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HIGHLY PURIFIED SARCOPLASMIC RETICULUM VESICLES ARE DEVOID OF Ca^{2+}-INDEPENDENT ('BASAL') ATPase ACTIVITY *

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Summary

On solubilization with Triton X-100 of sarcoplasmic reticulum vesicles isolated by differential centrifugation, the Ca^{2+}-ATPase is selectively extracted while approximately half of the initial Mg^{2+}-, or 'basal', ATPase remains in the Triton X-100 insoluble residue. The insoluble fraction, which does not contain the 100 000 dalton polypeptide of the Ca^{2+}-ATPase, contains high levels of cytochrome *c* oxidase. Furthermore, its Mg^{2+}-ATPase activity is inhibited by specific inhibitors of mitochondrial ATPase, indicating that the 'basal' ATPase separated from the Ca^{2+}-ATPase by detergent extraction originates from mitochondrial contaminants.

To minimize mitochondrial contamination, sarcoplasmic reticulum vesicles were fractionated by sedimentation in discontinuous sucrose density gradients into four fractions: heavy, intermediate and light, comprising among them 90—95% of the initial sarcoplasmic reticulum protein, and a very light fraction, which contains high levels of Mg^{2+}-ATPase. Only the heavy, intermediate and light fractions originate from sarcoplasmic reticulum; the very light fraction is of surface membrane origin. Each fraction of sarcoplasmic reticulum origin was incubated with calcium phosphate in the presence of ATP and the loaded fractions were separated from the unloaded fractions by sedimentation in discontinuous sucrose density gradients. It was found that vesicles from the intermediate fraction had, after loading, minimal amounts of mitochondrial and surface membrane contamination, and displayed little or no Ca^{2+}-independent

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* Part of this work has been reported previously (Hidalgo, C., Rosemblatt, M., Fernandez, J.L. and Ikemoto, N. (1979) Biophys. J. 25, 23a).

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Abbreviations: EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid; Mops, 4-morpholinepropanesulfonic acid; SDS, sodium dodecyl sulfate.

basal ATPase activity. This shows conclusively that the basal ATPase is not an intrinsic enzymatic activity of the sarcoplasmic reticulum membrane, but probably originates from variable amounts of mitochondrial and surface membrane contamination in sarcoplasmic reticulum preparations isolated by conventional procedures.

Introduction

Two ATP-hydrolyzing enzymatic activities have been shown to be present in the sarcoplasmic reticulum membrane, a Ca^{2+}-independent or basal ATPase that requires millimolar Mg^{2+} for activity, and the Ca^{2+}-ATPase that requires both micromolar Ca^{2+} and millimolar Mg^{2+} [1—3]. In the absence of Ca^{2+}, ATP is hydrolyzed slowly, with a specific activity at 32°C of approx. 15% of that measured in the presence of Ca^{2+}. This basal ATPase activity is not inhibited by ADP and is not blocked by thiol reagents under conditions which lead to total inhibition of the Ca^{2+}-ATPase enzyme [1,4]. The basal activity is completely abolished when the sarcoplasmic reticulum membranes are solubilized by cholate, deoxycholate or Triton X-100 [5—7]. In the absence of Ca^{2+} the interaction of ATP with the enzyme does not give rise to an acid-stable phosphorylated intermediate, in contrast to the Ca^{2+}-ATPase which in the presence of Ca^{2+} readily forms one or more acid-stable phosphorylated intermediates (for a review see Ref. 8). It has been shown that the basal ATPase is responsible for the early burst of phosphate liberation observed during the ATPase reaction, as determined by fast-quenching kinetic measurements [9], from which it was concluded that the basal ATPase activity is due to an alternate pathway of the Ca^{2+}-transport enzyme. However, it is not clear at present whether this basal ATPase is a separate enzyme from the Ca^{2+}-ATPase or whether both activities are two different forms of the same enzyme, as has been suggested recently on the basis of temperature and detergent effects on both basal and Ca^{2+}-ATPase activities [10].

In this work we present evidence showing that the basal ATPase activity present in sarcoplasmic reticulum is originated from contamination of sarcoplasmic reticulum with mitochondrial and surface membrane fragments, since highly purified sarcoplasmic reticulum vesicles are virtually devoid of Ca^{2+}-independent ATPase activity.

Materials and Methods

Sarcoplasmic reticulum preparation. The preparation of sarcoplasmic reticulum was essentially as described elsewhere [11]. However, since the method of preparation of sarcoplasmic reticulum is important in the context of this work, a detailed description of our method follows. Albino rabbits were killed by neck concussion and jugular incision, and the back and hind leg muscles were removed and immediately placed on ice. The rest of the procedures were all performed at 4°C. The combined muscles, cleaned and cut into small pieces, were homogenized in a Waring blender with 4 vols. of buffer I (0.3 M sucrose, 0.02 M Tris-maleate, pH 7.0) for 2 intervals of 30 s and 20 s,

respectively. To remove fat and connective tissue the suspension was centrifuged at 3000 × *g* for 20 min. The supernatant was recentrifuged for 20 min at 10 000 × *g* to remove contaminating mitochondria. The resulting supernatant was filtered through several layers of cheesecloth, and solid KCl was added to 0.5 M final concentration to solubilize the contaminating myofibrillar muscle proteins. The suspension was centrifuged at 150 000 × *g* (r_{av}) in a Beckman 45 Ti rotor for 30 min. The pellets, containing the sarcoplasmic reticulum vesicles, were resuspended in buffer I, and washed twice by centrifugation and resuspension as described above.

Solubilization of sarcoplasmic reticulum with Triton X-100. A fresh sarcoplasmic reticulum preparation was solubilized with Triton X-100 as described previously [12], using 10 mg/ml protein, 15 mg/ml Triton X-100, 10% glycerol, 7 mM β-mercaptoethanol, and 50 mM Tris-HCl, pH 8.5. Triton was added drop-wise, rapidly, under vigorous stirring, and the resulting slightly turbid solution was immediately centrifuged at 100 000 × *g* (r_{av}) for 45 min in a Beckman 40 rotor. After carefully removing all contaminating supernatant, the small brownish pellets were resuspended in buffer I. After protein determination, a second solubilization with Triton was carried out using a sarcoplasmic reticulum : Triton X-100 ratio of 2 : 1 (w/w) (instead of the 1 : 1.5 ratio of the first solubilization), and excluding β-mercaptoethanol. The resulting suspension was centrifuged as above, and the pellets, which will be referred to as the Triton X-100-insoluble fraction, were resuspended in buffer I and stored lyophilized at −20°C.

Fractionation of sarcoplasmic reticulum in sucrose density gradients. To separate different types of sarcoplasmic reticulum vesicle [13], discontinuous sucrose density gradients of 25% (1.5 ml), 35% (4.0 ml), 40% (5.0 ml) and 50% (1.5 ml) sucrose (w/v) (1.09, 1.13, 1.16, 1.19, density in g/cm^3 at 20°C, respectively) were used. All sucrose solutions contained 20 mM Tris-maleate buffer, pH 7.0. 1 ml of the sarcoplasmic reticulum preparation (10 mg protein) was layered at the top of the gradients and centrifuged overnight at 4°C in an SW 40 Ti rotor at 150 000 × *g* (r_{av}). The resulting fractions were diluted after collection approx. 10-fold with 20 mM Tris-maleate, pH 7.0, and concentrated by centrifugation at 150 000 (r_{av}) in a 45 Ti rotor for 30 min. The pellets were resuspended in buffer I.

