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ISOLATION OF CALCIUM PUMP SYSTEM AND PURIFICATION OF CALCIUM ION-DEPENDENT ATPase FROM HEART MUSCLE

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SUMMARY

The procedure for the isolation of the highly active fraction of sarcoplasmic reticulum from pigeon and dog hearts is described. The method is based on the partial loading of heart microsomes with calcium and oxalate ions and the precipitation of loaded vesicles in sucrose and potassium chloride concentration gradients. Preparations obtained possess high activity of Ca^{2+}-dependent ATPase and are also able to accumulate up to 10 μmol Ca^{2+} per mg protein. Purification of sarcoplasmic reticulum membranes is accompanied by a decrease in concentration of cytochrome $a+a_3$ and an increase in the content of [^{32}P]phosphoenzyme. The basic components in "calcium-oxalate preparation" from hearts are proteins with molecular weights of about 100 000 (Ca^{2+}-dependent ATPase) and 55 000 Calcium-oxalate preparation from pigeon hearts was used for subsequent purification of Ca^{2+}-dependent ATPase. Specific activity of purified enzyme from pigeon hearts is 12–16 μmol P_i/min per mg protein. Enzyme activity of purified Ca^{2+}-dependent ATPase is inhibited by EGTA and is not sensitive to azide, 2,4-dinitrophenol and ouabain. The data obtained demonstrate the similarity of calcium pump systems and Ca^{2+}-dependent ATPases isolated from heart and skeletal muscles.

INTRODUCTION

It has been demonstrated that relaxation of a muscle is provided by the function of the so-called "calcium pump" associated with sarcoplasmic reticulum membranes (for reviews see refs. 1–4). The main component of the calcium pump system is Ca^{2+}-dependent ATPase (EC 3.6.1.3.) an enzyme being able to transport calcium ions into the gaps of sarcoplasmic reticulum vesicles.

Microsomes isolated from white skeletal muscles are the main object for studying the properties of sarcoplasmic reticulum and Ca^{2+}-dependent ATPase. In the preparations of sarcoplasmic reticulum from fast contracting muscles the specific

Abbreviations: EGTA, ethyleneglycol bis(β-aminoethyleter)-*N,N'*-tetraacetic acid; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis-(5-phenyloxazolyl-2)-benzene.

ATPase activity as well as the ability to accumulate calcium ions is much higher than that in microsomes isolated from slowly contracting muscles including heart [4, 5].

So far there is no explanation for the nature of differences in calcium-accumulating systems from heart and skeletal muscles. It may be thought that the systems of relaxation in two types of muscles are qualitatively different in structure and properties. It would be pertinent to mention the hypothesis according to which not sarcoplasmic reticulum but mitochondria are the principal relaxing factors in slowly contracting muscles [6, 7]. Another explanation for the differences is that the systems of Ca^{2+} accumulation in heart and skeletal muscle being similar of identical in vivo are impaired to a different degree in the course of the purification procedure or may contain different amounts of impurities. Thus, according to electron microscopy data [8] the vesicles of microsomal fraction from heart muscle able to accumulate calcium ions represent only 5–10 % of all membrane vesicles of this fraction.

To solve these problems it is necessary to isolate and characterize the calcium pump system from heart muscle. The isolation of highly active preparations of sarcoplasmic reticulum and complete purification of Ca^{2+}-dependent ATPase from skeletal muscle have already been performed in several laboratories [9–11]. Similar attempts to isolate these systems from heart microsomes [12–14] were unsuccessful.

In this paper we describe a procedure for purification of the Ca^{2+} pump system from heart muscle which makes it possible to obtain preparations with high activity of Ca^{2+}-dependent ATPase. The method is based on the loading of microsomes with calcium oxalate [15] and subsequent precipitation of partly loaded vesicles in sucrose and KCl concentration gradient. The data are also presented on the isolation of pure and highly active Ca^{2+}-dependent ATPase from heart.

MATERIALS AND METHODS

Materials

Sucrose, deoxycholic acid, ammonium acetate and sodium dodecyl sulphate were obtained from Nutritional Biochemicals Corporation. Tris and imidazole were products of Merck. Sodium azide was obtained from Serva, $CaCl_2$ and oxalic acid from BDH. Dithiotreitol, ouabain, phosphoenolpyruvate and pyruvate kinase were purchased from Calbiochem. ATP was obtained from Reanal (Hungary). EGTA was from Koch-Light. [γ-^{32}P]ATP and $^{45}Ca^{2+}$ were obtained from Amersham. Deoxycholic acid was purified according to Meissner et al. [11]. Ammonium acetate (50 % saturation) was prepared in accordance with the MacLennan recommendations [9]. All solutions were made using triple distilled water.

