Tryptic Fragmentation of the Calcium Transport System in the Sarcoplasmic Reticulum

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Two protein fragments with a molecular weight of 50-60 000 daltons are formed when the calcium transport ATPase of the SR is mildly digested with trypsin. The initial fragmentation of the ATPase does not interfere with calcium transport, calcium dependent ATPase activity and phosphoprotein formation. The decay of the initially formed protein fragments after prolonged tryptic digestion is accompanied by the decline of the rate of calcium uptake and the calcium concentrating ability while the activity of the calcium activated ATPase is reduced only moderately. The initial tryptic fragmentation does not give rise to any change in the morphological appearance in the SR membranes. After prolonged digestion brush border or smooth surface structures are observed depending on the agent used for negative staining.

Introduction

The two major components of the membranes of the sarcoplasmic reticulum are the protein of the calcium transport ATPase and the phosphatidylcholin fraction of the membranal lipids[1–4]. The hydrolytic cleavage of both components has been reported to abolish readily the ability of the SR vesicles to store calcium[1,2,5–7]. In contrast the calcium activated transport ATPase is considerably more resistant. A slowly progressing inactivation, no change or even an activation may occur depending on the experimental conditions[6–8]. The alteration of the lipoprotein structure which accompanies the functional change has been studied by different groups[1,6,7,9]: While already at low degree of lipid hydrolysis marked structural changes of the SR vesicles have been observed[1,9], proteinases produce structural modifications only after extensive hydrolysis[6,7]. The main subject of this report is the initial tryptic breakdown of the protein moiety of the calcium transport ATPase into two peptides as it occurs when the SR vesicles are treated with low amounts of the enzyme.

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Unusual abbreviations:

EGTA, Ethyleneglycol-bis-(2-aminoethyl)-N′N′-tetraacetate; NEM, *N*-ethyl-maleimide; SDS, sodium dodecyl sulfate; SR, sarcoplasmic reticulum; FSR, fragmented sarcoplasmic reticulum.

Material and Methods

The vesicles of the SR were isolated and purified from rabbit skeletal muscle according to the procedure described by de Meis and Hasselbach[10]. The rate of calcium uptake and the accompanying hydrolysis of ATP were measured after the addition of 0.5 mM $^{45}CaCl_2$ to the media which contained ATP = Mg = Ox = 5 mM at short time intervals: 0.5; 1; 2 and 6 min[11]. Calcium uptake was terminated by filtration through Sartorius filters 0.45[12] and the ^{45}Ca activity in the filtrate was measured by liquid scintillation counting. Formation of phosphoprotein was performed as described by Makinose[13] at 0 °C. Instead of 5 mM ^{32}P-ATP 0.2 mM ^{32}P-ITP were used as phosphate donator. Protein was labelled with ^{14}C-NEM at pH 8.5 according to Hasselbach and Seraydarian[14]. The vesicle protein was digested with trypsin at pH 8 adjusted with 20 mM tris-HCl in the presence of 10 mM $CaCl_2$ at 20 °C. The reaction was terminated by the addition of trypsin inhibitor which was twice the amount of trypsin or by denaturation with TCA at a final concentration of 6%. The digested non-denaturated protein of the vesicles was sedimented at 165 000 x *g* for 30 min. The TCA denaturated protein was sedimented by low speed centrifugation and repeatedly washed with water. Protein pellets were dissolved in the respective media used for gel electrophoresis which was performed in 7% polyacryl-amide gels containing either acetic acid-phenol-water 1:1:1 or 0.1 M tris-bicine buffer pH 8.2-0.1% SDS[15]. For the separation in acetic acid-phenol-water both electrode chambers contained 10% acetic acid; for the separation of the protein dissolved in the SDS medium, the buffer fluid in the electrode chambers and in the gels were identical. After electrophoresis the gels were fixed by 20% sulfosalicyclic acid, stained with Coomassie (0.25%) or Amido Black

(1%), destained with an aqueous solution containing 5% methanol and 5% acetic acid, sliced and than treated with 1 M hyamine and H_2O_2 for 2 hours. Before adding the scintillation fluid (60 g naphtalene 4 g omnifluor (NEN), 20 ml ethyleneglycol, 100 ml methanol brought to 1 l with dioxane) the hyamine solutions were acidified with 1 N HCl. The scintillation counting was performed with a Packard 300 scintillation counter. As surface crosslinking reagent dextran-aminophenyl-diazotate was preparated according to the procedure described by PORATH *et al.*[16]. The vesicles were coupled with the reagent at pH 8.5. The diazo groups remaining free were blocked with either α-naphthol or α-naphthylaminesulfonate.

