Tryptic Cleavage of Sarcoplasmic Reticulum Protein

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ABSTRACT: The protein of Sarcoplasmic Reticulum (SR) membrane is separated by sodium dodecyl sulfate gel electrophoresis in three main components of molecular weight (MW) 106,000, 67,000, and 55,000. Other very faint bands may be also observed in heavily loaded gels. This simple electrophoretic pattern contrasts with those obtained from more complex membranes, such as sarcolemma or erythrocyte ghosts. The ATPase enzymatic activity is associated with the 106,000 MW protein, which accounts for 70% of the total SR protein and behaves homogeneously with respect to electrophoresis, -SH reduction, selective solubilization, and tryptic digestion. Therefore, this protein is either a simple polypeptide chain or a homogeneous lipoprotein complex. Brief exposure of SR vesicles to trypsin reduces all of the 106,000 MW protein into two fragments, which we have named F_1 (57,000 MW) and F_2 (46,000 MW). F_1 and F_2 are of similar molecular weight, but distinct from the minor native proteins of SR. This initial cleavage is not accompanied by any loss of enzymatic activity. Further exposure of the SR vesicles to trypsin causes slow degradation into smaller fragments and, ultimately, to loss of enzymatic activity. Electron microscopy of negatively stained SR permits observation of 35-Å granules projecting from the outer (cytoplasmatic) surface of the vesicles. Furthermore, on replicas of freeze fractured SR, a large number of 90-Å particles is found associated with the exposed concave surface of the outer (cytoplasmatic) half of the membrane bilayer. The particles are closely spaced, with an average density of 5700 per μ^2 of membrane surface. This density is very close to a figure calculated from the number of 106,000 MW protein molecules and the membrane area of a unit weight of SR. The convex fracture faces are almost totally deprived of particles, consistent with a stronger interaction of the particles with the outer, as opposed to the inner surface of the vesicles. Brief trypsin digestion of SR produces no alteration of the outer granules, while prolonged digestion leads to their gradual disappearance. Furthermore, trypsin digestion produces a decrease in the density of 90-Å particles on the concave fracture faces and appearance of particles on the convex fracture faces. In some fracture faces the particles appear aggregated as a consequence of trypsin digestion. The minor protein components of SR may be extracted by incubation with EDTA, leaving the 106,000 MW protein associated with the membrane. In such membrane preparations, both the outer granules and the 90-Å particles are still present, suggesting that these structures are related to the 106,000 MW protein. Structural changes and protein degradation are produced by tryptic digestion at a more rapid rate in EDTA treated SR, as compared to intact vesicles. The experiments indicate that: (1) the ATPase of SR may be identified with the 106,000 MW protein component, which is partially exposed to trypsin digestion on the outer surface of the vesicles. A portion of the enzyme is imbedded within the membrane and is more resistant to digestion; (2) the outer segments of the 106,000 MW protein appear as granules on negatively stained vesicles, and the inner segments as particles on the concave freeze fracture faces; (3) tryptic fragments of the ATPase molecule remain joined to each other and to the membrane through multiple weak interactions, retaining native conformation and enzyme activity. Prolonged digestion, however, causes enzyme inactivation.

Vesicular fragments of sarcoplasmic reticulum (SR) membrane display a very specific ability to accumulate Ca^{2+} through a process of active transport (Hasselbach and Makinose, 1961, 1963; Ebashi and Lipman, 1962; Weber *et al.*, 1966). In fact, the major protein component of the SR membrane is a Ca^{2+} sensitive ATPase which is tightly coupled to the cation pump (Martonosi and Halpin, 1971; MacLennan, 1970; McFarland and Inesi, 1970, 1971; Meissner and Fleischer, 1971). Understanding of structure and function in such a membrane system requires that (a) the distribution of the ATPase molecules within the membrane structure be determined, (b) its interaction with the lipids of the membrane be studied, and (c) techniques for its solubilization and fragmentation be acquired. In this regard, progress may be achieved by discrete membrane degradations, such as those obtained by the judicious use of lipolytic (Martonosi, 1964; Fiehn and Hasselbach, 1969) or proteolytic (Ikemoto *et al.*, 1968; Inesi and Asai, 1969; Martonosi, 1968; Migala *et al.*, 1973; Stewart and MacLennan, 1974) enzymes.

Trypsin was used in early experiments in which limited digestion of SR produced changes in the structural detail of the membrane outer surface, with little loss of enzyme activity (Ikemoto *et al.*, 1968; 1971). It was later shown that enzyme activity is retained by SR, even after limited cleavage of the main membrane protein (Migala *et al.*, 1973; Stewart and MacLennan, 1974).

Continuing our previous work on trypsin digestion, we have extended the experimentation to include studies of native and cleaved proteins by sodium dodecyl sulfate gel electrophoresis, and electron microscopic observations on negatively stained and freeze-fractured membranes. These observations and an approximate model for the assembly of ATPase protein in SR membrane are described here.

Methods

SR was prepared by homogenization and differential centrifugation of rabbit skeletal muscle (McFarland and Inesi, 1971).

Ca^{2+} accumulation by SR was measured by following the distribution of isotopic tracer in reaction mixtures containing

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20 mM MOPS[1] adjusted to pH 6.8, 80 mM KCl, 5 mM potassium oxalate, 3 mM ATP, 3 mM $MgCl_2$, 0.1 mM EGTA, 0.1 mM $CaCl_2$, and 25–40 μg of SR protein/ml. The incubation was interrupted by Millipore filtration (Martonosi and Feretos, 1964).

The permeability of the SR vesicles to Ca^{2+} was studied by following efflux of Ca^{2+} after sudden dilution of vesicles preloaded by overnight incubation (5°) in the presence of 20 mM MOPS (pH 6.8), 80 mM KCl, 2.5 mM $MgCl_2$, and 20 mM $^{45}CaCl_2$. Efflux was started by a 20-fold dilution (25°) of the "loaded" vesicles with a medium containing 20 mM MOPS (pH 6.8), 80 mM KCl, 2.5 $MgCl_2$, and 10 mM EGTA. The efflux was followed by filtering at various times 2.0-ml samples and determining the residual radioactivity in the filters (Millipore HA 0.45 μ).