Preparation of microsomes

Pigeon heart. Hearts were excised from decapitated pigeons, cleaned of fat, washed with distilled water and frozen in liquid nitrogen. Frozen hearts were kept at −70 °C for 2–3 months. Before homogenization 27–35 g of muscle were thawed in 180 ml of ice-cold isolation medium containing 0.29 M sucrose, 1–5 mM sodium azide and 30 mM Tris-HCl or 10 mM imidazole buffer (pH 8.3).

Hearts were homogenized in glass vessels of Virtis-45 homogenizer for 15 s at 4500 rev./min and 15–25 s at 31 000 rev./min. Cell debris, nuclei, myofibrils and mitochondria were sedimented in rotor 19 Spinco L3-50 centrifuge at 10 000 $\times g$ for 20 min.

Supernatant was passed through 4–6 layers of cheese-cloth and centrifuged at 32 000 × *g* for 60 min. The pellet obtained ("crude microsomes") was suspended in 1.17 M sucrose and 10 mM Tris · HCl buffer (pH 7.2), medium A, frozen and kept at −70 °C or used immediately for further purification.

To remove actomyosin the suspension was diluted to a protein concentration of 6–10 mg/ml with an equal volume of 1.2 M KCl, 4 mM EGTA, 4 mM ATP, 6 mM $MgCl_2$, 20 mM Tris-HCl buffer (pH 7.8) and after 25–40 min was centrifuged for 60 min at 73 000 × *g*. The precipitate ("KCl-microsomes") was suspended in medium A. All operations were carried out at 2–4 °C.

Dog heart. Heart ventricles removed from anesthesized mongrel dogs were washed with water, cut into 3–5 g pieces and frozen in liquid nitrogen. The procedure of microsomes isolation was analogous to that described above.

Skeletal muscle. Rabbits were sacrificed by decapitation after a blow on the neck. For microsome preparations white skeletal muscles of back and legs were used. The scheme of isolation was analogous to that described above. Isolation medium contained 0.15 M KCl/5 mM $NaHCO_3$ [16].

Precipitation of sarcoplasmic reticulum vesicles loaded with calcium oxalate

To the suspension of KCl-microsomes (600–800 mg protein in 50 ml of medium A) an equal volume of solution was added containing 20 mM ATP, 25 mM $MgCl_2$, 10 mM sodium azide, 0.3 mM KCl, 20 mM potassium oxalate, 60 mM Tris · HCl buffer (pH 6.7–7.0). The suspension was incubated at 20 °C with agitation, pH being monitored [17–19]. 0.1–0.2 ml portions of 0.1 M $CaCl_2$ solution were added until 15–60 % saturation of vesicles with calcium oxalate was achieved. Maximal calcium-oxalate capacity of preparations was determined by a pH-metric technique before the loading procedure. In the course of loading pH was kept at 6.5–6.7. When saturation was completed pH was adjusted to 6.6.

Suspension of loaded microsomes was cooled to 0 °C and layered by portions of 16 ml on linear gradient of KCl (0.1–0.6 M) and sucrose (0.72–1.75 M) containing

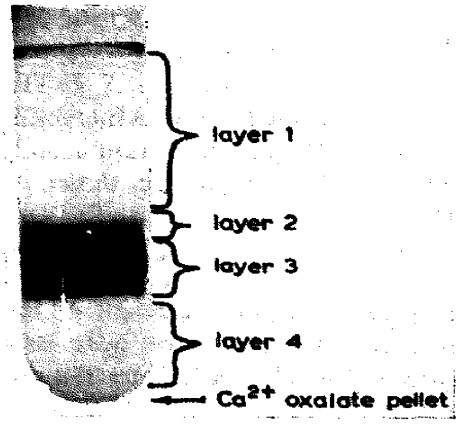

Fig. 1. Fractionation of pigeon heart microsomes loaded by 18 % with calcium oxalate. Five fractions were selected from a tube: clear upper layer 1, yellow layer 2, brown layer 3, supernatant layer 4 and Ca^{2+} oxalate pellet. For conditions of gradient preparation and vesicles loading with calcium oxalate see Materials and Methods.

1 mM ATP, 5 mM $MgCl_2$, 6.5 mM potassium oxalate, 5 mM sodium azide, 10 mM Tris · HCl buffer (pH 6.7). Centrifugation was carried out in 6 tubes of SW-27 Spinco rotor at 25 000 rev./min for 75 min. Precipitate and lower layer of supernatant (Fig. 1, layer 4) were collected. In a number of experiments the pooled fractions of the precipitate and layer 4 were diluted with 0,6 M KCl and recentrifuged at 73 000 $\times g$ for 60 min. The precipitate thus obtained will be referred to as "calcium-oxalate preparation" or purified heart microsomes.

