyme an active conformation.

Trypsinization of the Ca^{2+}-ATPase of skeletal muscle sarcoplasmic reticulum (SR)[1] has proven to be a useful tool for structural and functional studies, since the accessibility of the proteolytic cleavage sites reflects changes in conformation of the enzyme (Andersen & Jorgensen, 1985; Martonosi et al., 1987). Controlled trypsinization of the SR Ca^{2+}-ATPase in the presence of high concentrations of sucrose results in the rapid cleavage of a single site (T1) exposed to the cytosolic side of the membrane (Arg 505), thus giving rise to the formation of two fragments with M_r of 55 376 (fragment A) and 54 973 (fragment B) (Thorley Lawson & Green, 1975; Stewart & Maclennan, 1976). A second trypsinization site (T2), localized on the A fragment (Arg 198), becomes exposed only in the presence of Ca^{2+} ions at concentrations sufficient to saturate the high-affinity Ca^{2+} binding sites of the Ca^{2+}-ATPase (Thorley Lawson & Green, 1975; Stewart & MacLennan, 1976). Cleavage at site T2 yields subfragments A1 (residues 199−505) and A2 (residues 1−198), which display a molecular weight of 33 279 and 22 115, respectively. The cleavage at the T1 site is usually complete while only about 50% of the resulting A fragment is further split into A1 and A2 (Torok et al. 1988). The overall protein structure of the ATPase is well preserved after digestion as judged by the maintained ability of the doubly cleaved enzyme to translocate Ca^{2+} ions across the SR membrane even though at a reduced rate (Torok et al., 1988). To achieve further degradation of the ATPase, harsher conditions must be used: for instance, digestion at elevated temperature leads to the further digestion of the A1 fragment as well as to the cleavage of the extramembranal portion of the B fragment (Imamura & Kawakita, 1986, 1989; Saito et al., 1984). Alternatively, the use of elevated pressure during trypsinization induces a faster degradation of all primary fragments (Ronzani et al., 1990). While both conditions accelerate the rate of degradation of the Ca^{2+}-ATPase, none of them changes appreciably the original principal pattern of digestion.

In this paper, we characterized an altered pattern of ATPase trypsinization which can be induced simply by extraction of the SR membranes with EDTA combined with shearing forces. The change in trypsinization pattern was reversible and correlated with an inactive conformation of the enzyme.

MATERIALS AND METHODS

SR Preparation. SR membranes (the intermediate fraction) were prepared from white muscles of rabbit hind legs according to Eletr and Inesi (1972). The intermediate SR fraction (a mixture of longitudinal and cisternal systems) was homogenized in 20 mM Hepes, pH 7.2, and 0.25 M sucrose, frozen in liquid nitrogen, and stored at −70 °C until used.

Extraction and Reconstitution of SR Vesicles. If not stated otherwise, the extraction procedure comprised a 0.5 h incubation of the SR vesicles at a concentration of 2−3 mg/

† N.M. was a recipient of a Visiting Professor Fellowship from the Swiss Federal institute of Technology (1993).

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® Abstract published in *Advance ACS Abstracts,* October 15, 1994.

[1] Abbreviations: SR, sarcoplasmic reticulum; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid.

0006-2960/94/0433-13678$04.50/0

mL in 20 mM imidazole, pH 7.0−8.0, and 1 mM EDTA at room temperature combined with repeated homogenization (Potter homogenizer). Membrane-bound and extracted materials were subsequently separated by centrifugation at 100000*g*/1 h. The pellet was then resuspended in 20 mM imidazole, pH 7.0, and 0.25 M sucrose at a concentration of 10 mg/mL. Reconstituted membranes were obtained by supplementing the extracted membranes with various concentrations of divalent cations and thorough homogenization (Potter homogenizer). Some SR preparations were treated also with Affi Gel blue essentially according to Papp et al. (1986) to extract some additional accessory proteins such as phosphorylase (30 μg of Affi Gel blue/mg of protein).