Loading of sarcoplasmic reticulum with calcium phosphate. To load the vesicles with calcium phosphate the procedure described by Bonnet et al. [14] was followed with minor modifications. Each one of the fractions isolated by its density in sucrose was incubated at 0.1 mg/ml with 2 mM ATP, 0.3 mM $CaCl_2$, 50 mM potassium phosphate buffer, pH 7.5, 5 mM $MgCl_2$, and 0.15 M KCl (loading medium) for 20 min at 22°C. At the end of the incubation period, the vesicles were concentrated by centrifugation at 150 000 × *g* for 30 min, and the pellets were resuspended gently in loading medium. The loaded and unloaded vesicle populations in each fraction were then separated by centrifugation on discontinuous sucrose gradients of the following compositions: 35, 50 and 63% (w/v) sucrose (4 ml each) in loading medium. After layering approx. 1.5 ml of each fraction on top of the gradients, the tubes were centrifuged for 75 min at 150 000 × *g* (r_{av}) in an SW Ti 40 rotor at 10°C. The resulting fractions were collected from the bottom, diluted in 20 mM Tris-maleate, pH 7.0, concen-

trated by centrifugation, and resuspended in buffer I. Vesicles loaded with calcium phosphate were unloaded as described in Ref. 14 by incubating in 10 mM Mops, pH 6.0, 100 mM $MgCl_2$ at 10°C for 20 min at a protein concentration of 0.05 mg/ml. The vesicles were collected by centrifugation at 150 000 × *g* for 30 min. The amount of calcium phosphate remaining in the vesicles after the loading and unloading procedures was determined as described elsewhere [14].

Biochemical and chemical assays. Monoamine oxidase was measured by a modification of the method of Tabor et al. [15], by following the formation of benzaldehyde ($\epsilon_M = 1.32 \cdot 10^4$) spectrophotometrically at 250 nm at 25°C in an assay system containing $2.5 \cdot 10^{-3}$ M benzylamine and $5 \cdot 10^{-2}$ M phosphate buffer, pH 7.6. Monoamine oxidase specific activity is expressed as nmol of benzaldehyde produced/mg per min. Cytochrome *c* oxidase was assayed polarographically by measuring the oxygen consumption with a Clark electrode at 25°C, in an assay system containing $7.5 \cdot 10^{-2}$ M phosphate buffer, pH 7.2, $3.0 \cdot 10^{-5}$ M cytochrome *c*, $3.35 \cdot 10^{-3}$ M ascorbic acid, 200 μg fatty acid-free bovine serum albumin, and $3.0 \cdot 10^{-4}$ M *N,N,N',N*-tetramethyl-*p*-phenylenediamine [16].

ATPase activities were determined as Mg^{2+}-ATPase and 'total' ATPase, and Ca^{2+}-ATPase activity was calculated as the difference between them. The reaction medium to determine Mg^{2+}-ATPase activity contained 20 mM Tris-maleate, pH 7.0, 5 mM $MgCl_2$, 4 mM ATP-Tris, 100 mM KCl, 1 mM EGTA and 0.1 mg/ml of enzyme in a final volume of 1.0 ml. To measure the total ATPase activity, EGTA was excluded from the medium and 0.1 mM $CaCl_2$ was added. The enzyme was pre-incubated in the medium for 2 min at 32°C, and the reaction was started by addition of ATP. The reaction was stopped at different intervals by addition of 0.5 ml of 20% trichloroacetic acid, the tubes were centrifuged in a clinical centrifuge for 10 min to remove precipitated protein, and the supernatant was used to determine the amount of inorganic phosphate produced as described elsewhere [17]. If the ATPase assay was carried out with 1 mg/ml Triton X-100 in the reaction solution, 1% sodium dodecyl sulfate was added after stopping the reaction with trichloroacetic acid, and the centrifugation step was omitted. This prevents Triton X-100 interference with the colorimetric reaction.

Ca^{2+} uptake was measured at 22°C in a system containing 0.1 M KCl, 5 mM $MgCl_2$, 0.1 M Tris-maleate, pH 7.0, 5 mM potassium oxalate, and 0.1 mM $^{45}CaCl_2$. The assay was started by adding 0.025 mg/ml of sarcoplasmic reticulum protein, incubating for 2 min, and then adding 2 mM ATP to start the transport reaction. The samples were filtered through Millipore filters at different times, and the amount of radioactivity remaining in the filtrate was determined by liquid scintillation counting.

The phospholipid content of the membrane fractions was determined after hydrolysis to inorganic phosphate, as previously described [18]. To resolve the individual phospholipid components the extract was subjected to two-dimensional thin-layer chromatography on silica gel plates (Merck, 250 μm thickness) as described [18]. The individual phospholipid spots were visualized by exposing the plates to iodine vapor. After scraping the spots from the plates their phospholipid content was determined as described above. A factor of 22.5 was used to convert mg of phosphate to mg of phospholipid.

Protein concentration was determined by a modified Biuret method [19] using sodium cholate to solubilize the particulate protein or by using the method of Lowry et al. [20]. Bovine serum albumin was used as standard.

Gel electrophoresis. 8 or 10% cylindrical or slab polyacrylamide gels were used. The SDS-polyacrylamide gel electrophoresis was carried out according to the method of Weber and Osborn [21] or Laemmli [23], as described in the figure legends. From 20 to 100 μg of protein were loaded in each gel after heating at 45°C for 40 min at a protein concentration of 2 mg/ml in 1% SDS, 20 mM dithiothreitol. The gels were run at 5 mA per tube (Weber and Osborn [21]) or at 20 mA per slab (Laemmli [23]). Gels were stained with Coomassie blue, and destained in 10% acetic acid/10% methanol.

Reagents. Analytical grade reagents were used throughout the study. Only metal-free ATP-Tris, obtained from Sigma Chemical Co., St. Louis, was used in our determination of the Mg^{2+}-ATPase activity to insure no interference by metals in the assay. Triton X-100 was obtained from Sigma Chemical Co.