In those experiments where calcium-oxalate preparation was obtained for a subsequent purification of Ca^{2+}-dependent ATPase the procedure of gradient preparing was changed. 3 ml of a solution containing 1.75 M sucrose, 0.6 M KCl, 3 mM ATP, 5 mM $MgCl_2$, 6.5 mM potassium oxalate, 5 mM sodium azide and 10 mM imidazole buffer (pH 6.7) were poured into the tube. Gradients were layered over a 3 ml cushion. For gradient preparation the solution described above (7 ml) and 7 ml of the solution with changed concentrations of the following components were used: sucrose (0.72 M), KCl (0.1 M), ATP (8 mM) and $MgCl_2$ (10 mM). In this case centrifugation was done for 4–5 h.

Purification of Ca^{2+}-dependent ATPase

Ca^{2+}-dependent ATPase from sarcoplasmic reticulum of rabbit skeletal muscle was isolated according to MacLennan [9]. To obtain homogeneous preparation from heart sarcoplasmic reticulum a somewhat modified method was used. Calcium-oxalate preparation was suspended in 0.25 M sucrose, 10 mM Tris · HCl buffer (pH 8.0) at protein concentration 17 mg/ml. Dithiothreitol and KCl were added to a final concentration 3 mM and 1 M, respectively. 10 % sodium deoxycholate was added in a ratio 0.1–0.12 mg/mg protein. After 30 min the suspension was centrifuged at 190 000 $\times g$ for 20 min.

Supernatant (S_1) was carefully removed with syringe, precipitate (P_1) was suspended in 0.66 M sucrose, 1 mM histidine, 50 mM Tris · HCl buffer (pH 7.6) at a protein concentration of 10 mg/ml. The following components were added to P_1: dithiothreitol to final concentration 3 mM, 50 % saturated ammonium acetate, 0.2 ml/ml and sodium deoxycholate, 0.5 mg/mg protein. After 30 min the suspension was centrifuged in glass tubes at 1500 $\times g$ for 30 min.

Dense pellet (P_2) was suspended in 1 M sucrose, 1 mM histidine, 50 mM Tris–HCl buffer (pH 8.0). To supernatant S_2 0.01 ml/ml of 50 % saturated ammonium acetate was added. After centrifugation at 1500 $\times g$ for 30 min a small pellet (P_3) and transparent supernatant were formed. 50 % saturated ammonium acetate was added to S_3 in a final proportion of 0.27–0.3 ml/ml. The solution became turbid immediately. After 15 min of incubation, the suspension was centrifuged at 1500 $\times g$ for 20 min.

Supernatant (S_4) was removed, a yellowish pellet (P_4) containing Ca^{2+}-dependent ATPase was dissolved in 1 M sucrose, 1 mM histidine, 50 mM Tris–HCl buffer (pH 8.0).

The time required to complete the procedure from homogenization to isolation of purified enzyme is usually 2 days. In the first day all operations including preliminary purification of microsomes up to layering of loaded vesicles on sucrose and KCl gradient are performed. Centrifugation of calcium loaded vesicles was done over-night. Purification of Ca^{2+}-dependent ATPase is performed the next day. In

some cases when purification took several days, crude, purified, or P_1 preparations were suspended in medium A, frozen and kept at −70 °C. Starting from 250 g of muscle tissue one can isolate (in mg protein) about 500 mg of KCl-microsomes, 50–70 mg of calcium-oxalate preparation and 10–20 mg of Ca^{2+}-dependent ATPase.

Other techniques

Phosphoenzyme formation. Incorporation of ^{32}P [20] into sarcoplasmic reticulum proteins was carried out at 0 °C in 1 ml of medium containing 50 μmol KCl, 5 μmol $MgCl_2$, 50 nmol $CaCl_2$, 30 nmol [γ-^{32}P]ATP (about 7500 cpm per nmol), 15 μmol of imidazole buffer (pH 7.1), 0.6–1.2 mg protein. Reaction was stopped by the addition of 25 ml of 5 % trichloroacetic acid, 0.6 mM ATP, 0.8 mM KH_2PO_4. Protein was precipitated by centrifugation at 1500 × *g* for 15 min, washed twice with 25 ml of 5 % trichloroacetic acid, resuspended in 1.5 ml of 0.1 M NaOH, 2 % Na_2CO_3 and heated for 20 min at 70 °C. Aliquots were taken to determine the radioactivity and concentration of protein. Radioactivity was measured on a scintillation spectrometer Mark-2 in scintillation liquid containing 6 % naphthalene, 0.4 % PPO and 0.02 % POPOP in dioxane.