ATPase activity was measured by determining the production of inorganic phosphate (Fiske and Subbarow, 1925) in reaction mixtures identical with those used for Ca^{2+} accumulation. The reaction was stopped by addition of trichloroacetic acid to a final concentration of 5%.

Trypsin digestion of SR was carried out at 25° in the following reaction mixture: 1–3 mg of SR protein/ml, 0.015–0.200 mg of trypsin/ml, 20 mM MOPS (pH 6.8), and 80 mM NaCl or KCl. Incubation times varied between 10 sec and 180 min. The reaction was stopped with soybean trypsin inhibitor at a concentration twice as high as that of trypsin.

Solubilization of SR vesicles was obtained by diluting the above described mixture to 0.5 mg/ml protein concentration, in the presence of 1% sodium dodecyl sulfate. The samples were boiled for 5 min soon after solubilization.

In some instances reduction and carboxyamidomethylation of the SR protein were carried out as described by Louis and Shooter (1972), with mercaptoethanol and iodoacetamide at concentrations 2 to 50 times in excess of the protein –SH group.

Electrophoresis was performed in separating gels containing 7% acrylamide, 1.85% *N,N'*-methylenebisacrylamide, 4.5% 2-amino-2-hydroxymethyl-1,3-propandiol (Tris), 0.03% *N,N,N',N'*-tetramethylenediamine (Temed), 0.11 M HCl, 0.1% sodium dodecyl sulfate, and 0.7% ammonium persulfate; final pH 7.4. Stacking gels contained 4% acrylamide, 1.2% Tris, 0.11% Temed, 0.11 M HCl, 0.001% riboflavine, and 8% sucrose.

The running buffer contained 0.5% Tris, 0.164% sodium acetate, and 0.1% sodium dodecyl sulfate; final pH 7.4. Pyronine Y was used as a tracking dye and a current of 7–8 mA/gel was applied. After 7–8-hr electrophoresis the gels were fixed overnight in 50% trichloroacetic acid and subsequently stained for 20 hr with 0.25% Bromophenol Blue dissolved in 20% ethanol and 7% acetic acid.

Each gel was loaded with 25–60 μg of protein.

Protein concentrations were determined with the biuret reagent, and standardized by simultaneous Kjeldahl nitrogen determinations of identical SR samples.

Preparations for electron microscopy were made by placing droplets of membrane suspensions (approximately 0.1 mg of protein/ml) on copper grids covered with carbon–Formvar films and negatively stained with 1% uranyl acetate or 1% ammonium molybdate.

Freeze-fracture preparations were carried out as described by Deamer and Baskin (1969).

[1] Abbreviations used are: MW, molecular weight; MOPS, morpholinopropanesulfonate; EGTA, ethylene glycol bis(aminoethyl ether)-*N,N'*-tetraacetate; Temed, *N,N,N',N'*-tetramethylenediamine.

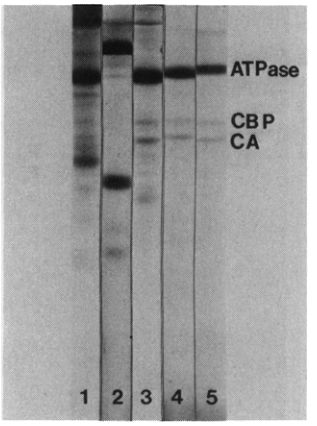

FIGURE 1: Gel electrophoresis of membrane proteins solubilized with sodium dodecyl sulfate. Each gel was loaded with 30–60 μg of protein: (1) rabbit erythrocyte ghosts; (2) rabbit white muscle sarcolemma (Soulakhe *et al.*, 1973); (3) rabbit SR, obtained from legs and back muscle; (4) rabbit SR prepared from selected white muscle of the hind legs; (5) same as in 4, but treated with mercaptoethanol and iodoacetamide at concentrations four times in excess of the –SH protein content. Note that while –SH reduction improves the resolution of other membranes' (Fairbanks *et al.*, 1971) electrophoretic patterns little change is produced in SR patterns. (Compare gel 5 to gel 4.) CBP and CA are the calcium binding proteins.

The samples were examined on a Philips EM 200 electron microscope with a double condenser illumination (300-μ platinum apertures), a 40 μ platinum objective aperture, and accelerating voltages of 60 and 80 kV.

Results

Protein Solubilization and Electrophoresis. The protein components of the SR membrane can be totally solubilized in sodium dodecyl sulfate and separated by gel electrophoresis (Martonosi and Halpin, 1971; McFarland and Inesi, 1971; Meissner and Fleisher, 1971).

The SR electrophoretic pattern consists of three components migrating with velocities corresponding to 106,000, 67,000, and 55,000 MW. Other faint bands may appear when the gels are heavily loaded, and are more prominent when SR is prepared in large scale and the choice of tissue is not limited to white muscles of hind legs (Figure 1). We have also found that when SR is stored in the absence of high concentration of sucrose, the membrane protein is slowly degraded into smaller fragments by proteolytic enzyme of probable bacterial origin (Inesi *et al.*, 1973).

The electrophoretic pattern of SR is simple, when compared to other complex membrane such as myolemma isolated from the same muscles as SR, or rabbit erythrocyte ghosts (Figure 1). This undoubtedly reflects the functional specificity of the SR membrane. The difference between the electrophoretic patterns of myolemma and SR membranes demonstrates the efficiency of the purification procedure.

The protein separations shown in Figure 1 (with the exception of gel 5) were obtained without previous reduction of –SH groups. It is interesting to notice that while such procedure generally increases the resolution of protein membrane components (Fairbanks *et al.*, 1971), in SR it produces only a slight change in migration velocity of the main protein. This protein is not divided in subunits by treatment with mercaptoethanol and iodoacetamide in amounts from 2 to 50 times in excess of the number of –SH groups in the protein (compare gels 4 and 5 of Figure 1). It appears therefore that the 106,000 MW protein is a single polypeptide chain or a lipoprotein complex which cannot be fragmented by reduction of –SH groups. In this re-

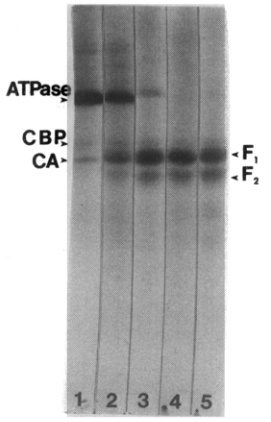

FIGURE 2: Electrophoretic patterns of SR digested lightly with trypsin. SR protein/trypsin = 200. Times of incubation: (1) 0; (2) 10 sec; (3) 60 sec; (4) 5 min; (5) 30 min. Each gel was loaded with 36 μg of protein. F_1 and F_2 are the two tryptic fragments of ATPase. Note how all the ATPase is rapidly cleaved in two fragments which are resistant to further digestion.