Results

Addition of Triton X-100 to sarcoplasmic reticulum under the conditions used previously to purify the Ca^{2+}-ATPase enzyme [12] results in solubilization of approx. 80—85% of the sarcoplasmic reticulum protein. Most of the soluble protein is the Ca^{2+}-ATPase enzyme, with calsequestrin and the high-affinity Ca^{2+}-binding protein as minor components. It has been extensively reported that addition of Triton X-100 to sarcoplasmic reticulum, at concentrations that lead to disruption of the sarcoplasmic reticulum permeability barrier, results in a 2- to 3-fold increase in Ca^{2+}-ATPase activity, whereas the basal ATPase is completely abolished by the detergent. However, the brownish insoluble material remaining after the first detergent treatment, which is largely devoid of Triton X-100, has 5.4 times higher Mg^{2+}-ATPase and 3.4 times lower Ca^{2+}-ATPase activity than the original sarcoplasmic reticulum (Table I). This indicates that sarcoplasmic reticulum contains an Mg^{2+}-ATPase activity that is lost in the presence of Triton X-100, but which can be recovered after removal of the detergent. A similar enrichment in Mg^{2+}-ATPase in a Triton-insoluble fraction of sarcoplasmic reticulum was described by McFarland and Inesi [7]. After the first Triton X-100 extraction of sarcoplasmic reticulum, a fraction of the Ca^{2+}-ATPase enzyme remains in the insoluble fraction (Table I and Fig. 1). In

TABLE I

DISTRIBUTION OF Ca^{2+}-ATPase AND Mg^{2+}-ATPase ACTIVITIES AFTER SOLUBILIZATION OF SARCOPLASMIC RETICULUM WITH TRITON X-100

The ATPase activities were determined as described in the text and are expressed as μmol P_i/mg per min. TR1 and TR2 represent the Triton X-100-insoluble fractions after the first and second Triton X-100 solubilization, respectively, under the conditions described in the text.

	Total ATPase	Ca^{2+}-ATPase	%	Mg^{2+}-ATPase	%	Ca^{2+}-ATPase/Mg^{2+}-ATPase
Sarcoplasmic reticulum	1.50	1.34	89	0.16	11	8.4
TR1	1.32	0.46	35	0.86	65	0.54
TR2	1.20	0.11	9	1.09	91	0.10

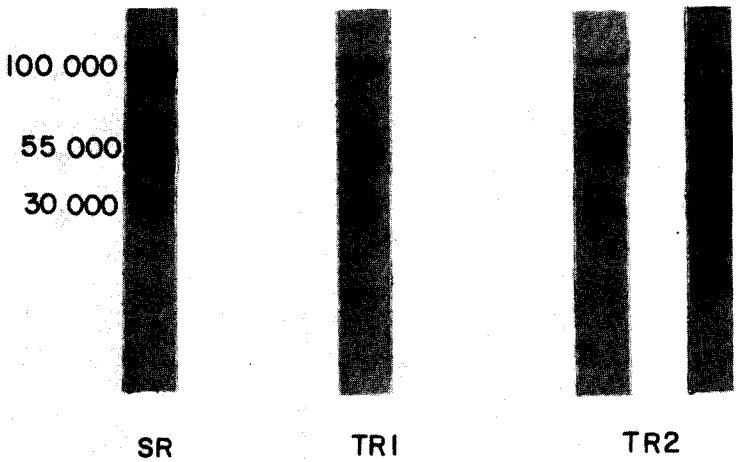

Fig. 1. Analysis of the protein compositions of sarcoplasmic reticulum (SR), and the first (TR1) and second (TR2) Triton X-100-insoluble fractions by SDS-polyacrylamide gel electrophoresis. Gel electrophoresis was carried out as described in the text according to the method of Weber and Osborn [21]. For sarcoplasmic reticulum and TR1, 40 μg of protein were loaded on the gels; for TR2, 40 and 80 μg of protein were loaded.

attempts to extract as much as possible of this residual Ca^{2+}-ATPase from the Triton-insoluble residue, a second detergent extraction was carried out. It was found (data not shown) that a weight-to-weight ratio of 1 : 2 Triton X-100 to protein yielded very low values of Ca^{2+}-ATPase, and relatively high values of basal ATPase remaining in the insoluble fraction (Table I). The material remaining after the second Triton X-100 extraction, designated hereinafter as the Triton X-100-insoluble fraction, represents from 5 to 10% of the starting sarcoplasmic reticulum protein. Analysis of the protein composition of this fraction (Fig. 1) by SDS electrophoresis in polyacrylamide gels, reveals that it is almost completely devoid of the 100 000 dalton band characteristic of the Ca^{2+}-ATPase enzyme, and that its two main components (approx. 40% of the total protein each) have apparent molecular weights in the range 50 000—55 000 and 30 000. Small percentages of lower molecular weight proteins are also visible (Fig. 1). The fact that the basal ATPase activity was enriched several-fold in the Triton X-100-insoluble fraction, together with the virtually complete elimination of the Ca^{2+}-ATPase enzyme from this fraction, indicates that the Mg^{2+}-ATPase activity remaining in the Triton X-100-insoluble fraction is indeed a different enzyme from the Ca^{2+}-ATPase. After detergent solubilization, 5—10% of the initial sarcoplasmic reticulum protein remains in the Triton X-100-insoluble fraction, with a 5- to 7-fold enrichment in basal ATPase activity, indicating that of the initial basal ATPase enzyme units present in sarcoplasmic reticulum approximately half are recovered in the insoluble fraction. However, this estimation is valid only if the basal ATPase activity remains unchanged after the removal of the detergent, since it is conceivable that the detergent treatment could either inhibit or stimulate the basal ATPase activity.

The Mg^{2+}-ATPase of the Triton X-100-insoluble fraction requires Mg^{2+} for activity, with an optimum stimulation at 5 mM $MgCl_2$ when assayed at 4 mM

TABLE II

EFFECT OF INHIBITORS OF MITOCHONDRIAL ATPase ON THE Mg^{2+}-ATPase ACTIVITY OF A TRITON-INSOLUBLE FRACTION OBTAINED FROM SARCOPLASMIC RETICULUM

The ATPase activities were determined as described in the text, except in the experiment labeled with an asterisk, which was performed using a coupled enzyme assay in an assay medium containing: 65 mM Mops-KOH, pH 8.0, 20 mM KCl, 25 mM $KHCO_3$, 100 μM KCN, 2.5 mM $MgCl_2$, 500 μM ATP, 1.7 mM phospho*enol*pyruvate, 2.7 mM NADH, 44 μg/ml of pyruvate kinase and 24 μg/ml lactate dehydrogenase. The reaction was carried out at 37°C with a protein concentration of 0.1 mg/ml. The Triton-insoluble fraction was obtained after two extractions of sarcoplasmic reticulum with Triton X-100 as described in the text.

Additions	Mg^{2+}-ATPase (μmol P_i/mg per min)	% inhibition
None *	1.0	
10 μg/ml oligomycin *	0.20	80
10 ng/ml oligomycin *	0.20	80
3 ng/ml oligomycin *	0.20	80
None	0.48	—
2 μg/ml oligomycin	0.04	87
None	0.48	
$2 \cdot 10^{-4}$ M azide	0	100
$1 \cdot 10^{-3}$ M 2,4-dinitrophenol	0.22	54
$1 \cdot 10^{-4}$ M 2,4-dinitrophenol	0.57	—19

ATP (not shown). Both Na^+ and K^+ inhibit the Mg^{2+}-ATPase activity; the enzymatic activity assayed in the presence of 100 mM KCl or NaCl is only 30 or 50%, respectively, of the values observed in the absence of monovalent cations. To determine the K_m for this Mg^{2+}-ATPase we tried to use an ATP-regenerating system, based on the oxidation of NADH coupled to the hydrolysis of ATP. However, the Triton X-100-insoluble fraction could by itself oxidize NADH in 100 mM Tris-maleate, pH 7.0. Further investigation revealed that the insoluble fraction contains an NADH dehydrogenase activity which is inactivated by boiling at 100°C for 5 min or by adding 100 μM KCN.