Gel electrophoresis. Electrophoresis of proteins in 5 % polyacrylamide gel at pH 7.3 was performed according to Weber and Osborn [21]. Concentration of sodium dodecyl sulphate in electrode buffer and gels was 0.1 % and 1 %, respectively. Before analysis, preparations were incubated at 37 °C with 1 % sodium dodecyl sulphate and 1 % mercaptoethanol for 30 min. Electrophoresis was carried out at room temperature for 4–5 h, the current of 3 mA per tube was applied. Methylene blue was used as a tracking dye. Gels were stained in 0.1 % Coomassie brilliant blue. For the determination of molecular weights, trypsin, lactic dehydrogenase, creatine kinase, pyruvate kinase, ovalbumin (44 000 and 88 000 dalton for dimer) and bovine serum albumin (67 000 and 134 000 for dimer) were used.

Electrophoresis of proteins from phosphorylated sarcoplasmic reticulum. Protein precipitated by trichloroacetic acid (see above) was suspended in 10 % sucrose and sedimented once more. The pellet was incubated with 1 % sodium dodecyl sulphate and 1 % mercaptoethanol at 20 °C for 30 min. Aliquots of 30–50 μg protein were layered on gels prepared according to Avruch and Fairbanks [22]. Electrophoresis of phosphorylated proteins occurred at pH 2.4 [22]. Gels were stained or frozen and sliced into 2.5 mm pieces. Cherenkov radiation of each slice was counted.

Cytochromes. Concentration of cytochrome $a+a_3$ was measured according to a double wave length scheme at 604–630 nm in spectrophotometer DW-2 Aminco-Chance. Molar-extinction coefficient of dithionite-reduced cytochrome $a+a_3$ was taken as 24 $mM^{-1} \cdot cm^{-1}$.

Protein estimation. The protein concentration was measured by a modified technique of Lowry et al. [23] using bovine serum albumin as a standard.

Enzyme assays. ATPase activity was registered by the appearance of inorganic phosphate in the medium [24] or by the rate of medium acidification [17–19] using pH-340 pH-meter (U.S.S.R.) connected to a Servogor recorder. A phosphate technique was used for determination of Ca^{2+}-dependent ATPase inhibited by EGTA and Mg^{2+}-dependent ATPase insensitive to EGTA. Hasselbach's "extra" and "basal" ATPases [1] were determined by a pH-metric technique*. The latter method has

* For footnote see page 473.

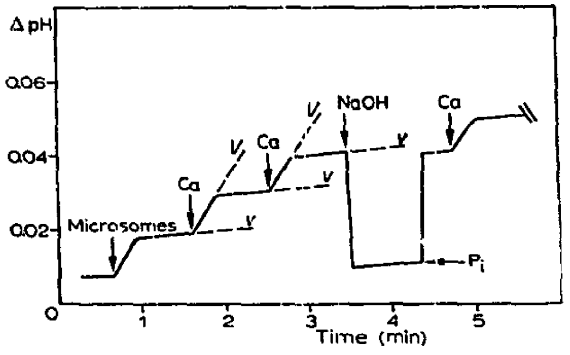

Fig. 2. Scheme illustrating the determination of calcium uptake and ATP hydrolysis by the pH-metric technique. Reaction is initiated by the addition of heart microsomes (0.3–1.0 mg protein) into a pH-meter cell containing 4.3 ml of incubation medium of the following composition: 100 mM KCl, 5 mM ATP, 6 mM $MgCl_2$, 6.5 mM potassium oxalate, 4 mM sodium azide, 10 mM Tris · HCl buffer (pH 6.75, 37 °C). V, the rate of acidification in the course of uptake of added 200 nmol of calcium aliquots. v, the rate of acidification after the removal of contaminant and added calcium from the incubation medium. The system is calibrated by the addition of 600 nmol of KH_2PO_4 (P_i). Calcium uptake is calculated using a maximal number of calcium additions which led to a burst of activity of extra ATPase. Activities of basal and extra ATPases are calculated using v and V-v values, respectively, and the rate of Ca^{2+} uptake by using the time interval needed for uptake.

certain advantages since in addition to ATPase one can also follow the rate and the value of Ca^{2+} uptake (Fig. 2).