Trypsin Digestion. Digestion with trypsin was performed essentially as previously described (Klip et al., 180) at room temperature in a 20 mM Tris-HCl buffer, pH 7.0, containing 100 mM KCl, 5 mM $MgCl_2$, 1 M sucrose, and either 2 mM $CaCl_2$ or 2 mM EGTA. Protein concentration was 2 mg/mL. If not stated otherwise, 1 h digestion with a 1/100 trypsin to protein ratio was performed.

Electrophoresis. SDS−PAGE on 10% or 12% slab gels was carried out according to Laemmli (1970). The Coomassie blue stained patterns were evaluated using an LKB 2202 Ultroscan laser densitometer coupled to an LKB 2221 integrator. Standard curves of ln(molecular weight) as a function of relative mobility were used to estimate the molecular weight of the proteins. The accuracy of such estimations was within 10%, based on predicted cleavage sites within the primary structure.

Ca^{2+} Uptake. The activity of the Ca^{2+}-pumping ATPase was measured at 37 °C using the Millipore filtration technique and ^{45}Ca as described (Chiesi & Inesi, 1979).

Ca^{2+}-ATPase Measurements. The Ca^{2+}-dependent ATPase activity was measured at 37 °C in a coupled enzyme assay (Hardwicke & Green, 1974). The reaction medium was composed of 100 mM KCl, 10 mM MOPS, pH 7, 2 mM $MgCl_2$, 1 mM EGTA, 0.5 mM Tris−ATP, 0.2 mM NADH, 3 mM phosphoenolpyruvate, and 1 unit/mL each of pyruvate kinase and lactate dehydrogenase. $CaCl_2$ was added to yield the desired free Ca^{2+} concentration. The reaction was started by adding SR membranes (about 50 μg/mL). The Ca^{2+} ionophore A23187 was always present in the medium (10 μM) to ensure linear reaction rates and to avoid complications due to changes in leakiness of the membranes to Ca^{2+}.

Phosphoenzyme Formation. SR membranes (0.2 mg/mL) were phosphorylated at 0 °C for 10 s in a buffer containing 20 mM MOPS, pH 7.0, 80 mM KCl, 5 mM $MgCl_2$, 0.1 mM $CaCl_2$ (or 1 mM EGTA), and 0.01 mM [γ-^{32}P]ATP (specific radioactivity 0.2 mCi/mmol) to a final volume of 0.1 mL. The reaction was stopped by the addition of 1 mL of a solution containing cold 7% TCA and 0.25 mM KP_i. The denatured proteins were collected by centrifugation and analyzed on acidic Weber and Osborne 6% gels and a running buffer adjusted at pH 6.3. Radioactivity was detected by autoradiography and counting of the radioactive bands.

Amino Acid Sequence Analysis. After trypsinization, SR proteins were separated by 12% Laemmli SDS−PAGE. The peptides from unfixed and unstained SDS−polyacrylamide gel were transferred electrophoretically onto poly(vinylidene difluoride) membranes (Millipore) according to Matsudaira (1987) for 4 h at 320 mA constant current and 4 °C in 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid, 10% methanol, pH 11, and stained with Coomassie blue. N-Terminal amino acid sequencing of excised peptide bands was performed using an Applied Biosystems 473A sequencer with a 120A on-line phenylthiohydantoin analyzer. The quantity of a given peptide was estimated from the average elevation above the background of the amino acid in each cycle (except for the first one).

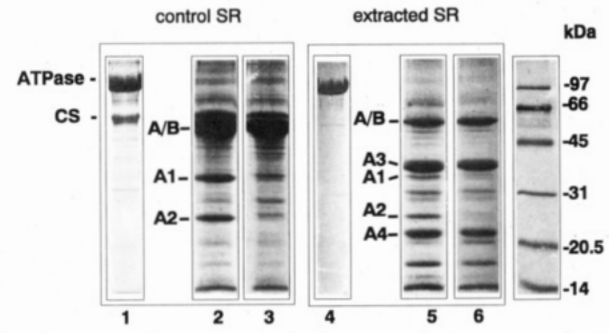

FIGURE 1: Trypsinization pattern of control and extracted SR. SR membranes were extracted in the presence of 1 mM EDTA at pH 8.0 and subjected to trypsinization in the presence of 1 M sucrose as described under Materials and Methods. The protein pattern was then analyzed by SDS−PAGE. Lanes 1−3, control SR membranes; lanes 4−6, extracted SR membranes. Lanes 1 and 4, before trypsinization. Lanes 2 and 5, after 1 h trypsinization in the presence of Ca^{2+}. Lanes 3 and 6, after trypsinization in the absence of Ca^{2+}. CS, calsequestrin; A/B, primary tryptic fragments; A1, A2, A3, and A4, secondary tryptic fragments deriving from A.