At this point, considering the possibility of mitochondrial contamination in the Triton X-100-insoluble fraction, we proceeded to study the effects of specific inhibitors of mitochondrial ATPase on its Mg^{2+}-ATPase activity, and to assay for the presence of mitochondrial marker enzymes in this fraction (Table II). We obtained 76% inhibition of the Mg^{2+}-ATPase activity of the Triton X-100-insoluble fraction with as little as 3 ng/ml oligomycin. Azide and high concentrations of 2,4-dinitrophenol inhibit the activity, whereas low concentrations of 2,4-dinitrophenol stimulated the Mg^{2+}-ATPase. When we assayed for the presence of monoamine oxidase and cytochrome *c* oxidase, markers for the outer and inner mitochondrial membrane, respectively, we obtained a monoamine oxidase activity of 4.7 nmol benzaldehyde/mg per min in the Triton X-100-insoluble fraction. Whilst no activity was detected in the initial sarcoplasmic reticulum, after solubilization with 1 mg deoxycholate per 10 mg protein, the sarcoplasmic reticulum showed a monoamine oxidase activity of 0.7 nmol benzaldehyde/mg per min; the activity in the insoluble fraction remained unchanged by addition of deoxycholate. The cytochrome *c* oxidase activity, expressed in ngatom oxygen/mg per min, was 3000 in the Triton X-100-

insoluble fraction. This activity is comparable to the activity present in a purified submitochondrial particle preparation. The cytochrome *c* oxidase values obtained for the sarcoplasmic reticulum fraction were much lower, with an average value of 100 ngatom oxygen/mg per min.

Analysis of the phospholipid content of the sarcoplasmic reticulum and of the Triton X-100-insoluble fraction revealed that the latter has 0.14 mg of phospholipid per mg of protein, approx. 1/3 the amount of phospholipids as the initial sarcoplasmic reticulum. At the same time, the analysis of the phospholipid composition indicates that the insoluble fraction has a high content of cardiolipin (21.1%), a phospholipid characteristic of the inner mitochondrial membrane. In contrast, the initial sarcoplasmic reticulum contains very little cardiolipin.

These results show that the fraction remaining as an insoluble residue after the two Triton X-100 extractions is highly enriched in inner mitochondrial membrane components, strongly suggesting that the Mg^{2+}-ATPase activity present in this fraction is of mitochondrial origin. To selectively eliminate mitochondrial contaminants from our sarcoplasmic reticulum preparation, and in order to assess whether sarcoplasmic reticulum devoid of mitochondrial contaminants would still have a basal ATPase component, we decided to use discontinuous sucrose density gradients for further purification, following a modified version of the fractionation procedure described by Meissner [13]. After overnight centrifugation in the discontinuous sucrose gradient system

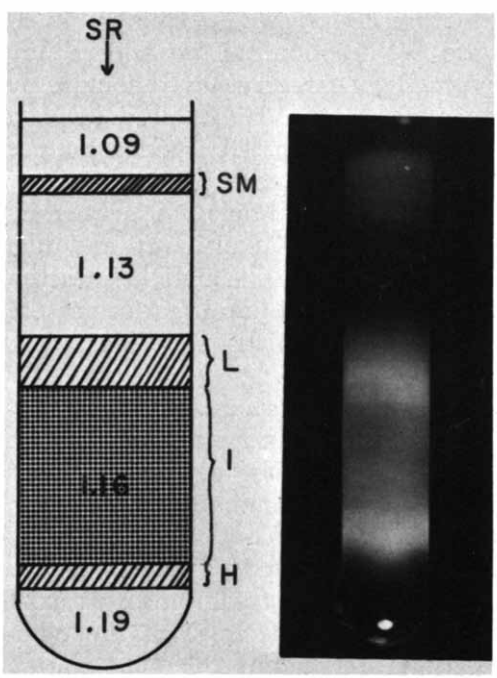

Fig. 2. Fractionation of sarcoplasmic reticulum (SR), on discontinuous sucrose density gradients. The bands formed at the interphase of the different sucrose solutions were named H, I, L and SM, as shown in the figure. For details, see text.

described in Materials and Methods, the initial sarcoplasmic reticulum segregates into four main fractions (Fig. 2): a brownish band at the interphase between the 50 and the 40% sucrose solution, which we have called the heavy or H fraction, and which comprises from 10 to 20% of the total sarcoplasmic reticulum protein loaded; a white turbid region, distributed evenly in the 40% sucrose solution, representing approx. 35 to 45% of the initial sarcoplasmic reticulum, which we designate as the intermediate or I fraction; a white and dense band at the interphase of the 40 and 35% sucrose solutions, the light or L fraction representing 20 to 30% of the initial sarcoplasmic reticulum; and a very light and discrete band, the SM fraction, at the interphase of the 35 and 25% sucrose solutions, which represents 5 to 10% of the original protein loaded. Of the four fractions obtained in this fashion, only the H, I and L fractions correspond to sarcoplasmic reticulum, as evidenced by their protein and lipid compositions, and by their enzymatic properties (as will be shown below). In agreement with the findings of Meissner [13], after polyacrylamide gel electrophoresis, the gel patterns of the H, I and L fractions indicate that the H and I fractions contain much more calsequestrin and high-affinity Ca^{2+}-binding protein than the L fraction, although still some calsequestrin was found in the L fraction (Fig. 3).

In contrast to the relatively simple protein composition of the H, I and L fractions, the SM fraction has a more complex protein composition (Fig. 3) and a cholesterol content 20-fold higher than sarcoplasmic reticulum. It also has 2—3 times higher phospholipid content per mg of protein than all the sarcoplasmic reticulum fractions [23], and is enriched in sphingomyelin. These findings strongly indicate that the SM fraction is enriched in membranes of surface origin. Further purification of the SM fraction can be achieved by removing the small amount of contaminating light sarcoplasmic reticulum by loading it with calcium phosphate in the presence of ATP [23,24]. Purified goat antibodies against this sarcoplasmic reticulum-free SM fraction do not cross-react with purified sarcoplasmic reticulum. Furthermore, the anti-SM goat antibodies bind specifically to the transverse tubule membranes, as revealed by cryostat sections of rabbit muscle stained first with anti-SM antibody, and then with fluorescent anti-goat IgG obtained from rabbit [24]. A detailed description of the composition, the enzymatic properties, and the origin of the SM fraction will be presented in detail elsewhere (Rosemblatt, M., Vergara, C., Hidalgo, C. and Ikemoto, N., unpublished results).