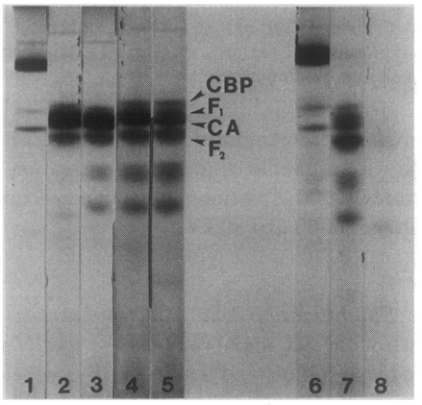

FIGURE 3: Electrophoretic patterns of SR digested heavily with trypsin. Gels 1–5 (each gel with 42 μg of protein): SR protein/trypsin = 40; incubation time = 0, 1 min, 10 min, 20 min, and 60 min. CBP and CA are the minor SR proteins. F_1 and F_2 are the ATPase fragments. Note the resolution of minor components and ATPase fragments and the slow disappearance of F_1, while F_2 is more persistent. Gels 6–8 (each gel loaded with 52 μg of protein): SR protein/trypsin = 20; incubation times: 0, 60 min, and 180 min. Note the low intensity of bands on gel 8, due to degradation of protein to peptides which are not retained by the gels. The residual ATPase activity in these samples is illustrated in Figure 7.

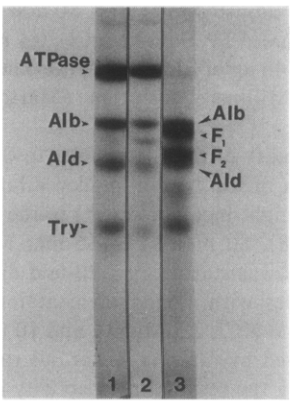

FIGURE 4: Examples of MW standardization for SR tryptic fragments. In these gels the standards added to the samples are albumin, aldolase, and trypsin: gel 1, purified ATPase and standards; gel 2, native SR and standards; gel 3, digested SR and standards.

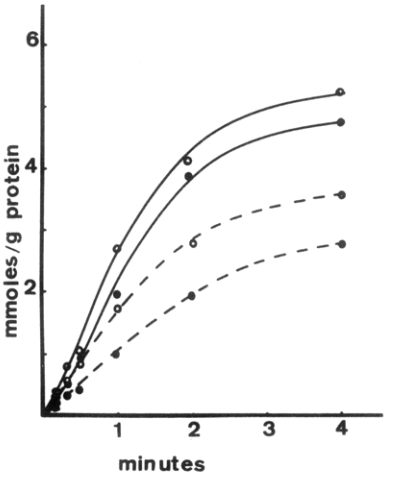

FIGURE 5: Ca^{2+} accumulation (——) and ATP hydrolysis (– – –) catalyzed by control (○) and trypsin treated (●) SR. Trypsin digestion was carried out for 20 min. SR protein/trypsin = 20, in the presence of 26% sucrose. Electrophoretic analysis of the trypsin digests revealed that all the ATPase was cleaved into two fragments. The reaction mixtures for Ca^{2+} accumulation and ATPase activity, in the presence of oxalate, are described in Methods.

spect our conclusions are in agreement with those of Stewart and MacLennan (1974) and in discrepancy with previous reports (Yu and Masoro, 1970; Martonosi and Halpin, 1971).

The 106,000 MW protein is the major component and, on the basis of studies on the incorporation of ATP terminal phosphate and on the enzymatic activity of purified SR proteins (MacLennan, 1970; Meissner and Fleischer, 1971), there appears to be no doubt that ATPase activity is associated with this protein. However, some disagreement is found in the literature with regard to the percentage of total SR protein accounted for by ATPase. In fact, figures varying from 16 to 90% were given by various authors (MacLennan, 1970; Martonosi and Halpin, 1971; Yu and Masoro, 1970; Meissner and Fleischer, 1971; Inesi *et al.*, 1973a). We find that if the SR preparation is pure the 106,000 MW protein accounts for approximately 70% of the membrane protein.

The two minor components of the SR electrophoretic pattern, which migrate with velocities corresponding to 67,000 and 55,000 MW, may be identified with the Ca^{2+} binding protein previously described (MacLennan and Wong, 1971; Ikemoto *et al.*, 1972; Ostwald and MacLennan, 1974). Minor discrepancies in the molecular weight of these proteins are found in the literature, probably due to the use of different proteins as molecular weight standards.

Tryptic Cleavage of SR Proteins. Incubation of SR with trypsin rapidly produces cleavage of the 106,000 MW protein in two unequal fragments migrating with velocities corresponding to 57,000 and 46,000 MW (Figures 2 and 3). The cleavage is completed within 1 min when the SR protein to trypsin ratio is 40, and within 5 min when the SR protein to trypsin ratio is 200. It is apparent that the 106,000 MW band behaves homogeneously with respect to trypsin cleavage, and that the two fragments which we named F_1 and F_2 derive from the same protein.

It should be pointed out that F_1 and F_2 are of similar molecular weight, but not identical with the Ca^{2+} binding proteins which, being quite resistant to the action of trypsin, remain visible on the electrophoretic gels of digested samples (Figures 2–4). F_1 and F_2 are also very resistant to digestion and, as opposed to the rapid initial cleavage, degradation of these fragments is completed over a period of 1–3 hr. F_1 is degraded first and its corresponding band on the electrophoretic gels slowly decreases in intensity, while F_2 is still pronounced (Figure 3).

