THE SEPARATION OF THE SOLUBILIZED PROTEINS OF THE SARCOPLASMIC RETICULUM ON DEAE-CELLULOSE AND ITS MODIFICATION

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1. Introduction

In the native membranes of the sarcoplasmic reticulum (SR) two different ATP hydrolyzing enzymes have been described, the basic and the extra-ATPase. Among other characteristics, the basic ATPase splits ATP in the absence of calcium ions [1–4] while the extra-ATPase needs for its activity a low concentration of ionized calcium. Therefore, the latter has also been named calcium dependent ATPase (c.f. [5]). It is presumably identical with the structure which translocates calcium from outside to inside of the SR vesicles consuming ATP and which incorporates inorganic phosphate into ADP when calcium moves from inside to outside following a concentration gradient [5, 6]. A most interesting property of the protein component of the energy converting structure is its high degree of functional flexibility. Its activity can be manipulated by removal and readdition of natural and artificial lipid compounds [4, 7, 8]. As long as natural lipids are used, none or only slight change of the activity pattern occurs but as recently shown by Walter [9] a treatment of the SR membranes with the artificial lipid compound, Triton X-100, produces a complete change of their activity pattern.

In this report the interchange of the calcium dependent ATPase activity as it results when the SR membranes, dissolved in Triton X-100, are fractionated on a DEAE cellulose column will be described, and it will be shown that the modification of the ATPase is accompanied by a transformation of its protein structure. The structural as well as the enzymatic changes are modified by calcium and magnesium ions.

Abbreviations:

NEM: N-Ethylmaleimide;
EGTA: Ethyleneglycol-2(2aminoethyl) tetraacetic acid.

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2. Methods

The SR vesicles were prepared from rabbit skeletal muscle according to Hasselbach and Makinose [10]. The vesicles were solubilized at 0° as described by Ikemoto [11]. The precipitate of the protein fraction of the Calsequestrin [13] was removed by low speed centrifugation. The DEAE DE 32 cellulose column used for the separation of the protein was equilibrated with a solution containing 0.05 mM Tris chloride pH 8.1, 5% glycerol and 0.2% Triton X-100. $CaCl_2$ or $MgCl_2$ were present as described in the legends. The column was loaded with 300–400 mg protein. The elution diagram was recorded at 280 nm with a Uvicord II ultraviolet absorptiometer (LKB, Sweden). All experiments were performed at 0°–4°. The ATPase assay contained 5 mM Mg-ATP, 20 mM histidine pH 7.0, and 40 mM KCl. The calcium concentration was adjusted by the addition of calcium and EGTA. The protein was determined according to Lowry et al. [15] and by UV measurements after correction for the Triton X-100 absorbance. In a number of experiments the protein was lightly labelled with [^{14}C]NEM to ascertain complete elution. The residual lipids in the protein fraction were determined by thin-layer chromatography on silica gel. No lipids could be detected in the perchloric

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acid precipitate of the eluted fractions. The lipids present in the Triton X-100 containing supernatant were absorbed on a DEAE-cellulose column at pH 8.1. After elution of the Triton X-100 with 50 mM Tris chloride, the lipids were eluted with 0.5 M sodium chloride. The water was evaporated and the dried lipids were dissolved in chloroform–methanol.

3. Results

The activity pattern of the calcium dependent ATPase of native SR membranes remains nearly unchanged [9, 11] when they are dissolved in Triton X-100. Enzymatically delipidated membranes whose calcium activated ATPase is inactive even can regain ATPase activity by the addition of Triton X-100 [7, 12]. In contrast, the activity of the basic ATPase is completely abolished by Triton X-100 [9]. SR membrane proteins after being solubilized by Triton X-100 can be absorbed by DEAE-cellulose and eluted by Triton X-100 containing solutions. The separation of the proteins is imperfect as long as the eluants contain less than 0.1% Triton X-100. Good separations are achieved when 0.2% Triton X-100 is present in the eluants (fig. 1). As a characteristic feature, the elution pattern of the membrane protein and its enzymatic property change characteristically when the protein and the lipid components of the membranes are modified. Furthermore, the elution and the enzyme pattern depend on the presence of alkaline earth ions in the eluant. Approx. 40–60% of the membrane protein from native as well as from phospholipase C digested vesicles are eluted with 50 mM Tris chloride pH 8.1 provided the eluant contains 4 mM $CaCl_2$ (Fraction I). Fraction I contains a calcium dependent ATPase of a high specific activity although the preparation is essentially free of lecithin and cephalin (fig. 3). It contains only traces of neutral lipids and traces of an acid lipid which on thin-layer chromatograms migrates with the sphingomyelin fraction. The protein is identical with the main component of the SR membranes observed in SDS and acetic acid–phenol–water gel electrophoresis having a molecular weight of approx. 100000 daltons. The second fraction eluted after applying an NaCl gradient (0–0.3 M) has no ATPase activity nor can its activity be restored by oleic acid.