Since the main purpose of the further fractionation in sucrose gradients was to obtain sarcoplasmic reticulum free of mitochondrial contamination, the four resulting fractions were assayed for cytochrome *c* oxidase activity to assess their purity. Although the extent of mitochondrial contamination varies from preparation to preparation, there are still low levels of contamination in the three fractions of sarcoplasmic reticulum origin, H, I and L. However, it is apparent that the H fraction has the highest values of cytochrome *c* oxidase, 198—290 ngatom oxygen/mg per min, indicating that most of the cytochrome *c* oxidase of the initial sarcoplasmic reticulum, 50—150 ngatom oxygen/mg per min, is now present in the H fraction. The corresponding values for the other fractions are (in ngatom oxygen/mg per min): 88—160 for the I, 35—125 for the L, and 12—50 for the SM fraction.

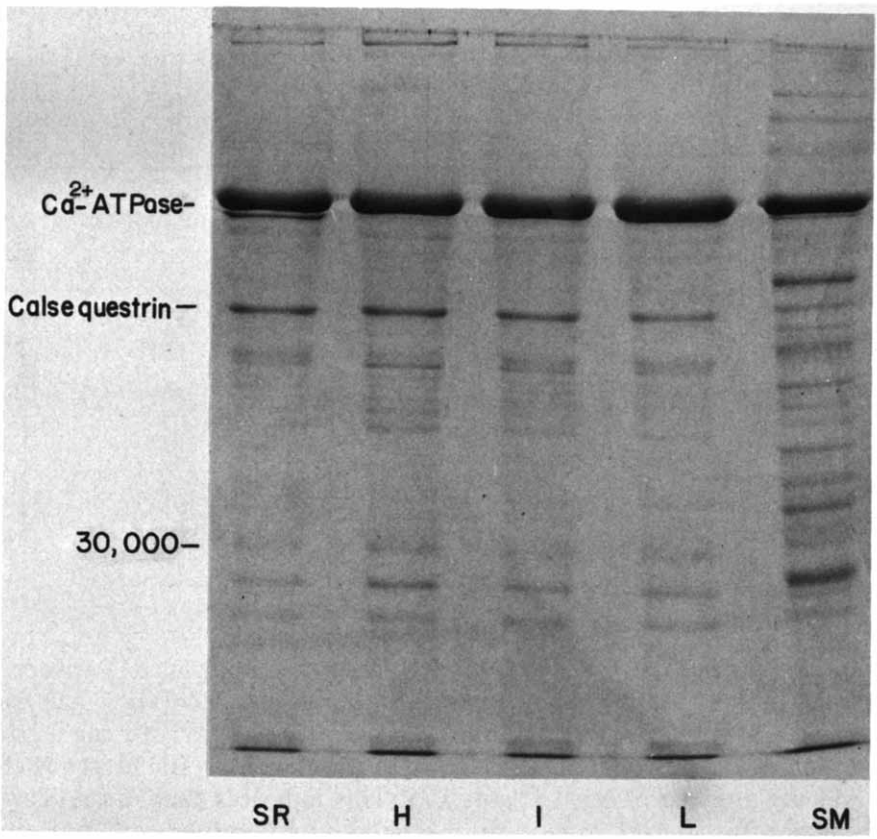

Fig. 3. Analysis of the protein composition of the fractions obtained after fractionation of sarcoplasmic reticulum (SR) in discontinuous sucrose density gradients. Gel electrophoresis was carried out according to the method of Laemmli [23], as described in the text. 20 μg of protein were loaded for each fraction. A slab gel with a thickness of 1.5 mm was used.

When the Mg^{2+}-ATPase activities of the four fractions were measured (Table III), it was found that the I fraction had the lowest activity, and that the SM fraction had very high levels of Mg^{2+}-ATPase. To test the extent of mitochondrial contribution to the measured Mg^{2+}-ATPase values, the assays were also carried out in the presence of aurovertin, which is a highly selective inhibitor of mitochondrial ATPase (Table III). The results confirm the finding that of the three fractions of sarcoplasmic reticulum origin, the H fraction has the highest mitochondrial contamination and the L fraction the lowest. It is also apparent that the Mg^{2+}-ATPase of the SM fraction is not of mitochondrial origin, since there was no inhibition by aurovertin. Oligomycin, rutamycin and azide all inhibit the Mg^{2+}-ATPase activity of the I fraction to the same extent, indicating that despite the purification in sucrose, this fraction is still contaminated with low levels of mitochondrial ATPase. In contrast, none of these inhibitors had any appreciable effect on the Mg^{2+}-ATPase of the SM fraction.

The difference between the two Mg^{2+}-ATPases, the one present in the SM fraction, and the one present in the H fraction and in the Triton X-100-

TABLE III

INHIBITION BY AUROVERTIN, OLIGOMYCIN, RUTAMYCIN AND AZIDE OF THE Mg^{2+}-ATPase ACTIVITIES OF SEVERAL SARCOPLASMIC RETICULUM FRACTIONS

The concentrations of inhibitors used were: 1.5 μM for aurovertin; 2 μg/ml for oligomycin and rutamycin, and 1 mM for azide.

Fraction	Inhibitor	Mg^{2+}-ATPase (μmol P_i/mg per min)		% Inhibition
		Control	Inhibitor present	
H	Aurovertin	0.18	0.12	33
I		0.16	0.13	19
L		0.38	0.33	13
SM		1.56	1.67	—7
I	Oligomycin	0.14	0.09	36
I	Rutamycin	0.14	0.09	36
SM		1.88	1.90	—1
I	Azide	0.14	0.09	36
SM		1.91	1.76	3

insoluble fraction (which most probably represents mitochondrial ATPase contamination), is further evidenced by the following findings. The Mg^{2+}-ATPase of the SM fraction increases slightly on addition of 5 mM Ca^{2+} to the assay medium containing 5 mM Mg^{2+}; the same activity is obtained in the presence of 5 mM Ca^{2+} in the absence of Mg^{2+} (Table IV). This indicates that the ATPase activity of the SM fraction not only is not inhibited by millimolar Ca^{2+}, but can be supported either by millimolar Ca^{2+} or millimolar Mg^{2+}. Although we refer to it as an Mg^{2+}-ATPase, it is in fact either a Ca^{2+}- or Mg^{2+}-ATPase. In contrast, 5 mM Ca^{2+} produces a 40% inhibition of the Mg^{2+}-ATPase activity of the Triton X-100-insoluble fraction measured with 5 mM Mg^{2+}. Addition of 1 mg/ml Triton X-100 to the SM fraction causes a drastic decrease in ATPase activity when assayed either with 5 mM $MgCl_2$ or with 5 mM $CaCl_2$ (Table IV). After Triton X-100 solubilization of sarcoplasmic reticulum, the insoluble residue contains only the Mg^{2+}-ATPase with characteristics of the mitochondrial enzyme. This strongly suggests that the detergent inactivates irreversibly

TABLE IV

EFFECT OF Mg^{2+}, Ca^{2+} AND TRITON X-100 ON THE ATPase ACTIVITY OF THE SM FRACTION

The assay media contained 0.1 M KCl, 20 mM Tris-maleate, pH 7.0, and 4 mM ATP, with or without 1 mg/ml Triton X-100 as indicated. The ATPase activities were measured as described in the text.