Millipore technique. In addition to the pH-metric method the value of calcium uptake was also determined by the Millipore filtration technique with $^{45}Ca^{2+}$ [25]. In these experiments measuring was performed at 25 °C in 10 ml of medium containing 0.1 M KCl, 1 mM $MgCl_2$, 1 mM ATP, 4 mM sodium azide, 5 mM potassium oxalate and 20 mM imidazole buffer (pH 6.8). To decrease the share of contaminating calcium powerful calcium-EGTA buffer was included in the medium (0.45 mM $^{45}CaCl_2$, 50–100 cpm per nmol and 0.45 mM EGTA). Reaction was started by addition of microsomal protein. 1 ml aliquots were taken in 2 min intervals and the samples were filtered through Millipore HAWP filters with a pore size of 0.45 μm. Correction was made for the adsorbtion of $^{45}Ca^{2+}$ on a filter. The composition of scintillation liquid for radioactivity determination was as described above.

RESULTS

Initial preparation

Microsomal fraction isolated from pigeon heart muscles contains according to electron microscopic data no intact mitochondria [26, 27]. Nevertheless, azide-

* It should be emphasized that the activity of basal ATPase is not identical in this paper to the activity of Mg^{2+}-dependent ATPase determined in the presence of EGTA. Basal ATPase represents the activity of Mg^{2+}-dependent ATPase plus some activity of Ca^{2+}-dependent ATPase due to a reverse flow of Ca^{2+} from sarcoplasmic reticulum vesicles into the medium. At a low degree of saturation of vesicles with calcium oxalate (low rate of reverse Ca^{2+} flow) the activity of basal ATPase does not differ significantly from Mg^{2+}-dependent ATPase measured in the presence of EGTA. This is also true when extra and Ca^{2+}-dependent ATPase activities are compared.

TABLE I

PURIFICATION OF SARCOPLASMIC RETICULUM FROM PIGEON HEART MICROSOMES

Ca^{2+} uptake and ATPase activity were measured as described in the legend to Fig. 2. Activity of azide-sensitive ATPase was determined as a difference of ATP hydrolysis in the absence and in the presence of 5 mM sodium azide. Levels of [^{32}P]phosphoenzyme and cytochrome $a+a_3$ were determined as described under Materials and Methods. The number of preparations tested is given in parentheses. The values following ± represent the standard error of the mean (S.E.).

Step of purification	Calcium uptake capacity (μmol Ca^{2+} per mg protein)	ATPase activity (μmol P_i/min per mg protein)			Level of cytochrome (nmol per mg protein)	Phosphoenzyme level (nmol P_i per mg protein)
		Extra	Basal	Mitochondrial (azide-sensitive)		
1. Homogenate		0.13 (1)	0.08 (1)	0.8 (1)		
2. Crude preparation of microsomes	2.0 ±0.29 (8)	0.47±0.06 (8)	0.176±0.05 (8)	0.75 (2)	0.43 (2)	0.58 (1)
3. Extraction with 0.6 M KCl	2.32±0.16 (7)	0.80±0.06 (8)	0.131±0.01 (8)	1.0 (1)	0.62 (2)	0.93 (1)
4. Purification in sucrose and KCl concentration gradient						
layer 2+3	1.54±0.32 (7)	0.5 ±0.06 (7)	0.175±0.08 (7)	0.15 (1)	0.80 (2)	
layer 4	16.4 ±1.28 (6)	4.0 ±0.09 (7)	0.30 ±0.14 (6)	0.32 (1)	0.20 (1)	
sediment	12.41±1.28 (8)	2.59±0.26 (7)	0.28 ±0.11 (6)	0.24 (1)	0.17 (2)	2.45 (1)*

* In this experiment the sediment and lower part of layer 4 were pooled.

TABLE II

CALCIUM UPTAKE BY HEART MICROSOMAL PREPARATIONS MEASURED BY TWO DIFFERENT METHODS

Purified heart microsomes were isolated after 20 % (dog) and 25 % (pigeon) loading with calcium oxalate. The loading procedure was carried out in the presence of $^{45}Ca^{2+}$. The amount of Ca^{2+} sedimented with pigeon purified preparation was estimated by the Millipore technique as described under Materials and Methods. The conditions of the pH-metric measurements are similar to those described in Fig. 2. The values in parentheses represent the total calcium capacity including calcium associated with purified microsomes after gradient centrifugation.