For electron microscopy, droplet material was negatively stained on collodion and carbon coated grids according to standard procedures and observed in the microscope a few minutes after air drying. A 1% potassium phosphotungstate or a 2% ammonium molybdate aqueous solution, both adjusted to pH 7 with KOH, and a 1% solution of uranyl acetate at pH 4.5-5 were used. For sectioned material, control and trypsin digested samples of FSR were fixed with glutaraldehyde for 45 min, adding the fixative to the test tube at a final concentration of 5%. Specimens were post-fixed for 1 hour with 1% OsO_4 in Na-Veronal and Na-acetate buffer, pH 7.2[17] and embedded in Epon 812. Sections were counterstained with uranyl acetate[18] and/or lead citrate[19]. A Siemens Elmiskop 101 electron microscope equipped with a specimen cooling device was used with a double condenser illumination, 300 μPt condensor and 30 μPt objective aperture, accelerating voltage of 80 kV and an emission current of 20 μA. Pictures were taken at magnification ranging from X 20 000 to 50 000.

Results

When the SR vesicles are incubated with trypsin even at low ratios of enzyme to membrane protein (1/200-1/500), already after a few minutes a striking change of the protein pattern occurs as revealed by gel electrophoresis (Figs. 1*, 2). The main protein band which is assumed to represent the transport ATPase has disappeared nearly completely and in acetic acid-phenol-water gel-electrophoresis a new band appears which moves with a velocity corresponding to a molecular weight of 50 000-60 000 dalton (Fig. 1). When prior to digestion the calcium transport and the calcium activated ATPase are inactivated by substituting 4-6 moles thiol groups per 100 000 dalton with ^{14}C-NEM[14,20], radioactivity can be recovered only in the low molecular weight fragments (Fig. 2a). Like the natvie protein the protein fractions are phosphorylated by ATP or ITP under conditions where calcium

Figs 1 and 4-16 see Tables pp. 180 a-c.

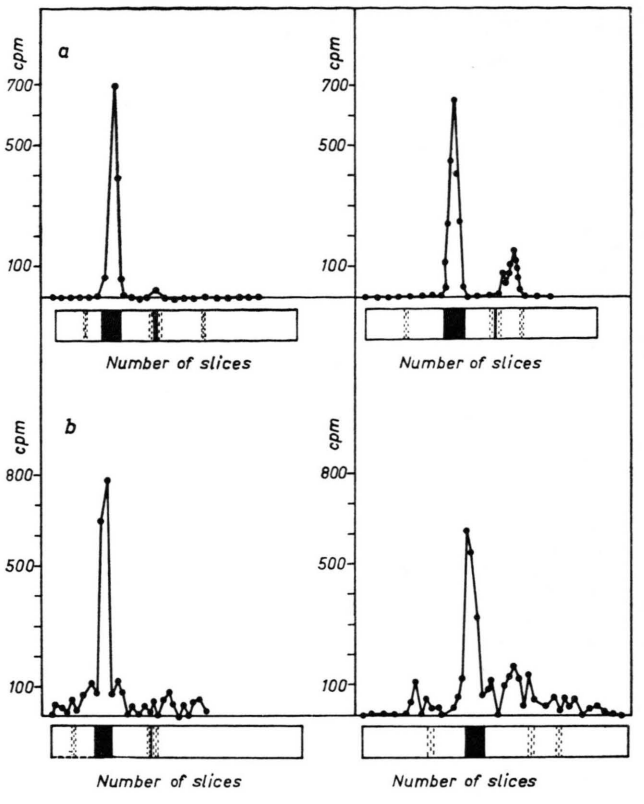


Fig. 2. Distribution of ^{14}C-NEM (a) and ^{32}P labelled (b) SR protein. The profiles are obtained from the preparations of Fig. 1. a. ^{14}C-NEM labelled preparation; left gel: ^{14}C-NEM labelled SR protein; right gel: ^{14}C-NEM labelled SR protein was digested with trypsin. b. SR protein phosphorylated with ^{32}ITP; left gel: Phosphorylated SR protein; right gel: Phosphorylation was performed after digestion.