Additions	ATPase activity (μmol P_i/mg per min)	
	No detergent	Plus Triton X-100
5 mM $MgCl_2$, 1 mM EGTA	1.51	0.06
5 mM $MgCl_2$, 0.1 mM $CaCl_2$	1.91	0.50
5 mM $CaCl_2$	2.02	0.16
5 mM $MgCl_2$, 5 mM $CaCl_2$	1.82	—

only the basal ATPase of surface membrane origin, whilst the basal ATPase of mitochondrial origin is still active provided the detergent is removed from the enzyme. Furthermore, the Mg^{2+}-ATPase activity of the SM fraction, measured in the absence of K^+, is indistinguishable from that measured in the presence of 10 or 100 mM K^+ (the values for one experiment are 2.50, 2.46 and 2.50 μmol P_i/mg per min, respectively). This is in contrast to the inhibition by K^+ and Na^+ of the Mg^{2+}-ATPase activity of the Triton X-100-insoluble fraction.

The ATPase activity of the SM fraction measured in the presence of 5 mM $MgCl_2$ and 0.1 mM $CaCl_2$ represents the sum of two ATPase activities, one stimulated by 5 mM $MgCl_2$ (or 5 mM $CaCl_2$), and one that requires micromolar $CaCl_2$ and millimolar $MgCl_2$, and that is designated as Ca^{2+}-ATPase in analogy with the Ca^{2+}-ATPase of sarcoplasmic reticulum. From the difference between the ATPase activities measured in 5 mM $MgCl_2$/0.1 mM $CaCl_2$ and in 5 mM $MgCl_2$/1 mM EGTA, a value of 0.4 μmol P_i/mg per min for the Ca^{2+}-ATPase of the SM fraction is obtained. It remains to be established whether the Ca^{2+}-ATPase of SM is the same or a different enzyme from the Ca^{2+}-ATPase of sarcoplasmic reticulum. Addition of 1 mg of Triton X-100 per ml of reaction solution containing 5 mM $MgCl_2$ and 0.1 mM $CaCl_2$ results in considerable inhibition of ATPase activity of the SM fraction (Table IV), to levels comparable to the Ca^{2+}-ATPase activity of SM. These results show that the Mg^{2+}-ATPase of the SM fraction is strongly inhibited by Triton X-100, whereas the Ca^{2+}-ATPase activity is neither inhibited nor significantly stimulated by the detergent. This indicates that Triton X-100 does not convert the Mg^{2+}-ATPase of the SM fraction into a Ca^{2+}-dependent enzyme, as has been suggested for sarcoplasmic reticulum [10].

In view of the finding that after fractionation in sucrose we still had mitochondrial contamination decreasing from the heavier to the lighter sarcoplasmic reticulum fractions, and presumably surface membrane contamination in the lighter sarcoplasmic reticulum fractions, we used the calcium phosphate loading procedure described by Bonnet et al. [14] to further purify our sarcoplasmic reticulum preparation. This procedure is based on the fact that when sarcoplasmic reticulum vesicles are incubated in the presence of calcium phosphate and ATP, they hydrolyze ATP and sequester calcium phosphate from the medium. Upon centrifugation in sucrose gradients of appropriate density, the heavier, loaded sarcoplasmic reticulum vesicles go to the bottom of the tube, and the lighter, unloaded vesicles remain at the top. Each sarcoplasmic reticulum fraction (H, I and L), isolated as described above, was incubated with calcium phosphate and ATP, and the vesicles loaded with calcium phosphate were separated from the unloaded vesicles by sedimentation in sucrose density gradients as illustrated in Fig. 4. It was found that 10—20% of the vesicles in the heavy, 50—70% of the intermediate, and 40—60% of the light fraction, were actually loaded with calcium phosphate. The fractions were named as shown in Fig. 4. After removal of the accumulated calcium phosphate by dilution and sedimentation, all of them displayed high Ca^{2+} uptake in the presence of oxalate, confirming the findings reported by Bonnet et al. [14] that the loading procedure does not result in inhibition of Ca^{2+} transport. Furthermore, no difference was observed between the vesicles loaded in the presence or absence of 2 μM ruthenium red (not shown), ruling out any mitochondrial contribution

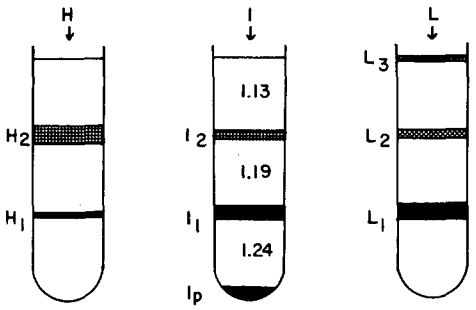

Fig. 4. Separation of the sarcoplasmic reticulum (SR) fractions obtained as illustrated in Fig. 2, by sedimentation in discontinuous sucrose density gradients after loading with calcium phosphate in the presence of ATP. For details on the loading procedure and the composition of the gradients, see text. The resulting fractions were named as follows. The loaded and unloaded vesicle populations from the H fraction were designated H_1 and H_2, respectively. The heavily loaded vesicle population from the I fraction, which formed a pellet at the bottom of the gradient, was designated I_p, and the intermediately loaded and the unloaded vesicle populations from I were designated I_1 and I_2, respectively. From the L fraction three populations of vesicles were obtained; one loaded, designated L_1 and two unloaded, L_2 and L_3.

in the loading process. Also, in the absence of ATP, the vesicles did not exhibit any calcium phosphate loading and remained on top of the gradients.

The values obtained for the Ca^{2+}- and basal ATPase activities of each fraction are presented in Table V. Of all the fractions loaded with calcium phosphate, the loaded vesicles obtained from the I fraction display the highest ratio of Ca^{2+}-ATPase to Mg^{2+}-ATPase activity. The more heavily loaded vesicles from fraction I, which are referred to as I_p, have 50-fold higher Ca^{2+}-ATPase than

TABLE V

DISTRIBUTION OF Ca^{2+}-ATPase AND Mg^{2+}-ATPase ACTIVITIES AMONG SEVERAL SARCOPLASMIC RETICULUM FRACTIONS ISOLATED ACCORDING TO THEIR DENSITY AND THEIR CALCIUM PHOSPHATE LOADING CAPACITY

The determination of ATPase activities and the procedures followed to obtain the different fractions are described in detail in the text. The symbols l and u, in parentheses, represent loaded and unloaded with calcium phosphate, respectively. The numbers represent averages values of two determinations ± S.D. Activities are expressed as μmol P_i/mg per min.