Animal	Microsomal preparation	Ca^{2+} uptake (μmol Ca^{2+}/mg protein)	
		pH-metric technique	Millipore technique
Dog	KCl-microsomes	7.05	6.75
	Purified	11.5	11.6
Pigeon	Crude	1.15	
	Purified	9.20 (14.75)	9.7 (15.2)

inhibited ATPase activity is higher that the activity of azide-resistant ATPase in crude as well as in KCl-microsomes (Table I). In addition the content of cytochrome $a+a_3$ in these preparations was also high (Table I). It was therefore concluded that in the initial preparations of heart microsomes the essential part of vesicles is presented by mitochondrial fragments.

Purification of heart sarcoplasmic reticulum

To remove the contaminations we have used the technique of loading of sarcoplasmic reticulum vesicles with calcium oxalate [14, 15], Fig. 1, Table I. Of certain advantage in this case is the high ability of microsomal fractions, from pigeon as well as dog hearts, to accumulate Ca^{2+} in the presence of oxalate (Tables I and II). Binding of calcium to mitochondrial fragments, under conditions of our experiments, was not significant since sodium azide present in the medium inhibits ATP-dependent calcium binding by mitochondria.

After centrifugation of microsomes loaded with calcium oxalate in the linear sucrose and KCl gradient, the compact clear pellet, supernatant layer 4 and broad dark layer, containing up to 75 % of initial starting protein, are formed (Fig. 1). When centrifugation time is increased the dark layer is in turn divided into brown and pale yellow layers (Fig. 1, layers 2 and 3).

As the fragments of sarcoplasmic reticulum are loaded with calcium oxalate the amount of protein in the pellet and layer 4 increases (Fig. 3). However, this is accompanied by the decrease in specific activity of axide-resistant ATPase in initial suspension as well as in calcium-oxalate preparation (Fig. 3).

Because of the strong inhibitory effect of large doses of calcium oxalate on ATPase activity the vesicles, loaded with calcium oxalate only by 15–20 %, were used for subsequent gradient centrifugations.

As a rule one-third of the initial activity of azide-resistant ATPase is associated with the pellet fraction. Cytochrome $a+a_3$ level is decreased in the pellet fraction by 3.7-fold (Table I). The decrease in specific activity of azide-sensitive ATPase was also

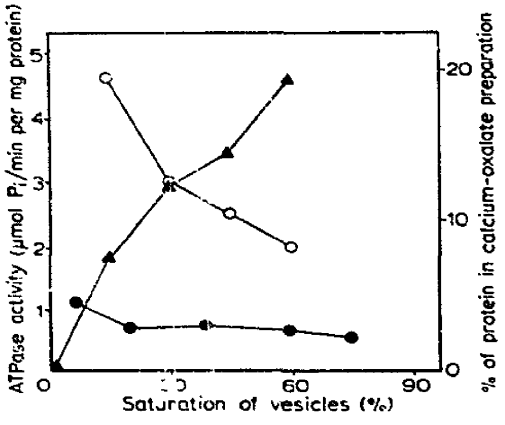

Fig. 3. Dependence of ATPase activity and protein content of calcium-oxalate preparation on vesicles loading with calcium oxalate. For conditions of measurements see legend to Table I. ▲—▲, % of protein in calcium-oxalate preparation; ●—●, activity of azide-resistant ATPase in initial microsomal suspension; ○—○, activity of azide-resistant ATPase in calcium-oxalate preparation.

found. This decrease, however is observed in all gradient layers and is probably related to the drop of mitochondrial ATPase activity during centrifugation. It is pertinent to emphasize here that it is the content of cytochrome $a+a_3$ but not the activity of labile mitochondrial ATPase that reliably indicates the presence of mitochondrial contaminations.

The highest activity of extra ATPase was found in layer 4, Fig. 1. The ability to accumulate calcium and specific activity of extra ATPase in layer 4 where smaller or less loaded vesicles are concentrated increases by 5-fold. ATPase activity of calcium-oxalate preparation from pigeon heart is only slightly lower than that of microsomal membrane fraction isolated from skeletal muscle [26, 27].

The increase in the ability of vesicles to accumulate Ca^{2+} was also found in the course of purification of dog heart microsomes (Table II). The data obtained by two different techniques show that the amount of Ca^{2+} which can be accumulated by vesicles increases by 1.6-fold in the purified preparation compared to crude microsomes. In this case we do not take into account the calcium bound to vesicles of calcium-oxalate preparation.

To determine the amount of calcium associated with calcium-oxalate preparation, immediately after gradient centrifugation, special experiment was performed (Table II). In this experiment microsomes from pigeon hearts were loaded with calcium oxalate by 25 %. The measuring of radioactivity associated with calcium-oxalate preparation have shown that it contains about 5 μmol Ca^{2+} per mg protein. Total calcium oxalate capacity of purified vesicles was 14.75 according to pH-metric data and 15.2 μmol Ca^{2+} per mg protein according to the Millipore filtration technique.