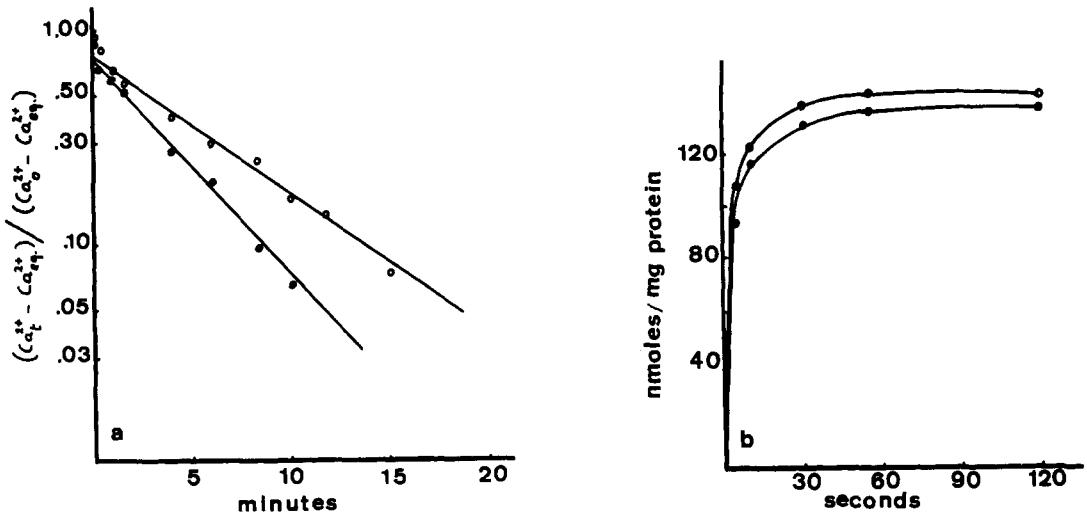

FIGURE 6: (a, left) Efflux of Ca^{2+} after sudden dilution of passively loaded SR vesicles: (○) control SR; (●) SR digested with trypsin as described in legend to Figure 5. The reaction mixture for efflux measurements is described in Methods. (b, right) Ca^{2+} uptake (in the absence of oxalate) by control SR (○) and SR digested with trypsin as described above (●). The reaction mixture for Ca^{2+} uptake contained 20 mM MOPS (pH 6.8), 80 mM KCl, 0.1 mM $CaCl_2$, 0.1 mM EGTA, 3 mM ATP, 3 mM $MgCl_2$, and 420–380 μg of protein/ml.

The first subfragments to appear display 35,000, 32,000, and 25,000 MW. Fragments of smaller size are not retained by the gels which are only faintly stained when loaded with samples thoroughly digested (Figure 3).

Effect of Trypsin on the Activity of SR. Depending on the intensity of trypsin digestion, inhibition of Ca^{2+} uptake and ATPase activity may be obtained in SR, while alterations of the membrane structural details are observed by electron microscopy (Ikemoto *et al.*, 1968; Inesi and Asai, 1969). It is most interesting that in experimental conditions in which all the SR protein identified with ATPase is cleaved in two fragments by trypsin digestion, only minimal alterations of the parameters mentioned above are produced.

Figure 5 shows that after 20-min digestion of SR with trypsin (SR protein weight/trypsin weight = 20) the ability of the vesicles to accumulate Ca^{2+} is only slightly reduced. A similarly low effect is produced on ATPase activity.

An estimate of membrane permeability to passive fluxes of Ca^{2+} may be obtained by following transmembrane Ca^{2+} fluxes after sudden dilution of vesicles preloaded by prolonged incubation in the presence of $^{45}CaCl_2$ (Figure 6a). In intact vesicles, Ca^{2+} efflux proceeds very slowly with first-order kinetics and equilibration of transmembrane Ca^{2+} gradients occurs with half-times ranging between 6 and 8 min. In vesicles digested with trypsin for 20 min (SR protein weight/trypsin weight = 20) the efflux is somewhat faster, with 4–5-min half-times to equilibration. In either case, the rates of efflux are 50–100 times slower than the rates of active transport and, therefore, have minimal effect on the steady-state levels of active Ca^{2+} accumulation by SR, even in the absence of oxalate (Figure 6b).

In the absence of sucrose, which exerts a protective effect (Ikemoto *et al.*, 1972), trypsin rapidly reduces the ability of SR to accumulate Ca^{2+} (Inesi and Asai, 1968; Migala *et al.*, 1973). On the contrary, ATPase is inactivated only after very prolonged digestions. In fact, complete inactivation of Ca^{2+} stimulated ATPase (Figure 7) is obtained when most of the ATPase protein is degraded to fragments smaller than 25,000 MW. Note that the enzymatic determinations displayed in Figure 7 were carried out with SR samples whose electrophoretic pattern is shown in Figure 3 (gels 6–8).

Electron Microscopic Observations on Negatively Stained SR. A granular layer on the outer surface of negatively stained SR vesicles has been previously described (Ikemoto *et al.*, 1968; Inesi and Asai, 1969). Regarding the detailed structure of these granules, it should be pointed out that at high magnifications all electron microscope images of thin specimens are phase images and have a characteristic granular appearance. This so called "phase grain" is caused by coherent scattering from the stain, specimen, and carbon support film. The grain size depends on the plane of focus, and is smallest near focus, increasing on both sides of true focus (Thon, 1966).