transport takes place (Fig. 2b). Separation of the digested vesicles in SDS-bicine reveals instead of one fragment, two fragments which migrate closely together (Fig. 1c). The small distance between the two fragments on the one hand and the instability of both the phosphorylated and the ^{14}C-NEM labelled protein on the other hand prevent to ascertain in which fragment the radioactive labels are contained. Like the intact ATPase molecule, its both fragments are crosslinked by dextran-aminophenyl-diazotate which due to its molecular weight of 40 000 can only react with the membrane constituents reaching the vesicular surface. The high molecular weight complex does not enter the polyacrylamide gels (Fig. 1d).

In spite of the described alteration of the protein structure, the calcium dependent ATPase as well as the ability of the vesicles to store calcium are only very little affected (Fig. 3). As in the native mem-

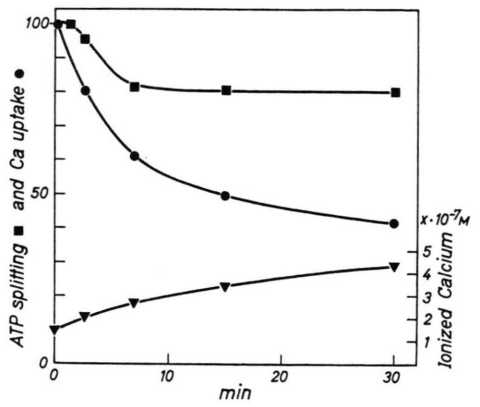


Fig. 3. Activity change of the SR vesicles in the course of trypsin digestion. 10 mg vesicle protein were digested as described in Methods with 0.05 mg trypsin. The digestion was terminated with trypsin inhibitor 0.1 mg at the time indicated on the abscissa. Subsequently, the initial rate of calcium uptake (●) the rate of ATP hydrolysis during calcium uptake (■) and the calcium remaining in the solution (▼) were measured. The rate of ATP splitting and the rate of calcium uptake are given as percentage of the activity of undigested vesicles which reached 0.8μM - 1.0μM P mg prot. $^{-1}$. min $^{-1}$, 1.2 μM - 1.6 μM Ca mg prot. $^{-1}$. min $^{-1}$, respectively. From the total calcium concentration remaining in the solution after an uptake period of 6 min and the added EGTA concentration, the free calcium concentration (▼) has been calculated. It is inversely related to the concentrating ability of the vesicles. (The stability constant of SCHWARZENBACH 38 was used.)

brane, the calcium dependent ATPase is half maximally activated at a free calcium concentration of approximately 0.3 μM and at an ATP concentration of 10 μM and both the characteristic activity concentration profiles remain unchanged[11,21,22]. Even the calcium concentrating ability of the vesicles which is their most vulnerable function is practically not reduced (Fig. 3).

During prolonged tryptic digestion the breakdown of the light fragments gives rise to peptides having molecular weights in the range of 10 000-30 000 daltons (Fig. 1c). At first, this breakdown results in a continuous decline of the initial rate of calcium uptake and the calcium concentrating ability. In contrast, after an initial drop, the activity of the calcium dependent ATPase remains constant for a considerable period of time (Fig. 3). It disappears a long time after the cessation of calcium uptake when high enzyme membrane ratios are applied.

The described initial fragmentation of the membrane ATPase by trypsin digestion does not lead to any recognizable morphological change of the membrane structure:

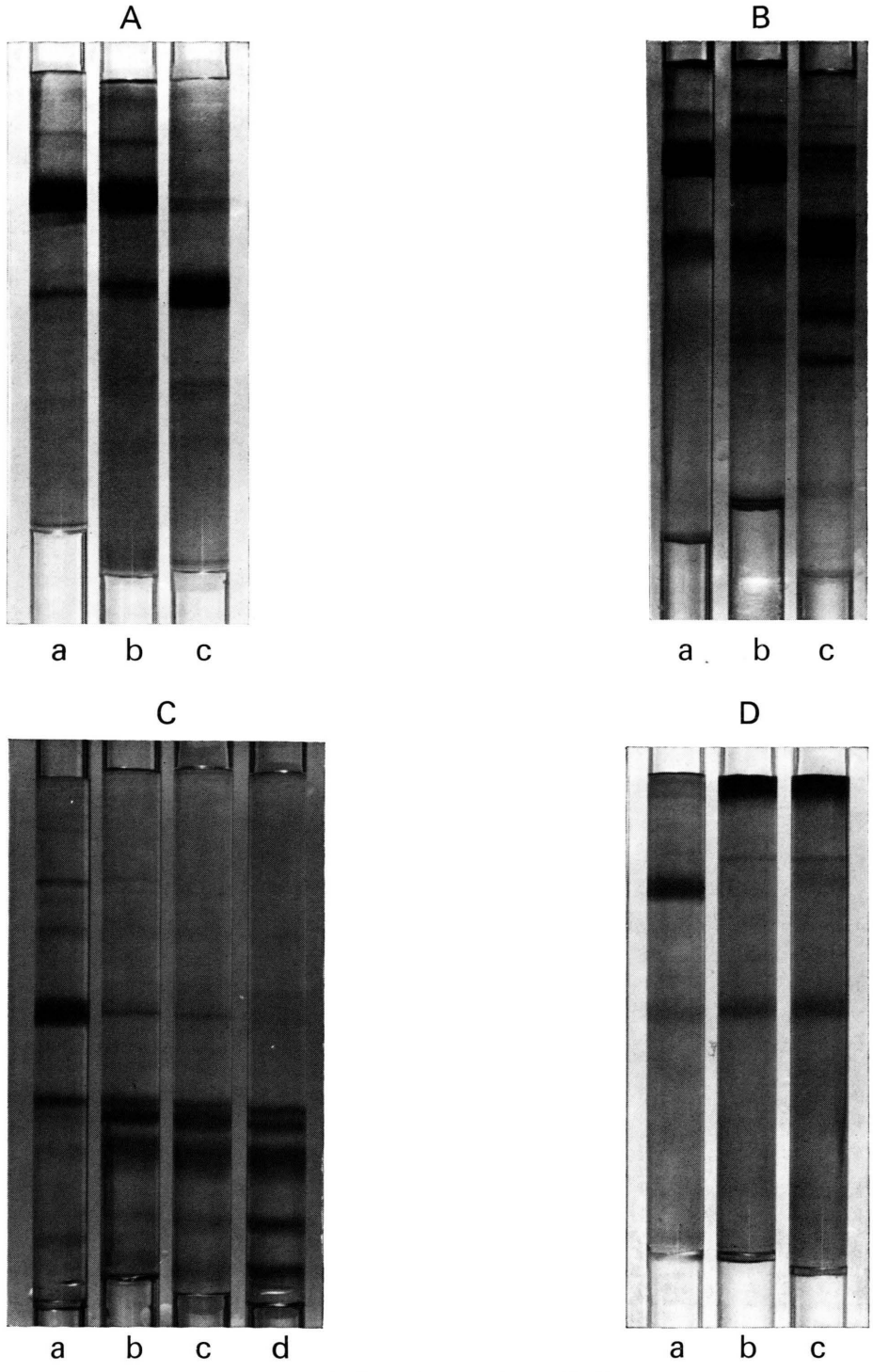
Figs 4-8 illustrate the morphological features of control SR membranes and their characteristic variability depending on the technique used[7,23-25]. No differences in size, shape and membrane structures can be detected between vesicles of shortly tryptic digested preparations and the companion controls as it can be observed by comparison of Fig. 7 with Fig. 9 of preparations stained with potassium phosphotungstate, and of Fig. 8 with Fig. 10 from preparations stained with uranyl acetate.