RESULTS

Novel Trypsinization Pattern of the SR Ca^{2+}-ATPase. As reported by many laboratories, the controlled trypsinization of the SR Ca^{2+}-ATPase in the presence of high concentrations of sucrose resulted in the rapid formation of the two primary fragments A and B of M_r 55 376 and 54 973, respectively. These primary fragments were not resolved by normal Laemmli SDS−PAGE and comigrated with an apparent molecular weight of about 50 000 (just below the band of calsequestrin as shown in Figure 1). The second trypsinization site (i.e., T2) became exposed in the presence of Ca^{2+} ions and gave rise to subfragments A1 and A2 displaying molecular weights of 33 279 and 22 115, respectively (see Figure 1, lane 2).

When SR membranes were extracted at pH 8.0 in the presence of 1 mM EDTA (Figure 1, lane 4, shows that the procedure extracted most of calsequestrin) and subsequently trypsinized, the digestion pattern was significantly changed. In the presence of Ca^{2+} ions, the most remarkable difference was the appearance of two novel major fragments of apparent molecular masses of about 35 and 20 kDa in addition to A1 and A2 (see Figure 1, lane 5). The difference was even more pronounced when the digestion was carried out in the presence of 2 mM EGTA, i.e., when the T2 site was not accessible to trypsin and hence the A1 and A2 fragments were not generated (Figure 1, lane 6). The unusual 35 and 20 kDa fragments were blotted after separation by SDS−PAGE, and their amino acid sequence was analyzed. The N-terminus of the 35 kDa fragment was found to be blocked, indicating that it corresponded to the acetylated N-terminus of the Ca^{2+}-ATPase. The amino acid sequence of the 20 kDa fragment was found to be SerLeuProSerValGluThr-Leu..., which is in agreement with that following arginine

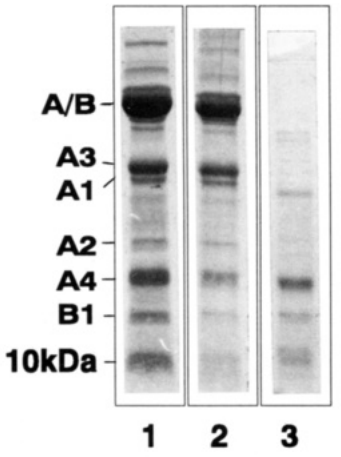

FIGURE 2: Solubility characteristics of tryptic fragments. SR membranes were extracted in the presence of 1 mM EDTA at pH 7 and then trypsinized in the presence of 2 mM $CaCl_2$. The suspension was then centrifuged at 100000*g* for 1 h and the protein composition analyzed by SDS−PAGE (12% gels). Lane 1, trypsinized SR before centrifugation. Lanes 2 and 3 represent the pellet and the supernatant after centrifugation, respectively.

334 (see Figure 5), according to the established amino acid sequence of the fast-twitch skeletal SR Ca^{2+}-ATPase (SERCA1) (Brandl et al., 1986). Thus, these two fragments resulted from the alternative cleavage of the primary A fragment of the Ca^{2+}-ATPase at Arg 334. Accordingly, we will refer to this novel cleavage site as T3 and to the resulting fragments as A3 (residues 1−334; M_r 35 279) and A4 (residues 334−505; M_r 20 117). As can be seen from Figure 1 (lanes 5 and 6), cleavage at Arg 334, in contrast to cleavage at Arg 198, was completely Ca^{2+}-independent. Mapping of the novel trypsin cleavage site onto the predicted secondary structure of the Ca^{2+}-ATPase molecule (see Figure 5) shows that only the larger A3 fragment is anchored to the membrane (it comprises the first four N-terminal intramembrane domains) while the smaller A4 fragment (which contains the phosphorylation domain of the ATPase) is not membrane-bound. Figure 2 shows that, as expected, when the EDTA-extracted SR preparation was centrifuged after tryptic digestion, the A4 fragment was released into the supernatant.