	Ca^{2+}-ATPase	Mg^{2+}-ATPase	Ca^{2+}-ATPase/Mg^{2+}-ATPase
Initial sarcoplasmic reticulum	0.90 ± 0.03	0.24 ± 0.06	3.8
H	0.60 ± 0.09	0.13 ± 0.04	4.6
H_1 (l)	0.51 ± 0.01	0.14 ± 0.04	3.6
H_2 (u)	0.44 ± 0.09	0.18 ± 0.03	2.4
I	0.87 ± 0.04	0.11 ± 0.01	7.9
I_p (l)	1.00 ± 0.03	0.02 ± 0.01	50.0
I_1 (l)	0.98 ± 0.10	0.05 ± 0.01	19.6
I_2 (u)	0.69 ± 0.03	0.10 ± 0.01	6.9
L	0.71 ± 0.11	0.18 ± 0.05	3.9
L_1 (l)	1.01 ± 0.05	0.08 ± 0.04	12.6
L_2 (u)	1.10 ± 0.11	0.16 ± 0.02	6.9
L_2 (u)	0.38 ± 0.02	0.28 ± 0.04	1.4
SM	0.40 ± 0	1.90 ± 0.77	0.21

Mg^{2+}-ATPase activity. The latter is so low that within experimental error it is virtually negligible. The less heavily loaded fraction of I, designated I_1, has 20-fold higher Ca^{2+}-ATPase than Mg^{2+}-ATPase, whereas the unloaded I fraction has a ratio of only 7. In the case of the L fraction, although there are higher levels of Mg^{2+}-ATPase than in the I fraction, it is clear that the loaded fraction L_1 has lower Mg^{2+}-ATPase than the unloaded fractions L_2 and L_3. It is interesting to note in this regard that fraction L_3 has lower density than the starting L fraction, which probably indicates that the L fraction was still contaminated with some of the very light surface membrane fragments. In favor of this view is the fact that after electrophoresis on polyacrylamide gels the gel patterns of the L_3 fraction and the SM fractions are quite similar (not shown). This would suggest that the Mg^{2+}-ATPase activity of the L fraction arises mainly from surface membrane contamination, since the L fraction has lower mitochondrial contamination than the H or I fractions.

The small proportion of loaded vesicles from the H fraction, which is the most heavily contaminated with mitochondrial fragments, do not show a significant decrease in Mg^{2+}-ATPase after loading and on the contrary, loading results in a slight decrease in Ca^{2+}-ATPase activity.

The Ca^{2+}-ATPase and Mg^{2+}-ATPase activities of the SM fraction not subjected to the loading procedure are also included in Table V for comparison purposes. It is interesting to note that the Ca^{2+}-ATPase of the SM fraction is much lower than the Mg^{2+}-ATPase activity, which is very high, a fact not found with any of the other fractions isolated.

The gel patterns of the loaded I fractions, I_p and I_1, show three main bands, the Ca^{2+}-ATPase, the high-affinity Ca^{2+}-binding protein and calsequestrin (not shown). Very little if any protein is present in the 30 000 dalton region. The loaded vesicles from the L fraction, L_1, show almost exclusively only one band in gels, the 100 000 dalton Ca^{2+}-ATPase. This is in agreement with the protein composition reported by Meissner [13] for the heavy and light sarcoplasmic reticulum vesicles.

Discussion

By solubilizing unfractionated sarcoplasmic reticulum vesicles with Triton X-100, the Ca^{2+}-ATPase activity was enriched in the soluble fraction whereas a Ca^{2+}-independent Mg^{2+}-ATPase remained in the Triton-insoluble residue. We have collected a large amount of evidence indicating that the Mg^{2+}-ATPase present in the Triton-insoluble fraction originates from mitochondrial fragment contaminants and is probably the mitochondrial F_1-ATPase. Both are inhibited by highly specific mitochondrial inhibitors, such as aurovertin, azide, oligomycin and rutamycin. Although azide is considered to be a specific inhibitor of mitochondria, it has also been reported to inhibit basal ATPase activity in sarcoplasmic reticulum [6]. These observations could indicate the presence of mitochondrial contamination in previous reports [6]. Oligomycin is a very specific inhibitor of the F_1-ATPase only when present at low concentrations (2 μg/ml); high concentrations of oligomycin (20 μg/ml) have been reported to inhibit other systems such as the $(Na^+ + K^+)$-ATPase [25]. Since Triton X-100 is known to counteract the effects of oligomycin and it was possible that some

detergent still remained in the Triton X-100-insoluble fraction, 100% inhibition was not expected. Nevertheless, 80% inhibition with as little as 3 ng/ml oligomycin strongly suggests that the Mg^{2+}-ATPase in the Triton-insoluble fraction is F_1-ATPase. The phospholipid analysis of the Triton X-100-insoluble fraction indicates that it contains a low amount of phospholipids, suggesting that this fraction is composed of membrane fragments. Since cardiolipin, a characteristic component of mitochondrial membranes, is present in a significant amount in the Triton X-100-insoluble fraction, we conclude that the extraction of sarcoplasmic reticulum with Triton X-100, as described above, results in the selective removal from sarcoplasmic reticulum of mitochondrial membrane fragments, which remain as an insoluble fraction. Accordingly, we conclude that despite the careful removal of mitochondria during the preparation of sarcoplasmic reticulum by differential centrifugation, our initial sarcoplasmic reticulum preparation was still a heterogeneous mixture of membranes, some of which arose from mitochondrial fragments which are presumably tightly bound to the sarcoplasmic reticulum vesicles, and which were then selectively enriched in the Triton X-100-insoluble fraction. Further supporting this conclusion is the fact that the SDS-polyacrylamide gel pattern for the Triton-insoluble fraction is remarkably similar to the protein pattern of the well-characterized F_1-ATPase, which has three major subunits of 54 000 (α), 50 000 (β) and 30 000 (γ) daltons representing approx. 90% of the protein, and two minor subunits of 17 300 and 5700 daltons. A glycoprotein component of M_r = 30 000 has been isolated from the Triton-insoluble fraction of sarcoplasmic reticulum [26]. It has been reported [27] that this glycoprotein reacts very quickly with a complex of fluorescamine and cycloheptaamylose when forming part of the sarcoplasmic reticulum vesicles, suggesting that the glycoprotein is externally located. The glycoprotein is also readily labeled by *N*-[^{14}C]ethylmaleimide or by a maleimide spin label [28]. The finding that the Triton X-100-insoluble fraction, which is highly enriched in the 30 000 dalton glycoprotein component, is heavily contaminated with mitochondrial markers casts doubts as to the origin of this glycoprotein. Further experiments are needed to establish unambiguously whether the 30 000 dalton glycoprotein is indeed a component of the sarcoplasmic reticulum membrane, or whether it is of mitochondrial origin.

In order to decrease the extent of mitochondrial contamination of sarcoplasmic reticulum which (depending on the preparation) accounts for approx. 50% of its basal ATPase activity, we then proceeded to further purify sarcoplasmic reticulum by density gradient sedimentation. Meissner [13] has reported that highly purified sarcoplasmic reticulum with very low values of Mg^{2+}-ATPase activity can be obtained by this procedure. However, a low extent of mitochondrial contamination persists after fractionation of the sarcoplasmic reticulum vesicles by density. For this reason, we used calcium phosphate loading in an attempt to further purify the sarcoplasmic reticulum vesicles by their ability to transport Ca^{2+}.