Alteration of SR membranes is discernible by negative staining technique only after a prolonged digestion which leads to the solubilization of 20-30% of the membrane protein. As reported also by IKEMOTO, SRETER, NAKAMURA, and GERGELY[6] and by INESI and ASAI[8] alteration begins with a gradual lacking of the particulate fringe, whereas the fine granularity of the membrane surface appears less and less prominent (Figs 11-16). However, at this stage significant differences can be observed on the membrane surface depending on the stain applied. The granularity on the surface of tryptic digested vesicles depends on the applied stain. The granularity of vesicles negatively stained with phosphotungstate (Figs 12 and 15) is finer than that of vesicles stained with uranyl acetate (Figs 11, 13, 14) and vesicles stained with molybdate are definitely smooth (Fig. 16). Changes produced by tryptic digestion in presence of calcium are undistinguishable from those observed when hydrolysis is carried out without adding calcium.

In suitable sections of glutaraldehyde-osmium fixed preparation the trilamilar aspect of the vesicular membrane is preserved (*cf.*[25]) and electron-dense dots of the outer leaflet appear somewhat larger than in the inner rim.

Discussion and Conclusions

The first attempt to estimate the molecular weight of the calcium transport ATPase was based on the assumption that the enzyme must be a main constituent of the membrane protein. The respective considerations were supported by the kinetic properties of the enzyme and the electron microscopic visualization of its functional important thiol groups[23,27,28]. The estimated size of approximately 100 000 dalton has been substantiated by recent gel chromatographic observations[3,29]. Lately, it has become evident that



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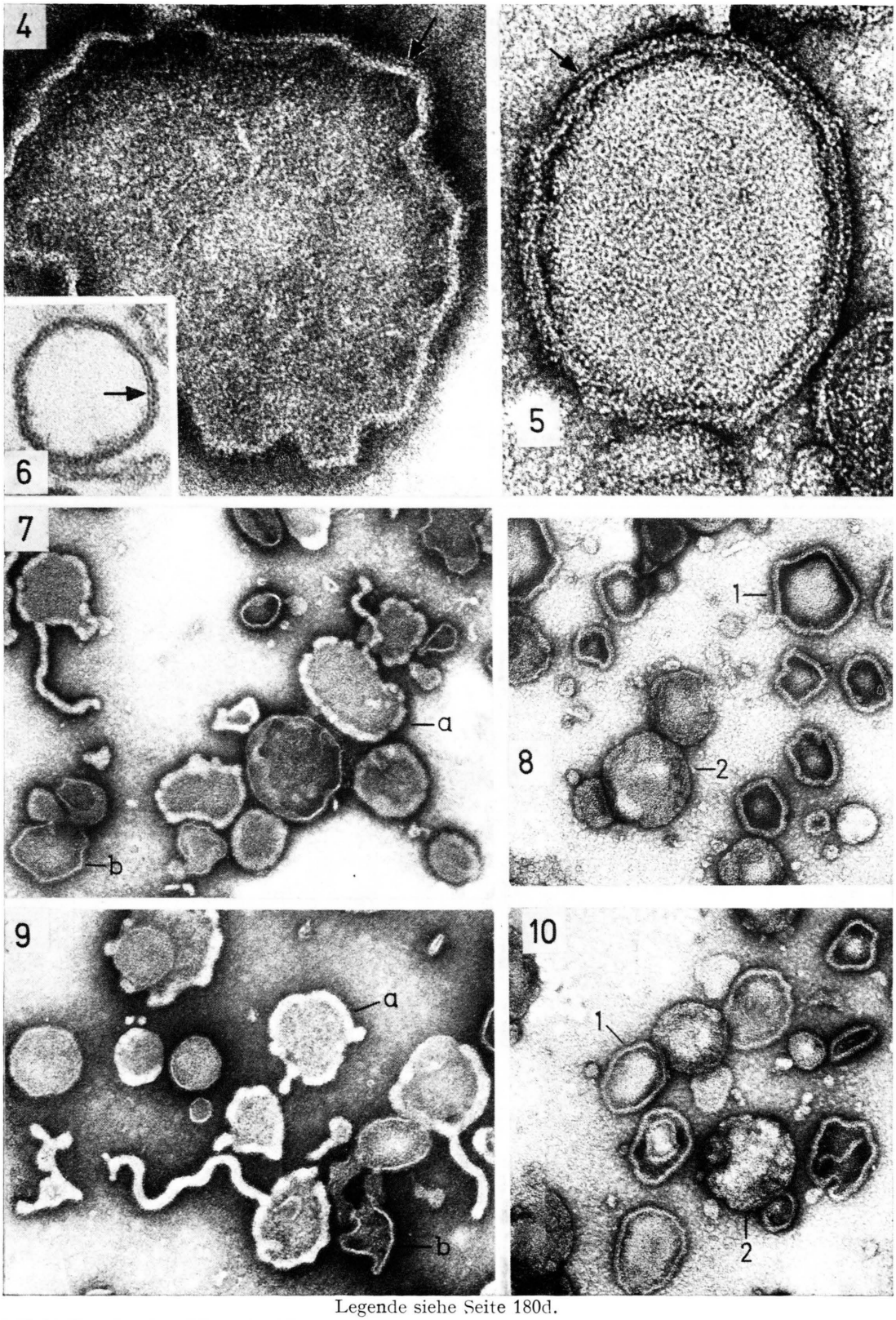