In Plate 1, for example, the moderately underfocused image of an SR vesicle is shown, which bears a periodic granularity around its surface. However, the background contains granules of significant size, when compared to the dimensions of the structural detail on the membrane outer surface. The superimposition of the background granules on the membrane detailed structure prevents an accurate determination of size and shape

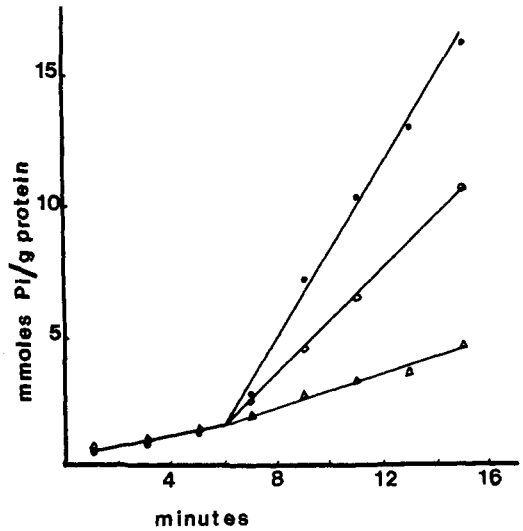

FIGURE 7: ATPase activity of control SR (●) and SR digested with trypsin (SR/trypsin = 20) for 60 min (○) and 180 min (△). Electrophoretic analysis of these samples is shown in Figure 3 (gels 6–8). The reaction mixture for ATPase contained: 20 mM EGTA and SR (38 μg of protein/ml). Soon before starting the reaction, a small amount of Triton-X-100 (SR protein:Triton = 1) was added to avoid net Ca^{2+} accumulation and maintain constant rates of reaction (McFarland and Inesi, 1970). The reaction was started with 3 mM ATP. After 6 min, 0.2 mM $CaCl_2$ was added.

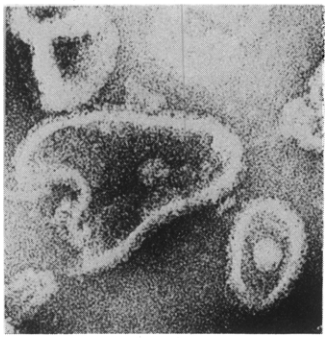

PLATE 1: Sarcoplasmic reticulum vesicles of rabbit skeletal muscle, negatively stained with 1% uranyl acetate (pH 4). Underfocus phase effects emphasize the granularity of the membrane surface. See text. × 142,500.

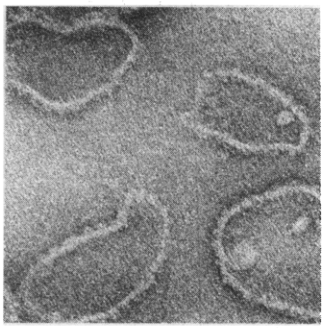

PLATE 2: SR vesicles, negatively stained with 1% ammonium molybdate (pH 7.2). × 142,500.

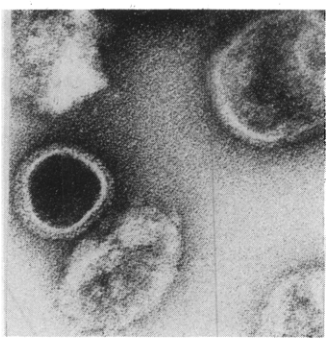

PLATE 3: SR vesicles after 20-min digestion with trypsin (SR: trypsin = 20, in the presence of 26% sucrose). Negatively stained with 1% uranyl acetate. × 142,500.

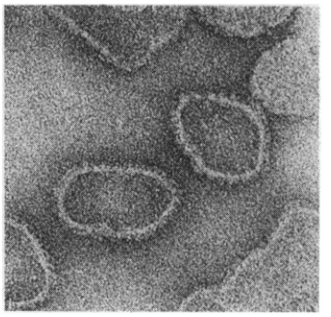

PLATE 4: SR vesicles after 20-min digestion with trypsin, negatively stained with 1% ammonium molybdate. × 142,500.

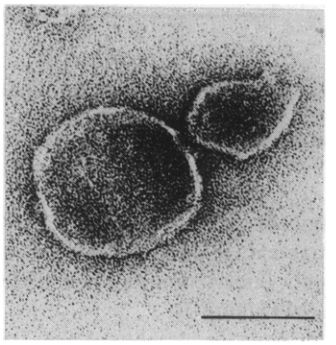

PLATE 5: SR vesicles preincubated in the presence of EDTA, then digested with trypsin as described in the legend to Figure 2. the vesicles were negatively stained with 1% uranyl acetate. The reference line represents 1000 Å. × 142,500.

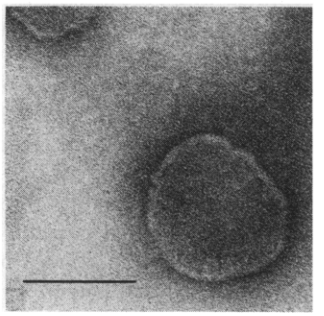

PLATE 6: SR vesicles extracted with EDTA, then digested with trypsin, as described in the legend to Figure 2. The vesicles were negatively stained with 1% ammonium molybdate. × 142,500.

of the structure. This may account in part for the slightly different images obtained with various stains (*cf.* Plates 1 and 2).

At any rate, after examining numerous plates, it is safe to conclude that the outer surface of the SR vesicles contains periodic structures of about 35 Å transversal diameter. The particles are closely spaced, but an accurate repeat distance cannot be determined since the negative stained vesicles are not sectioned and the visualized particles likely originate from two or three horizontal planes superimposed on each other.

Limited trypsin digestion of SR vesicles, in conditions producing cleavage of all the main protein in two fragments, but only slight changes in activity, does not alter the electron microscopic appearance of the outer granular layer (Plates 1–4).

On the other hand, prolonged digestion with high concentrations of trypsin (SR protein:trypsin = 20 or 40) induces progressive disappearance of the outer granules. The outer surface of the SR digested in these conditions acquires a smooth appearance which is identical with that of the vesicles reproduced in Plates 5 and 6.

Electron Microscopic Observations on Freeze-Fractured SR. Deamer and Baskin (1969) first demonstrated the presence of 90-Å particles on the fracture faces of SR vesicles. Their suggestions on the identification of these particles with the ATPase protein has received further support by studies in which appearance of particles and increase in ATPase activity were correlated in developing muscles (Boland *et al.*, 1974).