Under all other conditions which are illustrated by fig. 2, the main protein fraction appears not until the NaCl gradient has been applied. Like fraction I, fraction II is essentially free of phospholipids. In SDS as well as in acetic acid–phenol–water gel electrophor esis fraction II exhibits exactly the same properties as fraction I (fig. 3). However, fraction II has no or only very little ATPase activity. The loss of enzymatic activity after substitution of most of the SH groups in the SR membranes is evident [3]. However, the fact that the NEM substituted protein is eluted with the calcium containing eluant as fraction II indicates a change of its structure, since the elution pattern of other proteins like the Calsequestrin of MacLennan [13] is not changed by NEM substitution. Similar structural change must have taken place when the calcium ions in the eluant are replaced by magnesium ions or after the digestion of most of the phospholipids by phospholipase A. The latter change occurs even when 4 mM calcium were present throughout the procedure. The protein fraction separated in the pres-

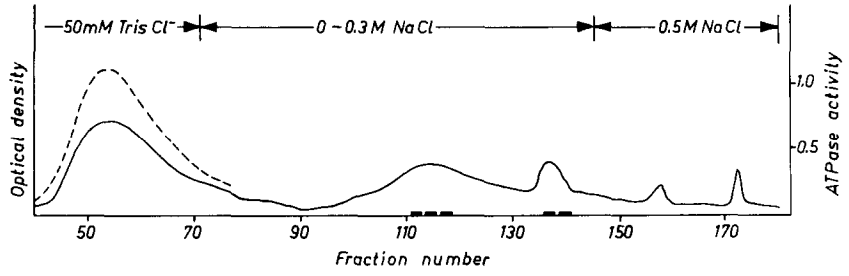

Fig. 1. Elution of solubilized SR membranes from a DEAE DE 32 cellulose column with eluants containing 0.2% Triton X-100 and 4 mM $CaCl_2$. The basic eluant contained 0.2% Triton X-100, 4 mM $CaCl_2$, 5% glycerol, 50 mM Tris chloride pH 8.1 and the specified NaCl concentrations. The column (2.5 × 50 cm) was loaded with 400 mg solubilized vesicle protein after the Calsequestrin[13] fraction has been removed by centrifugation. 70 fractions of 4.5 ml were eluted with the basic eluant. For the elution of fraction 70 to 145 a NaCl gradient 0–0.3 M is superimposed. The lipid fraction was removed by an eluant containing additionally 0.5 M NaCl. (- - - -): Represents the calcium dependent ATPase activity μmole P × min^{-1} × $(ml\ eluate)^{-1}$ (left ordinate). $[Ca^{2+}]$ = 0.1 mM was present in the ATPase assay. T = 20°. No ATPase activity in fractions 110–120 and fractions 135–140 was found.