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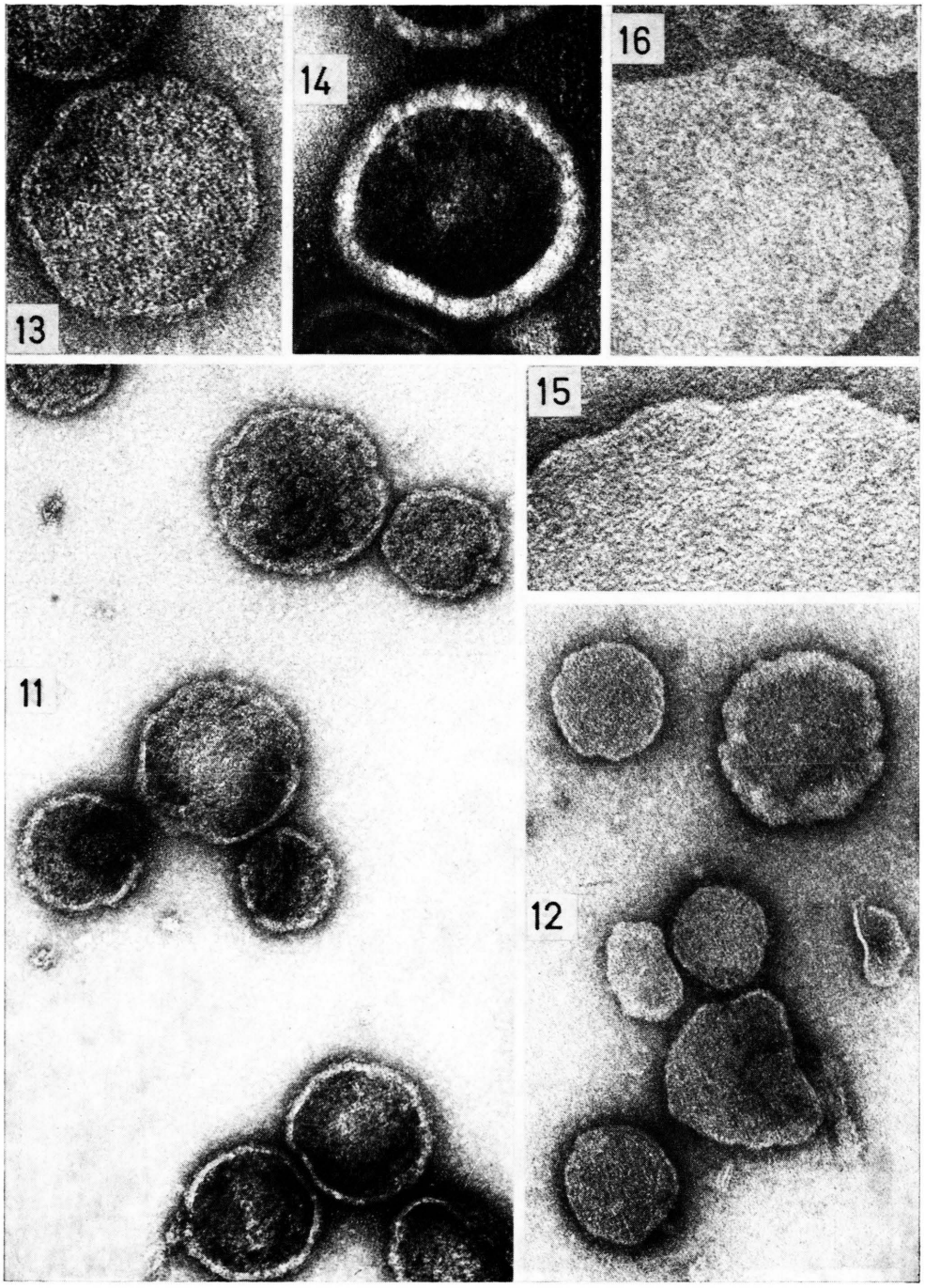