Protein composition

In Fig. 4 the data are presented on the variation of protein composition in the course of heart "calcium pump" purification. Crude microsomes (Fig. 4, gel a) and KCl-microsomes (gel b) represent a heterogeneous mixture of proteins.

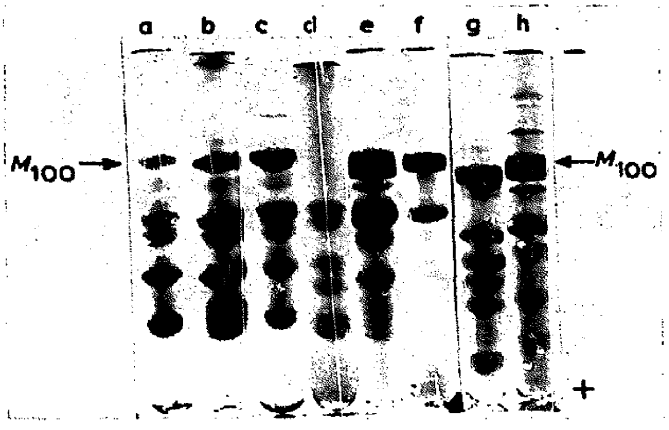

Fig. 4. Protein composition of the calcium pump system from skeletal and heart muscles. Polyacrylamide gel electrophoresis at neutral pH was performed as described under Materials and Methods. Gels: a, crude pigeon heart microsomes; b, KCl-microsomes from pigeon heart; c, layer 2 of the gradient, pigeon heart; d, layer 3 of the gradient, pigeon heart; e, calcium-oxalate preparation, pigeon heart; f, sarcoplasmic reticulum from rabbit skeletal muscle; g, KCl-microsomes from dog heart; h, calcium-oxalate preparation of dog heart.

After centrifugation in sucrose and KCl concentration gradient the proteins associated with "calcium pump" are separated from most of the low molecular weight proteins (Fig. 4, gels c–e, h). The basic components of heart calcium-oxalate preparation (gels e and h) as well as those of skeletal sarcoplamsic reticulum (gel f) are 2 proteins with molecular weights of 100 000–105 000 and 55 000–59 000 (M_{100} and M_{55}, respectively). Small amounts of low molecular weight proteins are also present in calcium-oxalate preparations. It is not clear at the moment whether they are contaminations or an essential part of heart calcium pump system.

[^{32}P]Phosphoenzyme formation

The [^{32}P]phosphoenzyme level in sarcoplasmic reticulum preparation serves as a good indicator of the degree of its purity [11]. As Table I shows the amount of ^{32}P bound per mg of protein increases in the course of the purification of heart sarcoplasmic reticulum up to 2.5 nmol ^{32}P per mg protein. If one assumes that by forming [^{32}P]phosphoenzyme bond each molecule of Ca^{2+}-dependent ATPase, with a molecular weight of 100 000, binds one molecule of phosphate, the specific activity of ATPase in calcium-oxalate preparation (Table I) can be increased by further purification at least up to 10 μmol P_i/min per mg protein.

In Fig. 5 the data are presented on electrophoretic separation of proteins from heart calcium-oxalate preparation incubated with [γ-^{32}P]ATP. Most of the label was bound to a 100 000 dalton protein component. It is important to note that protein M_{55} did not bind the label at all. This fact demonstrates that M_{55} protein is not a part of the degraded ATPase molecule [28] but rather corresponds to a calcium-binding protein isolated from sarcoplasmic reticulum of skeletal muscle [11, 29].

Isolation of Ca^{2+}-dependent ATPase from heart sarcoplasmic reticulum

The successful purification of a highly active and non-contaminated system

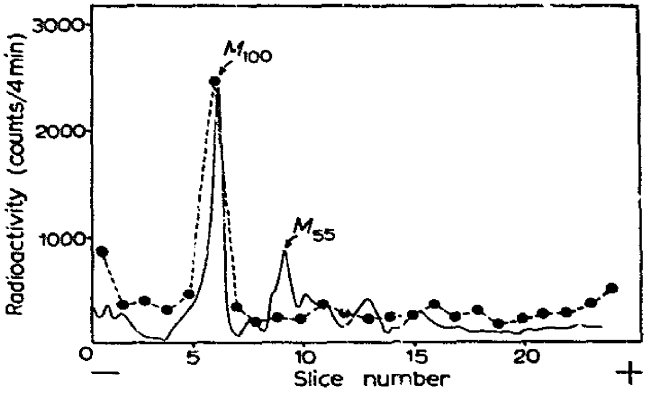

Fig. 5. Polyacrylamide gel electrophoresis of phosphorylated protein of calcium-oxalate preparation from pigeon heart. For conditions of incubation with [γ-^{32}P]ATP and electrophoretic separation of proteins at pH 2.4 see Materials and Methods. ●– – –●, radioactivity of gel slices; —, densitogram of polyacrylamide gel scanned on Integraph CM (Bende Hobein, Zurich) at 600 nm.