The cleavage produced by freeze fracturing occurs within the hydrophobic interior and parallel to the plane of the membrane (Branton, 1966). The exposed faces of SR vesicles are therefore related to the outer (originally cytoplasmatic) half, and to the inner half of the membrane. "Inner" and "outer" are used here with respect to the vesicles and therefore appear convex and concave, respectively. As expected, the ratio of convex to concave fracture faces is 1. We found that in well-pre-

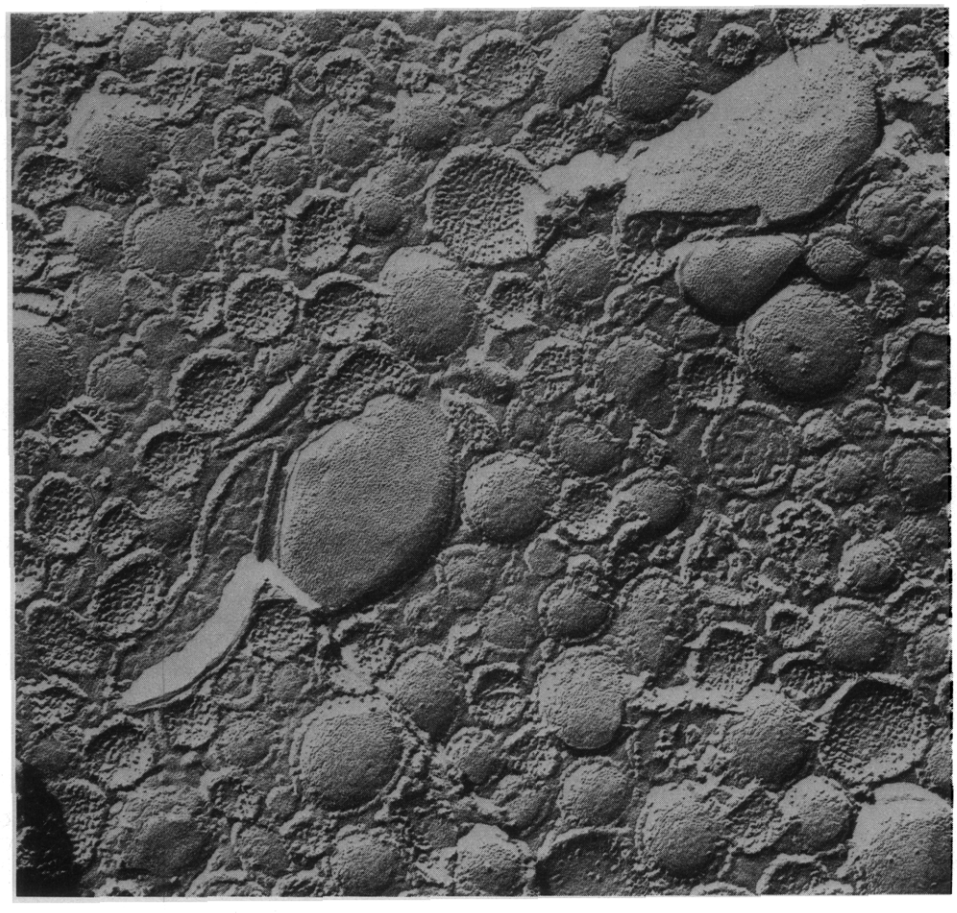

PLATE 7: Freeze-fractured SR membranes showing particles associated with the concave faces. Average particle densities are 5700/μ^2 for concave faces and negligible for convex faces. × 92,000.

served SR, the previously noted (Deamer and Baskin, 1969) 90-Å particles are almost exclusively located on the concave fracture faces (Plate 7), indicating a stronger interaction of the particles with the outer, as compared to inner half of the membrane. The particles are closely spaced, with an average density of 5700 particles per μ^2 of membrane surface.

As a consequence of trypsin digestion, we noted the appearance of particles on the convex fracture faces. This change is produced after mild trypsin digestion which cleaves the ATPase in F_1 and F_2. After extensive trypsin digestion producing inhibition of ATPase, a larger number of particles are found on the convex fracture faces, while the density of particles on the concave faces is much decreased (Plate 8). In addition, as noted by Stewart and MacLennan (1974), the particles of some vesicles digested with trypsin undergo phenomena of aggregation, with appearance of clusters or strings on the fracture surfaces (Plate 8, insert).

These observations indicate that trypsin digestion produces alterations on the protein particles, which interfere with their interaction with the outer ("cytoplasmatic") polar surfaces of the membrane bilayer, permitting their complete solubilization in the hydrophobic interior of the membrane and random diffusion to the inner surface.

Digestion of SR Vesicles Preincubated with EDTA. Duggan and Martonosi (1970) first reported that incubation of SR in the presence of EDTA causes solubilization of minor protein components, while the ATPase remains with the membrane vesicles. We found that by prolonged incubation with EDTA in the presence of sodium azide (Inesi *et al.*, 1973a) considerable purification of the SR ATPase may be accomplished. However, as a consequence of treatment with EDTA, the SR vesicles loose their ability to accumulate Ca^{2+} and the remaining ATPase is not stimulated by Ca^{2+}.

Figure 8 shows electrophoretic separations of protein fractions after sodium dodecyl sulfate solubilization of the protein extracted from SR by 48-hr incubation with EDTA and the protein remaining in the vesicles after EDTA extraction. It is clear that EDTA causes solubilization of most of the minor protein components. On the contrary, the 106,000 MW protein remains with the membrane vesicles. As mentioned above, the

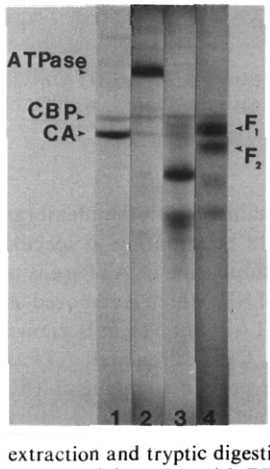

FIGURE 8: EDTA extraction and tryptic digestion of SR: gel 1, minor protein components extracted from SR with EDTA; gel 2, protein remaining in SR membrane after EDTA extraction; gel 3, tryptic digest of EDTA extracted SR (SR/trypsin = 4; 5-min digestion); gel 4, native SR digested with trypsin in comparable conditions. Each gel was loaded with 21–46 μg of protein.