Another characteristic feature of Ca^{2+}-ATPase digestion in extracted SR membrane preparations was a faster disappearance of the B fragment as compared to untreated membranes. Comparison of lanes 2, 3 and lanes 5, 6 in Figure 1 shows that the band corresponding to the mixture of A and B fragments was more readily degraded in the case of the extracted SR membranes. Since degradation of the A band was similar in both control and extracted SR (compare the yield of A1, A2 in lane 2 and of A3, A4 fragments in lane 6 of Figure 1), a faster digestion of the B fragment must have been responsible for the decrease in intensity of the A/B protein band on the gel. The increased lability of the B fragment was further demonstrated by the formation of smaller molecular mass fragments displaying apparent molecular masses of about 15 kDa (Figure 1, lanes 5, 6, and Figure 2, lane 1) and 10 kDa (Figure 2, lane 1). The N-terminal sequence of the 15 kDa fragment was also analyzed and shown to be AlaAlaValGlyAsnArgMetPheVal..., which is, in fact, identical to that following residue Arg 505 (i.e., the N-terminal portion of the primary B fragment). The yield of the 15 kDa fragment estimated from N-terminal sequence analysis (80 pmol) was very similar to that of fragment A4 isolated from the same hydrolysate (60 pmol). The 15 kDa fragment, to which we will refer to as B1, and the small 10 kDa fragment are lost in the supernatant after centrifugation (see Figure 2).

Extractions of SR Membranes Leading to the Altered Tryptic Pattern. Treatment of SR membranes with EDTA at alkaline pH has long been known to result in the removal of peripheral membrane proteins (Duggan & Martonosi, 1970). Since we have observed that upon such treatment the pattern of tryptic digestion of the SR ATPase was dramatically altered, we compared the effect of various extraction conditions on the subsequent trypsinization. We found that the extraction of membranes with 1 mM EDTA at neutral pH or extraction at pH 8.0 without EDTA was already sufficient to evoke the change in the pattern of ATPase trypsinization provided that the membranes were extensively homogenized before centrifugation. The effect (as judged by the relative amount of the alternative A3, A4 fragments versus that of the common A1, A2 fragments) was more pronounced when both EDTA and elevated pH treatment were used, or when the extraction was repeated several times. The use of equimolar concentrations of EGTA in place of EDTA yielded identical results. Shearing forces were necessary for the change to occur since omission of the homogenization steps during extraction resulted in no (or only a minimal) change in the trypsinization pattern. Homogenization itself was found to be necessary but not sufficient to induce the change in trypsinization pattern. Treatment of SR vesicles with Affi Gel blue is also known to remove extrinsic proteins normally contaminating the SR preparations such as phosphorylase (Papp et al., 1986). Trypsinization of Affi Gel blue-treated SR membranes yielded a normal digestion pattern.

Effect of Extraction on the Function of the Ca^{2+}-ATPase. The cleavage of the Ca^{2+}-ATPase at the T1 and T2 sites yielded fragments containing transmembrane domains (i.e., A1, A2, and B) and the Ca^{2+}-ATPase-preserved high degree of integrity, as revealed by its maintained ability to form in a Ca^{2+}-dependent way a phosphorylated intermediate (not shown). In fact, the enzyme is still capable of transporting Ca^{2+} ions even though at a reduced rate (Torok et al., 1988). The extraction procedures which changed the trypsinization pattern of the Ca^{2+}-ATPase, on the other hand, induced a complete loss of the Ca^{2+}-transport activity of the SR membranes. Some residual Ca^{2+}-dependent ATPase activity could still be measured, indicating that EDTA treatment rendered the SR membrane leaky to Ca^{2+} ions. The hydrolytic activity, therefore, was measured in the presence of a Ca^{2+} ionophore to eliminate complications due to variations in the Ca^{2+} permeability of the membranes. When SR membranes were repeatedly homogenized and extracted in the presence of 1 mM EDTA at pH 7, the Ca^{2+}-dependent ATPase activity was reduced to 31 ± 10% (mean ± SEM using three different SR preparations) of that of control SR membranes (which were treated following an identical protocol but in the absence of EDTA). To get more information on the inhibition of the ATPase reaction, the ability of the extracted membranes to form the phosphorylated high-energy intermediate of the ATPase was investigated. The steady-state level of Ca^{2+}-dependent phosphoenzyme was reduced to about one-third in the extracted vesicles. Figure 3 shows that the amount of acyl phosphate intermediate associated with the ATPase of extracted mem-