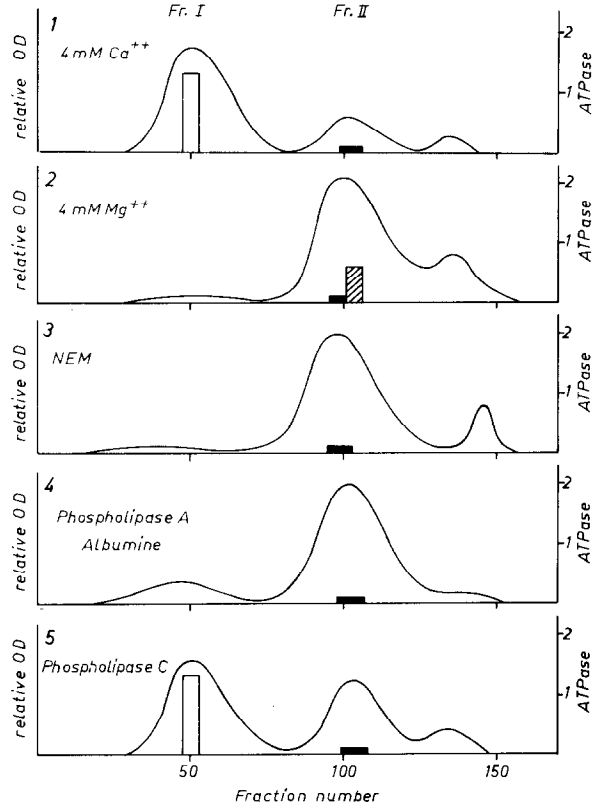

Fig. 2. The variability of the elution pattern of solubilized SR protein. The solubilized protein was absorbed on a DEAE DE 32 cellulose column as described in Methods. The basic eluant contained 0.2% Triton X-100, 5% glycerol, 50 mM Tris chloride pH 8.1. Fraction I is eluted with the basic eluant; for elution of fraction II a NaCl gradient 0–0.3 M is superimposed. 1.) Native SR vesicles. 4 mM $CaCl_2$ were present in all solutions. Calcium activated ATPase activity is present only in fraction I. 2.) Native SR vesicles. The SR vesicles were solubilized in presence of 4 mM $CaCl_2$. The eluant contained 4 mM $MgCl_2$ instead of 4 mM $CaCl_2$. Most protein appears as fraction II. It has no ATPase activity but its activity is reconstituted on addition of oleic acid 0.2 mg/mg protein. The reconstituted ATPase is calcium independent. 3.) Inactivated SR vesicles.The thiol groups of the vesicular protein (0.1 μmole/mg) were substituted by NEM. The eluant contained 4 mM $CaCl_2$. 4.) Delipidated SR vesicles. The SR vesicles were digested with phospholipase A and the hydrolysis products were removed with albumin [4]. The ATPase activity is lost and cannot be restored. 5.) Delipidated SR vesicles. The vesicles were treated with phospholipase C. Most protein is eluted as fraction I and its calcium dependent ATPase is active. $[Ca^{2+}]$ = 0.1 mM was present in the ATPase assay. □: Calcium dependent ATPase; ▨: calcium independent ATPase reactivated by oleic acid; ▬: no ATPase activity. Right ordinates: ATPase activity μmole P × mg^{-1} × min^{-1}. T = 20°.

ence of 4 mM $MgCl_2$ regains ATPase activity when the preparation is supplemented with oleic acid. This ATPase activity, however, does not depend on calcium ions.

4. Discussion

The described observations indicate that the ATPase of the SR membranes can exist in a number of different functional and structural states whose appearance depends on the lipid components in contact with the membrane protein and the presence of alkaline earth ions. Different functional states have previously been demonstrated by the replacement of the natural lipids by stoichiometrical amounts of oleic acid, and lysolecithin [4, 7]. Like oleic acid or lysolecithin, Triton X-100 can replace the natural lipids effectively, as far as ATP splitting is concerned [7, 12]. In contrast to the activating effect of low amounts of Triton X-100, high amounts (3–5 mg/mg protein) by which the membranes are solubilized and which remove practically all phospholipids, rapidly produce a complete inactivation of the calcium dependent ATPase [11]. However, this inactivation of the solubilized membranes can largely be prevented when millimolar concentrations of calcium ions are added to the solubilized protein [11]. It is the presence of calcium ions which stabilizes the enzyme since the preparation soon becomes inactive when they are removed or replaced by magnesium ions even after the Triton/protein ratio has been reduced tenfold. The transition of the enzyme from the active to the inactive state obviously is connected with a transformation of its structure. This change is revealed by the observation that the active enzyme is eluted from the DEAE cellulose column with 50 mM Tris chloride pH 8.1 alone, while the inactive one requires 0.15 M NaCl for elution when 0.2% Triton X-100 is present in the eluant. Since the enzyme in the active and in the inactive state displays identical gel electropherograms, the transformation obviously is brought about by an exposition of more acid groups. In spite of the presence of calcium ions the same transformation takes place when the thiol groups of the membrane protein are substituted by NEM. It seems likely that this structural change gives rise to the aggregation of the SR vesicles when their surfacial thiol