We found that a low extent of mitochondrial contamination persisted in the H fraction even after loading. Based on the assays to determine the presence of mitochondrial marker enzymes and on the effects of mitochondrial ATPase inhibitors such as rutamycin, aurovertin, oligomycin and azide, this H fraction

seems to contain the highest level of the same mitochondrial basal ATPase that was present in the Triton X-100-insoluble fraction.

The intermediate fraction that was loaded with calcium phosphate represents sarcoplasmic reticulum vesicles of the highest purity that we have isolated, as determined by the very high Ca^{2+}-ATPase to Mg^{2+}-ATPase ratio exhibited. This implies that the basal ATPase activity present in sarcoplasmic reticulum is not an intrinsic property of the sarcoplasmic reticulum membrane, but rather that it arises from other membrane contaminants. Analysis of the loaded I fractions by SDS-polyacrylamide gel electrophoresis reveals that the 30 000 dalton band that is highly enriched in the Triton X-100-insoluble protein fraction has virtually disappeared. The two-band components present in the 55 000 dalton region could now be assigned unambiguously to calsequestrin and the high-affinity binding protein since any possible overlap with the α and β subunits of the F_1-ATPase can now be discarded.

The SM fraction exhibits the lowest cytochrome *c* oxidase activity but has the highest Mg^{2+}-ATPase and the lowest Ca^{2+}-ATPase activity. The Mg^{2+}-ATPase from the SM fraction is a different enzyme from the mitochondrial ATPase present in the H or in the Triton X-100-insoluble fractions. The SM Mg^{2+}-ATPase is not inhibited by aurovertin, oligomycin, rutamycin, or azide. Furthermore, Na^+ and K^+ did not inhibit the SM Mg^{2+}-ATPase, in contrast to the ATPase isolated in the Triton X-100-insoluble fraction. In further contrast, the SM Mg^{2+}-ATPase is stimulated by either Ca^{2+} or Mg^{2+}, and is irreversibly inactivated by Triton X-100. It is interesting to note in this regard that a Ca^{2+}- or Mg^{2+}-activated ATPase has been recently found in a surface membrane fraction of low density obtained from chicken pectoralis muscle [29]. This enzyme was localized by ultrastructural cytochemistry, and was found to be present both in plasma membrane and transverse tubular membranes but not in sarcoplasmic reticulum. The activity was lost when the membrane was exposed to Triton X-100 or to deoxycholate. These properties of the ATPase from the surface membranes of chicken muscle are very similar to those of the Mg^{2+}-ATPase activity present in our SM fraction, further supporting the notion that the SM fraction is of surface origin and different from sarcoplasmic reticulum. It has also been reported [30] that the basal ATPase is associated with a cholesterol-rich fraction of rabbit skeletal muscle, indicating that it is present in microsomes of surface membrane origin.

In conclusion, all these data suggest that the Ca^{2+}- and basal ATPases present in sarcoplasmic reticulum preparations obtained by differential centrifugation, which is the procedure routinely used in many laboratories to obtain sarcoplasmic reticulum, are, in fact, three different enzymes. It appears that the basal ATPase activity present in the sarcoplasmic reticulum vesicles obtained by conventional procedures arises from varying degrees of both mitochondrial and surface membrane contaminants. Once the mitochondrial membrane fragments bind to the sarcoplasmic reticulum vesicles, it is very hard to further remove them by differential centrifugation or by sucrose gradient sedimentation. Since the highly purified and active sarcoplasmic reticulum obtained by calcium phosphate loading is practically devoid of Ca^{2+}-independent ATPase activity, it appears clear now that the basal ATPase of sarcoplasmic reticulum is not an intrinsic activity of the Ca^{2+}-ATPase system of sarcoplasmic reticulum.

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References

1 Hasselbach, W. and Makinose, M. (1961) Biochem. Z. 333, 518—528
2 Hasselbach, W. and Makinose, M. (1963) Biochem. Z. 339, 94—111
3 Makinose, M. and Hasselbach, W. (1965) Biochem. Z. 343, 360—382
4 Hasselbach, W. and Seraydarian, K. (1966) Biochem. Z. 345, 159—172
5 Yamamoto, T. and Tonomura, Y. (1967) J. Biochem. 62, 558—575
6 Walter, H. and Hasselbach, W. (1973) Eur. J. Biochem. 36, 110—119
7 McFarland, B.H. and Inesi, G. (1970) Biochem. Biophys. Res. Commun. 41, 239—243
8 Tada, M., Yamamoto, T. and Tonomura, Y. (1978) Physiol. Rev. 58, 1—79
9 Froehlich, J.P. and Taylor, E.W. (1976) J. Biol. Chem. 251, 2307—2315
10 Inesi, G., Cohen, J.A. and Coan, C.R. (1976) Biochemistry 15, 5293—5298
11 Ikemoto, N., Sreter, F.A. and Gergely, J. (1971) Arch. Biochem. Biophys. 147, 571—582
12 Ikemoto, N. (1975) J. Biol. Chem. 250, 7219—7224
13 Meissner, G. (1975) Biochim. Biophys. Acta 389, 51—68
14 Bonnet, J.P., Galante, M., Brethes, D., Dedieu, J.C. and Chevallier, J. (1978) Arch. Biochem. Biophys. 191, 32—41
15 Tabor, C.W., Tabor, H. and Rosenthal, S.M. (1954) J. Biol. Chem. 208, 645—661
16 Schnaitman, C.A., Erwin, V.G. and Greenwalt, J.W. (1967) J. Cell Biol. 32, 719—735
17 Fiske, C.H. and Subbarow, Y. (1928) J. Biol. Chem. 66, 375—400
18 Hidalgo, C., Ikemoto, N. and Gergely, J. (1976) J. Biol. Chem. 251, 4224—4232
19 Gornall, A.G., Bardawill, G.J. and David, M.M. (1949) J. Biol. Chem. 177, 751—766
20 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
21 Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406—4412
22 Rosemblatt, M., Hidalgo, C., Vergara, C. and Ikemoto, N. (1979) XIth International Congress of Biochemistry, Toronto, Canada, Abstract No. 05-2-R125
23 Laemmli, U.K. (1970) Nature 227, 680—685
24 Rosemblatt, M. and Ikemoto, N. (1979) J. Cell Biol. 83, 278a
25 Van Groningen, H.E.M. and Slater, E.C. (1963) Biochim. Biophys. Acta 73, 527—530
26 Ikemoto, N., Cucchiaro, J. and Garcia, A.M. (1976) J. Cell Biol. 70, 290a
27 Hidalgo, C. and Ikemoto, N. (1977) J. Biol. Chem. 252, 8446—8454
28 Hidalgo, C. and Thomas, D.D. (1977) Biochem. Biophys. Res. Commun. 78, 1175—1182
29 Malouf, N.M. and Meissner, G. (1979) Exp. Cell Res., in the press
30 Flaherty, J.O., Barrett, E.J., Bradley, D.P. and Headon, D.R. (1975) Biochim. Biophys. Acta 401, 177—183