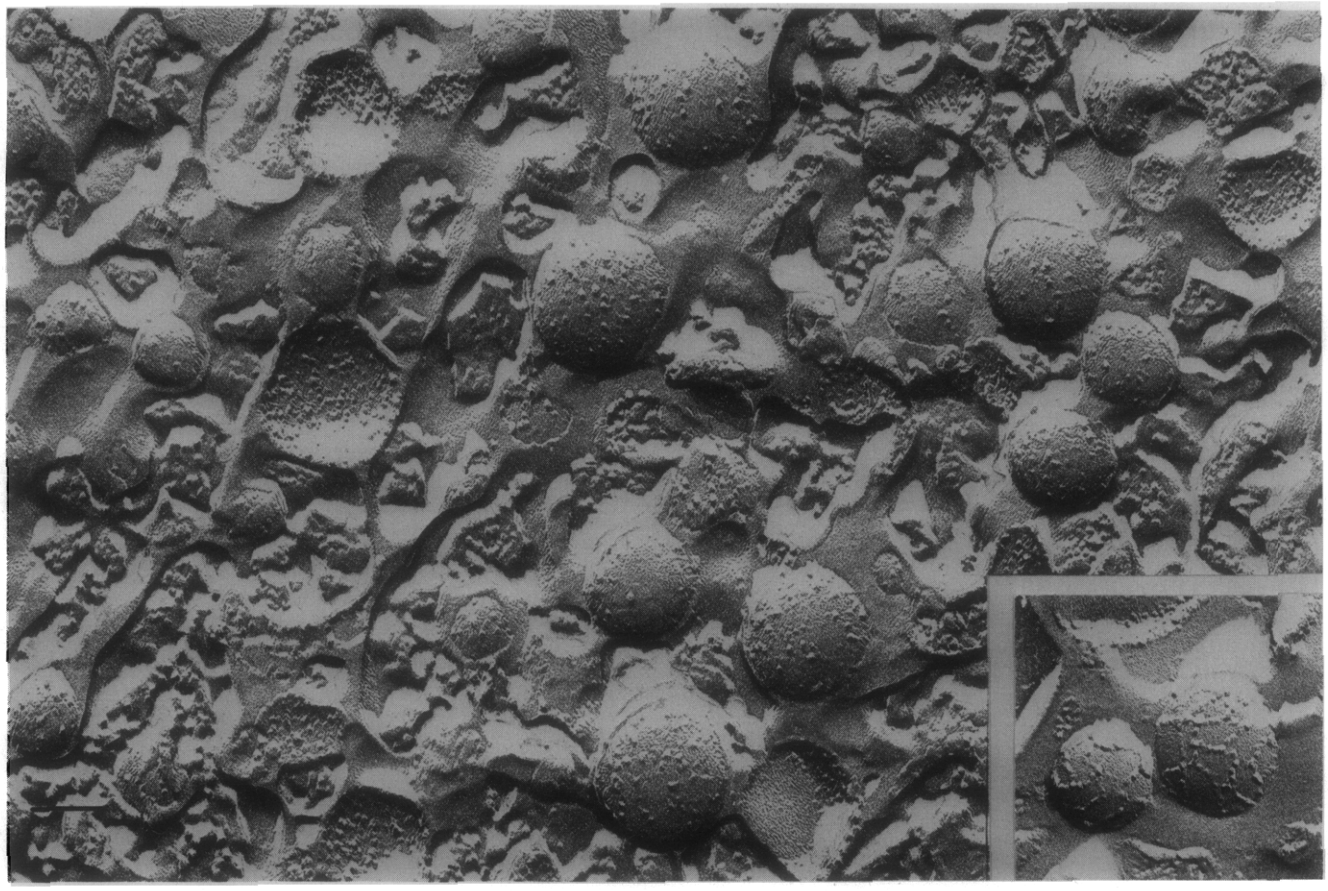

PLATE 8: SR membranes extracted with EDTA and treated with trypsin (SR protein/trypsin = 40) for 5 min before freeze fracturing. Note the distribution of particles on the concave *and* convex faces. Aggregation of particles is shown in the insert. Average particle densities are $3300/\mu^2$ for concave faces and $1500/\mu^2$ for convex faces. × 92,000.

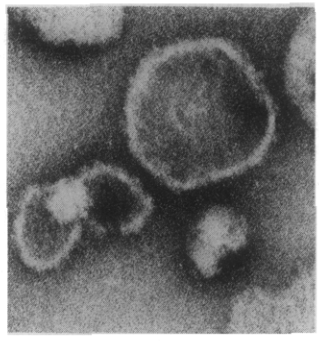

PLATE 9: SR vesicles extracted with EDTA as described in the text. The vesicles were negatively stained with 1% uranyl acetate. × 142,500.

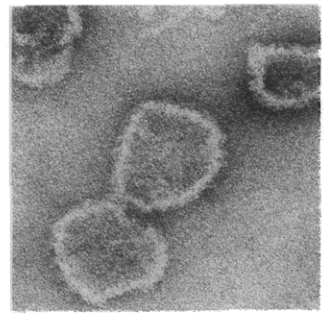

PLATE 10: SR vesicles extracted with EDTA, as described in the text. The vesicles were negatively stained with 1% ammonium molybdate. × 142,500.

ATPase activity remaining with the membrane is not stimulated by Ca^{2+}. It would be tempting to speculate that, in native membranes, Ca^{2+} stimulation of ATPase is mediated by one of the minor proteins of SR, which is removed as a consequence of incubation with EDTA. However, it is known that the 106,000 MW protein retains Ca^{2+} stimulated ATPase activity even in the absence of other protein components (MacLennan, 1970), if purified by other methods.

An alternative explanation is that removal of tightly bound Ca^{2+} by prolonged incubation with EDTA permits the occurrence of an irreversible structural modification of the ATPase molecule, which interferes with the normal mechanism of enzyme activation. A similar modification of ATPase was obtained by Hasselbach and Migala (1972) by passing Triton-solubilized protein through DEAE-cellulose columns, suggesting that "basic" and "Ca^{2+} activated" ATPase correspond to two conformations of the same enzyme.

Electron microscopic observation of EDTA treated SR reveals that negatively stained and freeze-fractured preparations retain the outer granules and the 90-Å particles, respectively. We noticed, however, that in EDTA treated vesicles the elements of the outer granular layer are not well outlined (Plates 9 and 10, to be compared to Plates 1 and 2), as if removal of divalent cations or minor protein components modifies the interaction of negative stains with the outer surface of the membrane.