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Fig. 1. Separation of different SR protein preparations by gel electrophoresis.

A. Separation in acetic acid-phenol-water: a. Normal SR protein band (1) ~ 100 000 dalton, band (2) ~ 60 000 dalton; b. SR protein labelled with ^{14}C-NEM, 4 moles per 100 000 dalton; c. ^{14}C-NEM labelled protein was digested with trypsin for 5 min (trypsin-membrane protein ratio 1/200).

B. Separation in acetic acid-phenol-water: a. Normal SR protein; b. phosphorylated SR protein; c. the protein was digested with trypsin (trypsin-membrane protein ratio 1/200) and subsequently phosphorylated with ^{32}P-ITP.

C. Separation in tris-bicine buffer: a. Normal protein; b. c. d. protein digestion for 2 min (b), for 5 min (c) and 30 min (d) with trypsin (trypsin-membrane protein ratio 1/200). Note that the less intense band (2) which corresponds to a molecular weight of ~ 60 000 dalton remains unchanged by digestion.

D. Separation in acetic acid-phenol-water: a. Normal SR protein; b. SR protein shortly digested with trypsin and subsequently treated with the surface reagent dextran-aminophenyldiazotate; c. SR protein crosslinked with dextran-aminophenyl diazotate.

Fig. 4. Control vesicle of rabbit FSR negatively stained with 1% potassium phosphotungstate, pH 7.0. Membrane surface displays a fine regular granularity. Vesicular profile is limited by a particulate fringe (arrow). X 240 000.

Fig. 5. The same as in Fig. 1 negatively stained with 1% uranyl acetate, pH about 4.5. Small particles with a diameter of 40 Å (arrow) appear to protude from the membrane profile to which are connected by a short stalk. X 240 000.

Fig. 6. Thin epon section of a glutaraldehyde-osmium fixed control vesicle of rabbit FSR. When unit membrane is well resolved, dense dots of the outer rim are larger than in the inner leaflet (arrow). Uranyl acetate and lead citrate stain. X 240 000.

Fig. 7. Survey electron micrograph of a control preparation of rabbit FSR negatively stained with 1% potassium phosphotungstate. Almost all vesicles display a particulate fringe, however, vesicles with different degree of electron density can be observed; (compare a with b). X 72 000.

Fig. 8. The same preparation a in Fig. 7 negatively stained with 1% uranyl acetate. A particulate rim is well discernible on the profile of most vesicles (1) while others display a diffuse fine granularity (2). X 72 000.

Fig. 9. FSR preparation after digestion with trypsin — 2 mg/100 mg membrane protein — for 5 min at 25° C in presence of calcium. No difference from the control can be observed (see Fig. 7). Negative stain with 1% potassium phosphotungstate. X 72 000.

Fig. 10. FSR vesicles digested with trypsin as in Fig. 6, negatively stained with 1% uranyl acetate. The picture cannot be distinguished from that of the companion control (see Fig. 8). X 72000.

Fig. 11. Structural changes in the FSR vesicles after digestion for 40 min with trypsin. Conditions of digestion as described in Fig. 9. The particulate rim on the vesicular profile is lacking. The fine granularity of the membrane appears less prominent than in control preparation. Vesicular border of individual vesicles displays a various degree of electron density (see also Figs 13 and 14). Negative staining with 1% uranyl acetate. X 120 000.

Fig. 12. The same preparation as in Fig. 11, negatively stained with 1% potassium phosphotungstate. On comparison with Fig. 11 the fine granularity of the vesicular membrane is still less prominent than in uranyl stained preparation. X 120 000.