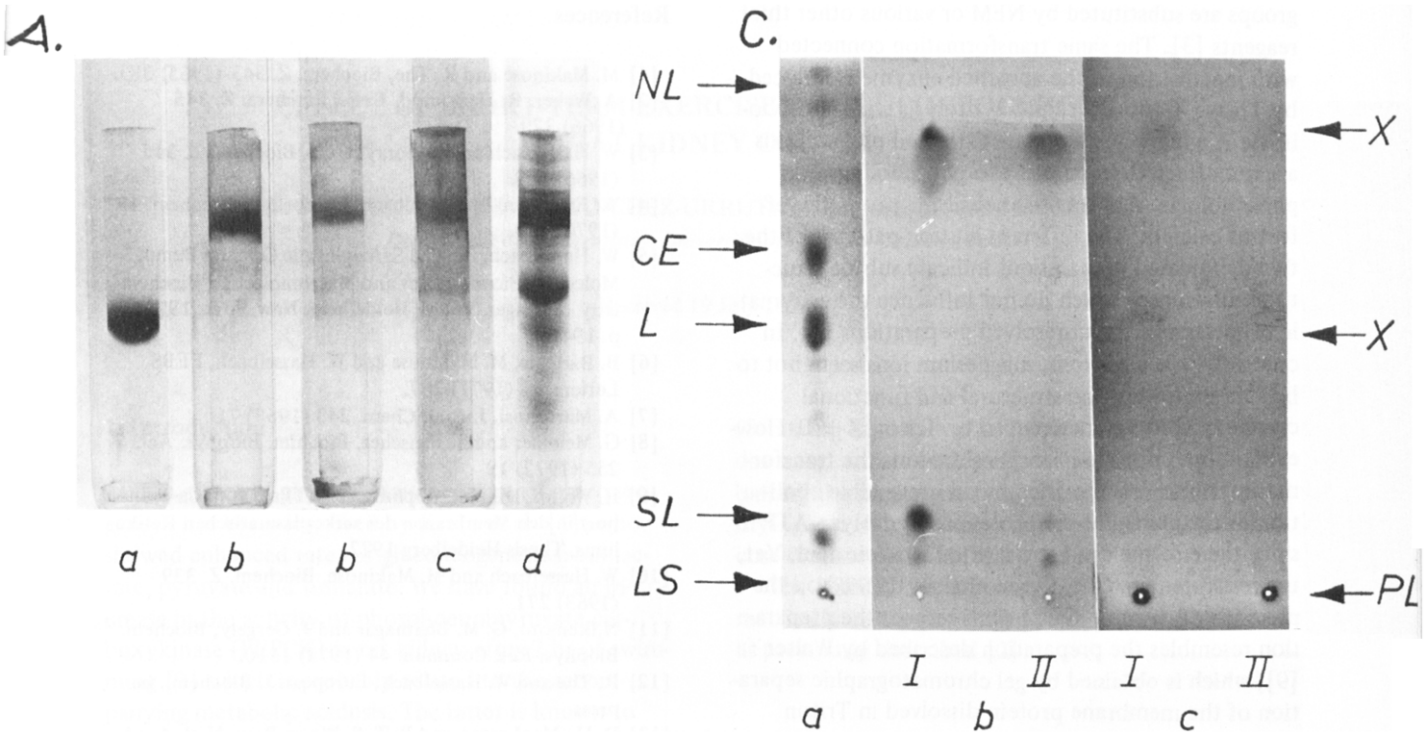

Fig. 3. The separated SR proteins and their lipids. A) SDS gel electropherograms of a) albumin, b) fraction I, c) fraction II and d) total SR protein. SDS polyacrylamid gel electrophoresis was performed with 7.0% gels containing 0.1% SDS and 0.05 M glycerophosphate, pH = 7.4. B) Phenol–acetic acid–water gel electropherograms of a) total SR protein, b) fraction I, c) fraction II. The protein was precipitated with 5% perchloric acid, washed with water and dissolved in phenol–acetic acid–water, 1:1:1. Electrophoresis was performed with 7.0% gels soaked in phenol–acetic acid–water, 1:1:1. C) Thin-layer chromatograms of the lipids of the separated protein fractions. a) For comparison lipids of the SR membranes; b) lipids of protein Fraction I and II; a) and b) developed in chloroform–methanol–ammonia (23%) 17:7:1; c) lipids of fraction I and II developed in chloroform. The protein fractions were prepared as described in fig. 1. The isolation of the lipids is described in Methods. The spots are revealed by iodine vapour. NL: neutral lipids; CE: cephalin; L: lecithin; SL: sphingomyelin; LS: lysolecithin; PL: phospholipids; X: unidentified compounds.

groups are substituted by NEM or various other thiol reagents [3]. The same transformation connected with inactivation of the absorbed enzyme is induced by Triton X-100 after lipid hydrolysis with phospholipase A while phospholipase C treated preparations are not affected. In contrast to phospholipase C, phospholipase A seems to abolish the protective effect of calcium. The different elution patterns of the two delipidated preparations indicate subtile structural differences which do not influence the enzymatic properties of the unresolved preparations [4]. In contrast to calcium ions, magnesium ions seem not to be able to prevent the structural and functional change of the enzyme induced by Triton X-100. However, in the presence of magnesium ions the transformation is not accompanied by an irreversible abolition of the ability of the protein to hydrolyze ATP since the enzyme can be restituted by oleic acid. Yet, the restituted enzyme does no longer depend on the presence of calcium ions. In this respect the preparation resembles the preparation described by Walter [9], which is obtained by gel chromatographic separation of the membrane protein dissolved in Triton X-100 on Sepharose 6B in the presence of 0.25 M KCl.

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