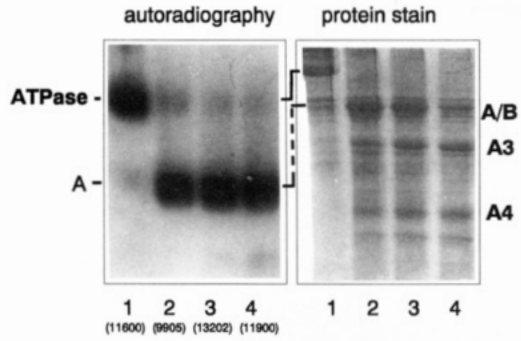

FIGURE 3: Acyl phosphate intermediate formation by extracted, trypsinized SR membranes. SR membranes were extracted in the presence of EDTA (lane 1) and then trypsinized for various times (5, 15, and 60 min corresponding to lanes 2, 3, and 4, respectively) in the presence of EGTA to protect site T2 and avoid formation of the A1 and A2 fragments. The steady-state levels of the Ca^{2+}-dependent phosphoenzyme intermediate were then measured in the various fractions using radioactively labeled [γ-^{32}P]ATP and the acidic Weber Osborne gel system as described under Materials and Methods. The radioactive protein bands were cut out of the gel and counted (the cpm values are given in parentheses). For protein staining, the same samples were separated by normal Laemmli SDS−PAGE and stained with Coomassie blue.

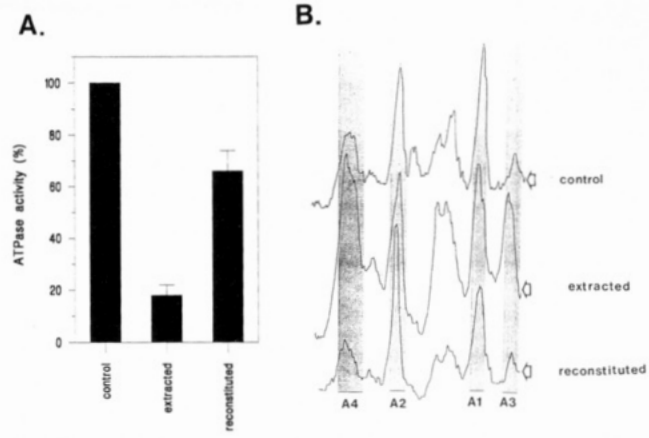

FIGURE 4: Restoration of the ATPase activity of extracted SR membranes. (A) Ca-dependent ATPase activity was measured by the coupled enzyme assay in the presence of a Ca^{2+} ionophore as described under Materials and Methods. Extracted: SR membranes were extracted by homogenization in the presence of EDTA. Reconstituted: extracted SR membranes were homogenized in the presence of 5 mM $MgCl_2$ before ATPase measurement. Details are given under Materials and Methods. (B) The same membranal fractions as described in panel A were trypsinized under standard conditions in the presence of Ca^{2+} ions and separated by 10% SDS−PAGE. Densitometric scans of the gel regions ranging from 18 to 40 kDa are presented.

branes was the same as that associated with fragment A obtained after extensive trypsinization (see quantitation of radioactivity of lanes 1 and 2−4). Trypsinization was carried out in the presence of EGTA. Under such conditions, digestion of normal ATPase molecules stops at the level of the primary fragments A and B while ATPase molecules exposing the cleavage site T3 are further digested down to A3 and A4 (see Figure 1). Hence, Figure 3 provides evidence for the presence of two populations of Ca^{2+}-ATPase molecules in the EDTA-treated SR membranes: A portion of ATPase molecules was resistant to the extraction procedure, did not expose the novel trypsinization site T3, and was capable of normal acyl phosphate intermediate formation. The population of ATPase molecules which was modified by EDTA treatment exposed the novel T3 trypsinization site and was incapable of Ca^{2+}-dependent phosphoenzyme formation.