Figs 13-16. Different aspects of membrane surface of FSR vesicles digested with trypsin in presence of 10 mM calcium for 1 hour. Conditions of digestion as described in Fig. 9. X 240 000.

Fig. 13. A fine granularity is still discernible on the vesicular surface. Negative staining with 1% uranyl acetate.

Fig. 14. The surface granularity is masked by stain whereas the profile is well outlined. Negative staining with 1% uranyl acetate.

Fig. 15. The vesicular profile appears smooth. Negative staining with 1% potassium phosphotungstate.

Fig. 16. The vesicular surface as well as its profile is definitely smooth. Negative staining with 2% ammonium molybdate.

this relatively small molecule acts as a system which can convert not only chemical into osmotic but also osmotic into chemical energy[30,31]. During the conversion of chemical into osmotic energy the system is activated by ionized calcium outside and inhibited by calcium ions inside the closed SR vesicles. During the conversion of osmotic into chemical energy the system is activated by calcium inside and inhibited by calcium outside the vesicles. These properties suggest that the energy converting structure of the SR ATPase reaches through the membrane. The finding that after crosslinking the surfacial protein by dextran-aminophenyl diazotate only the protein fraction with a molecular weight of approximately 60 000 dalton enters the gel, strongly supports the assumption that the enzyme spans the SR membranes. The rapid and complete fragmentation of the calcium transport protein by trypsin leading to fragments of approximately half the size of the native molecule indicates that it is composed of two polypeptide chains of similar molecular weight. The linkage which connects the two fragments is obviously of minor functional importance, since only very little loss of activity is observed.

Since evidence has been given that calcium transport and the calcium dependent ATPase require the simultaneous interaction of two calcium ions and one molecule of ATP[32,33] and since, furthermore, the protein of the calcium transport ATPase has two high affinity calcium binding sites [34,35], it is tempting to speculate that the membrane ATPase may be composed of two identical subunits interconnected with each other. However, since two tryptic fragments can be detected by SDS gel electrophoresis in bicine buffer, this assumption seems less likely. Therefore, one may assume either that all functional important sites are located only in one fragment or these sites are present in both fragments but the interaction is not disturbed by tryptic cleavage. At the moment, it is not possible to distinguish between these two alternatives.

In every case, if there is an interaction between the two parts of the molecule it must be very stable as demonstrated by the finding that neither phosphoprotein formation nor calcium accumulation nor calcium activated ATP splitting are affected when the molecule is cleaved. Even, the hitherto described activity increase caused by tryptic digestion of the calcium[7,8] dependent ATPase could not be demonstrated in these experiments. Most likely in those experiments the measurement of the rate of ATP hydrolysis during the initial phase of calcium uptake of intact SR vesicles yielded to low values (*cf.*[28]).

The interconnecting linkage can either be a peptide or an ester-bond because trypsin hydrolyzes both kinds of bonds (*cf.*[36]). Since it is unlikely that trypsin penetrates in appreciable amounts through leaks into the interior of the vesicles during the short incubation period, it is very probable that the bond is located at their outer surface. The rapidity with which the molecule is fragmented into two units favours the assumption that the linkage must be much more accessible than all other trypsin sensitive bonds. Presumably the same bond is split during disaggregation of the delipidated membrane by succinylation. The main components of the succinylated protein separated by gel chromatography in SDS containing solutions has a molecular weight as estimated by classical methods of 50 000-60 000 dalton[28].

Since the covalent linkage connecting the two molecule moieties seems to be not involved in the mechanism of calcium transport, one may suspect that it might have some implications for the structural properties of the membrane. However, the morphological appearence of the membranes neither after staining with uranyl acetate nor with phosphotungstate is affected if the linkage between the two halves of the molecule is cleaved during a short trypsin digestion. However, after a more progressive digestion during which a number of small protein fragments are produced, the action of the membrane stains, uranyl acetate on the one hand and phosphotungstate or molybdate on the other hand gives rise to different structural features: Brush borders and smooth surfaces. This observation indicates that the interaction of the residual lipid protein complex in the digested membranes with the different stains gives rise to the formation of different structural peculiarities[37]. These differences seem not to depend on the presence or the absence of calcium ions during digestion as it has been suggested by IKEMOTO *et al.*[7].

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