The importance of the electron microscopic observations on EDTA treated vesicles is to indicate that both the outer granules and the 90-Å particles are structural features of the 106,000 MW protein. In fact this protein is the only one remaining in EDTA treated SR. Furthermore, the protidic nature of the outer granules and of the 90-Å particles is shown by their sensitivity to trypsin.

We found that treatment with EDTA renders SR more susceptible to trypsin digestion. In fact, the 106,000 MW protein is rapidly cleaved into subfragments smaller than F_1 and F_2, as shown by sodium dodecyl sulfate gel electrophoresis (Figure 8). However, the low rate Ca^{2+} insensitive ATPase remains scarcely affected by such cleavage, as if this type of enzyme activity were less dependent on the complex protein structure which is required for Ca^{2+} activation.

With regard to membrane structure, trypsin digestion produces disappearance of the outer granular elements (Plates 5 and 6) and changes the distribution of the 90-Å particles which in some vesicles appear in aggregated clusters on convex fracture faces. Although these alterations are identical with those produced by trypsin in intact vesicles, the time of incubation with trypsin required for their appearance is much shorter in EDTA treated than in native SR.

Discussion

Several experiments on the enzymatic activity of purified preparations (MacLennan, 1970; Meissner and Fleischer, 1971) and on reconstitution of functional membranes (Racker, 1972; Meissner and Fleischer, 1973) have by now provided strong evidence for the identification of the major protein component of SR (106,000 MW) with the ATPase involved in Ca^{2+} transport. The detergents required for the solubilization of this protein indicate that hydrophobic interactions contribute to its stabilization within the membrane structure.

The 106,000 MW protein is rapidly cleaved by trypsin in two fragments of MW 57,000 and 46,000, respectively. These two fragments (F_1 and F_2) are resistant to further digestion and their degradation occurs slowly over a period of 1–3 hr. The rapid rate of the initial cleavage suggests that part of the 106,000 MW protein is exposed to the outer surface of the vesicles and is sterically favorable to trypsin binding. On the other hand, the much slower degradation of the two fragments F_1 and F_2 may be understood if a significant portion of the protein molecule is imbedded within the membrane thickness and, therefore, protected from trypsin attack.

Such a view is consistent with the electron microscopic observations on freeze-fractured preparations, in which the 90-Å particles appear extended within the membrane interior, but are associated almost exclusively with the concave, rather than the convex, fracture faces. It appears then that in SR the protein particles interact both with the hydrophobic aliphatic chains and with the outer ("cytoplasmatic") polar surface of the lipid bilayer. The protein portion exposed on the outer surface evidently contributes to the appearance of the "outer granules" in conditions of negative staining.

This model confers amphiphilic properties to the protein particles with consequent stabilization of their relationship with the lipid bilayer. Digestion with trypsin perturbs this stabilization, allowing the protein particles to diffuse across the membrane and to group in aggregated clusters (Plate 8). A similar observation was recently reported by Stewart and MacLennan (1974).

Identification of the 90-Å particles observed in freeze-fracture preparations with the ATPase was first proposed by Deamer and Baskin (1969). Later, Boland *et al.*, (1974) provided support to this view by demonstrating a parallel increase in ATPase activity and number of particles in developing muscle.

The density of the ATPase molecules on the plane of the membranes was previously calculated (Inesi, 1972) from the number of 106,000 MW protein molecules and the membrane area of a unit weight of SR. An approximate figure of 100 Å for the average repeat distance of protein molecules was thus obtained. This figure is very close to the 130 Å observed in the freeze-fracture faces, and is consistent with the identification of the particles with the 106,000 MW protein molecules. Furthermore, the intercalation of the ATPase protein within the lipid bilayer is in agreement with the calculated contribution of both protein and lipids to the membrane area (Inesi, 1972).

Our results and interpretations may be helpful in considering two interesting studies recently reported by Worthington and Liu (1973) and Dupont *et al.* (1973), in which Fourier representations of X-ray diffraction patterns obtained from packed SR vesicles indicate an asymmetric electron density distribution across the lamellar structure, consistent with a prominent concentration of protein on one side of the membrane. Unfortunately the identification of the peaks of the electron density profile with either side of the membrane is based on uncertain criteria, and the two studies offer opposite suggestions regarding the prevalent location of protein on the outer or inner side of the membrane.

With regard to functional features of SR, it is clear that limited tryptic digestion of SR vesicles can be carried out with minimal reduction of Ca^{2+} uptake and ATPase activity. In this regard, our experiments are in agreement with those recently reported by Migala *et al.* (1973).

Following digestion in the appropriate experimental conditions, solubilization of the membrane with sodium dodecyl sulfate and electrophoresis reveal that *all* the ATPase protein is cleaved by trypsin in two fragments, while no alteration of functional parameters or membrane structure is produced. Since only a minimal amount of protein is released from SR vesicles, in these experimental conditions, it must be assumed that the protein fragments remain joined to each other and to the membrane through multiple weak interactions. This explains the scarcity of structural alterations following limited trypsin digestion.

To explain the persistence of ATPase activity, it must be assumed that either the enzymatic site is contained exclusively in one of the fragments, or various fragments contributing reactive residues to the enzymatic site remain joined through noncovalent bonds. The fragments are subsequently dissociated by sodium dodecyl sulfate solubilization.

The experiments on EDTA treated SR indicate that removal of tightly bound Ca^{2+} produces a structural alteration of the 106,000 MW protein, which modifies the enzymatic behavior of ATPase and renders it more susceptible to trypsin degradation.

In conclusion controlled trypsin digestion cleaves discrete protein fragments of SR ATPase, while enzymatic activity is retained. This is a useful initial step in studies of protein structure. In addition, observations made during digestion of SR vesicles in various experimental conditions indicate that projections of the ATPase protein are exposed on the outer side of the membrane, while its major portion is extended within the lipid bilayer. The relationship of this protein with the membrane structure is stabilized by polar and hydrophobic interactions.

This model is consistent with previous spectroscopic studies showing that structural perturbations are induced on the lipids by protein conformational changes and on the protein by lipid

phase transitions, in both polar and hydrophobic regions of the SR membrane (Davis and Inesi, 1971; Inesi *et al.*, 1973b).

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