Reversal of Ca^{2+}-ATPase Inactivation and of the Change in Trypsinization Pattern. With the following experiments, we investigated whether the change in trypsinization pattern induced by extraction of the membranes with EDTA was reversible. To this purpose, SR membranes were first extracted by repeated homogenization in the presence of EDTA, pH 7.0. A portion of the extracted vesicles was then supplemented with 5 mM $MgCl_2$, and the mixture was again extensively homogenized and centrifuged (reconstituted membranes). Control, extracted, and reconstituted SR membranes were then submitted to 1 h digestion with trypsin in the presence of 2 mM Ca^{2+}. A densitometric analysis of the gel region containing the A1−A4 fragments is shown in Figure 4B. (As already shown in Figure 1, such cleavage conditions produced mainly the A1 and A2 fragments from control SR membranes, while after extraction the two novel additional tryptic fragments A3 and A4 were formed.) The reconstitution treatment evoked a reversal of the change in digestion pattern of the ATPase. In this particular experiment, the presence of prominent bands in the region of about 30 kDa was apparent in the extracted membranes. These bands, which probably derive from the B fragment together with B1 and the 10 kDa fragment, were not always clearly distinguishable and appeared sometimes as a smear on the gel (see for instance Figure 2).

The reversal of the change in structure induced by the reconstitution procedure was paralleled by a partial recovery of the ATPase activity as shown in Figure 4A. Simple homogenization of the extracted SR membranes in the presence of $MgCl_2$ was sufficient to achieve a consistent restoration of the ATPase activity. Partial reversal of the inhibition was already apparent at concentrations above 20 μM and was nearly maximal at 100 μM (not shown). Similar results obtained by using similar concentrations of Ca^{2+} ions. The results clearly showed that the effect on ATPase conformation evoked by treatment of the membranes with ion chelating agent could be reversed, at least in part, by homogenization in the presence of divalent cations.

DISCUSSION

Functional Consequences of the Extraction Procedure. Cleavage of the SR ATPase at the tryptic site T3 and formation of the soluble tryptic fragment A4 have been previously reported (Andersen et al., 1986). The A4 fragment, however, could be detected in very small amounts and only after prolonged incubation with trypsin at alkaline pH and in the presence of detergents. The authors proposed that this might represent partial denaturation of the protein under those drastic proteolytic conditions. In this study, it is shown that a simple extraction procedure is sufficient to expose this alternative trypsinization site (Arg 335) on the surface of the Ca^{2+}-ATPase of SR membranes, giving rise to the formation of stoichiometric amounts of the alternative fragments A3 and A4. This indicates either that the treatment induces a conformational change of the Ca^{2+}-ATPase or that it removes an extrinsic protein normally interacting with the Ca^{2+}-ATPase and protecting the trypsinization site. The latter possibility is rather unlikely. The postulated protein should be stoichiometrically related to the ATPase and thus represent a major component of the SR membrane. No such

protein could be identified in the supernatant after extraction of the SR membranes. The fact that the change in trypsinization pattern can be reversed by simple readdition of divalent cations to the extracted membranes, on the other hand, supports the hypothesis of a conformational change of the ATPase coupled to a rearrangement of the cytosolic domains. The conformation of the ATPase which favors cleavage at Arg 334 is functionally incompetent. The Ca^{2+}-dependent hydrolytic activity and even the formation of the acyl phosphate intermediate are blocked. At the moment, it is not established whether the ATPase still maintains the capability to bind Ca^{2+} to the transport sites. The changes in conformation responsible for the inactivation of the enzyme are reversed by readdition of divalent cations in the high micromolar range. It is noteworthy that removal or readdition of this special set of divalent cations occurs only in combination of shearing forces (such as repetitive homogenization of the SR membranes). It is possible, therefore, that divalent cations (Ca^{2+} and/or Mg^{2+} ions) are bound to an occluded site deeply buried within the ATPase to which EDTA can have access only during mechanical stress. Occupancy of this site is an absolute necessity for maintaining an active conformation of the enzyme. The results, however, could also mean that only the metal-free form of the ATPase is vulnerable to shearing forces.

Assignment of the Tryptic Fragments. The fragments A3 and A4 clearly derive from cleavage of the primary A fragment at site T3 (Arg 334). According to N-terminal amino acid sequence data, the starting point of fragment B1 is Ala 506. Considering its apparent molecular mass of 15 kDa and position in the primary structure, fragment B1 most probably extends to Arg 638 or 639 (in the latter case it would display a molecular weight of 14 660). Since the sum of fragments A3 and A4 represents the entire primary fragment A, it is obvious that the short fragment of 10 kDa cannot be a product of further tryptic cleavage of A, A3, or A4 and, therefore, must originate from the primary fragment B. Hence, we will refer to its as fragment B2. The primary fragment B is composed of two very different moieties: the C-terminal halve consists of a large cytoplasmic loop of about 25 kDa comprising the ATP binding and hinge domains, and an intramembrane bundle of α-helical segments of about 30 kDa. The clear hydrophilic character of B2 indicates that it originates, as B1, from the cytoplasmic loop. B2 cannot represent the same region as B1, since the yield of the latter is similar to that of A4. Therefore, the most probable origin of B2 is the C-terminal portion of the large cytoplasmic loop (a very preliminary assignment of B2 to the region 639−728 could be done). The fate of the residual membrane-bound portion of the primary fragment B remains unknown and is the subject of further study. According to the most recent topological models, this part of the polypeptide chain (which we will name B3) forms two or three hairpin structures connected by very short cytoplasmic loops (MacLennan & Toyofuku, 1992; Sachs et al., 1992). To achieve trypsin cleavage at those points, a very high enzyme to ATPase ratio (1/4) must be applied (Sachs et al., 1992). It seems unlikely that this type of protein fragmentation could take place under the mild conditions of trypsinization used in the present study. In addition, fragment Thr729−Gly994 was found to be stable after prolonged tryptic digestion of SR vesicles (Imamura & Kawakita, 1986), and even in the case of a 2 h treatment of SR by the nonselective proteinase

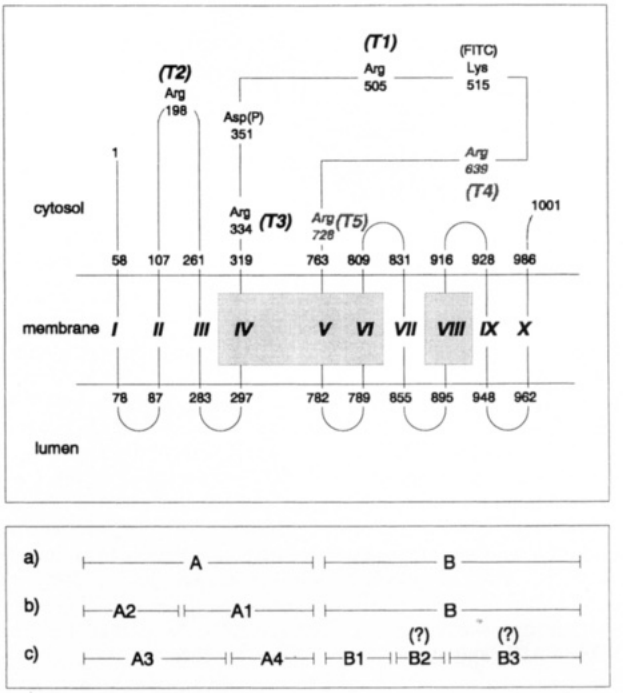

FIGURE 5: Topology of the Ca^{2+}-ATPase and trypsinization pattern. The model, which is based on that of Clarke et al. (1989a), shows the established (T1, T2, T3) and postulated (shaded T4 and T5) trypsinization sites on the SR Ca^{2+}-ATPase. The region between digestion sites T1 and T3 represents the phosphorylation domain; that between T1 and T4 is the nucleotide binding domain. The shaded areas within the membrane denote regions involved in Ca^{2+} binding. (a) The primary fragments A and B (molecular weights of 55 376 and 54 973) are obtained after cleavage at the T1 site. (b) After occupation of the transport sites with Ca^{2+} ions, the cleavage site T2 becomes exposed and yields subfragments A1 and A2 (molecular weights of 33 279 and 22 115). (c) After inactivation by EDTA treatment site T3 becomes available to trypsin so that subfragments A3 and A4 (molecular weights of 20 117 and 35 279) are obtained. Sites T4 and T5 on the B fragment also become available, yielding fragment B1 and putative fragments B2 and B3 of about 10 and 30 kDa, respectively.

K, a stable C-terminal fragment of about 30 kDa was identified by specific antibodies (Matthews et al., 1990). Therefore, it could be proposed that the protein band of about 30 kDa, which was sometimes apprent in the hydrolysate of extracted membrane, might represent B3. The band was not always clearly distinguishable and appeared sometimes as a smear on the gel. In this respect, one should note that Coomassie is often unable to stain peptides thought to be membrane-embedded (Sachs et al., 1992).

Theoretically, up to 87 fragments would be produced by full trypsinization of the cytoplasmic loops of the ATPase exposed on the surface of the SR membranes. In the extracted SR membranes, trypsin cleavage is very efficient but restricted exclusively to sites T1, T3, T4, and T5, indicating that these few target peptide bonds are freely exposed to the cytoplasm while the rest of the cytosolic portion of the protein must form globular domains. This finding could reflect, at least partially, the characteristics of the real domain organization of the cytoplasmic moiety of the ATPase. The trypsin cleavage sites (defined and predicted) shown in Figure 5 might be considered as markers of the boundaries between structural and functional domains. The site T3 is located at the beginning of the catalytic phosphorylation domain (328−505) and very close to the C-terminal end of the stalk stretch following transmembrane

segment IV. The T1 site has always been considered as a boundary between the phosphorylation and nucleotide binding domains (MacLennan & Toyofuku, 1992). The C-terminal end of the ATP binding region is thought to be located close to the expected T4 site (638 or 639). Interestingly, the proposed location of T4 is in close proximity to the epitope (657−672) for the monoclonal antibody A52 which was clearly indentified as exposed on the surface of the Ca^{2+}-ATPase (Clarke et al., 1989b). Presumably only a very small rearrangement within this portion of the ATPase is required to make the T4 site accessible for trypsin attack. The hypothetical site T5 (728) is expected to mark the boundary between the hinge domain and the stalk region preceding membrane segment V (Clarke et al., 1989a). This highly restricted character of the trypsinization pattern clearly demonstrates that the extraction procedure does not lead to an unfolding of the cytoplasmic functional domains but somehow slightly disrupted their interaction, thus resulting in the exposure of new trypsin-sensitive peptide bonds to the cytoplasm.

Implications. Trypsinization of extracted SR membranes results in an almost complete removal of the ATP binding and hydrolyzing domains from the large cytoplasmic loop of the Ca-ATPase molecule while leaving entire the intramembrane moiety as well as the cytoplasmic loop between the second and third transmembrane segments. It is likely, therefore, that the high-affinity Ca binding site located near the middle of the intramembrane domain (Clarke et al., 1989a) remains functionally competent. If so, the described approach opens the interesting perspective of studying the properties of the cation binding (and ion-transporting) site while it is physically separated from the other functional domains. Promise for that comes from the recent observation that the intramembrane moieties of the Na,K-ATPase (Karlish et al., 1990) and the H,K-ATPase (Rabon et al., 1993), deprived of the exposed domains by trypsin cleavage, are still capable to occlude Rb^+ ions. The developed approach also could be exploited to more precisely localize the thapsigargin binding site on the SR Ca-ATPase which, according to recent data, is formed presumably by peptide fragments from the N-terminal one-third of the protein (Sumbilla et al., 1993).

ACKNOWLEDGMENT

We are grateful to Dr. Ernesto Carafoli (Department of Biochemistry, Swiss Federal Institute of Technology, Zurich), in whose laboratories part of this research was performed, for his support and helpful discussions. We thank Dr. P. James and W. Staudemann for amino acid sequencing.

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