of "calcium pump" was a key moment for the subsequent isolation of Ca^{2+}-dependent ATPase in a homogeneous form. Modification of the MacLennan technique [9] including solubilization of enzyme with sodium deoxycholate and subsequent precipitation with ammonium acetate was used for this purpose (Table III, Fig. 6).

By treatment of calcium-oxalate preparation with a low concentration of of sodium deoxycholate it was possible to partially remove protein M_{55} which went to supernatant S_1 (Fig. 6, gel b) and to decrease the amount of calcium associated with microsomes. The latter is very important since the presence of Ca^{2+} though protecting ATPase molecules from inhibitory action of detergents [10] increases the number of bands in polyacrylamide gel [26, 27]. This may be due to the activation by Ca^{2+} [30] of proteases associated with membranes of heart sarcoplasmic

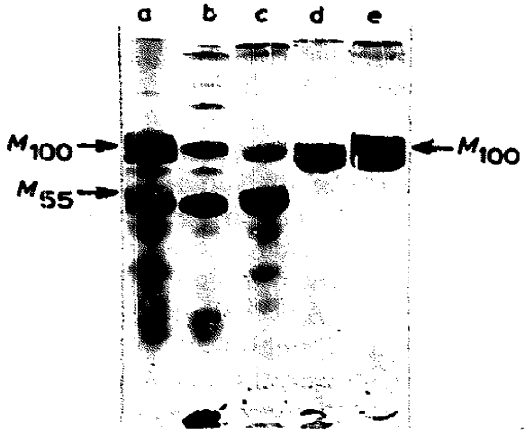

Fig. 6. Changes in the protein composition in the course of the isolation of Ca^{2+}-dependent ATPase from pigeon heart. Polyacrylamide gel electrophoresis at neutral pH was performed as described under Materials and Methods. Gels: a, calcium-oxalate preparation; b, preparation S_1 (see text and Table III); c, preparation P_2; d, preparation P_4, Ca^{2+}-dependent ATPase; e, Ca^{2+}-dependent ATPase from skeletal muscle microsomes.

TABLE III

PURIFICATION OF Ca^{2+}-DEPENDENT ATPase FROM PIGEON HEART MICROSOMES

ATPase activity was measured at 37 °C by P_i liberation [24] in a 1 ml incubation mixture containing 0.1 M KCl, 5 mM ATP, 6 mM $MgCl_2$, 5 mM sodium azide, 15 mM imidazole buffer (pH 7.0). Reaction was initiated by protein addition and terminated after 3 min by the addition of 1 ml 6 % $HClO_4$.

Step of purification	Volume (ml)	Total protein (mg)	Total activity (units*)	Specific activity (units per mg)	Yield of activity (%)
1. Calcium-oxalate preparation + 0.12 mg deoxycholate/mg protein	1.45	22.9	127.0	5.5	
2. Primary purification of calcium-oxalate preparation					
Pellet P_1	1.65	16.3	103.0	6.3	
Supernatant S_1	1.25	5.2	12.6	2.4	
3. Solubilization of Ca^{2+}-dependent ATPase by 0.5 mg deoxycholate/mg protein					
Pellet P_1 + 0.5 mg deoxycholate/mg protein	2.1	16.3	162.0	10.0	100
Pellet P_2	1.0	4.9	21.7	4.4	
Pellet P_3	0.5	0.67	5.3	7.9	
Supernatant S_3	1.8	10.8	130.0	12.0	81
4. Precipitation of Ca^{2+}-dependent ATPase					
Pellet P_4	1.2	7.4	112.0	15.1	69
Supernatant S_4	1.7	2.2	9.0	4.1	

* 1 unit equals the production of 1 μmol P_i per min at 37 °C.

reticulum [31]. To block endogeneous proteases [31] dithiothreitol was added to the medium at various stages of the Ca^{2+}-dependent ATPase purification.

To prevent the decrease of ATPase activity at further stages of enzyme fractionation glass centrifuge tubes were substituted for organic ones. Low speed centrifugation for 30 min was found to be sufficient to precipitate proteins in the presence of ammonium acetate. Decreasing the pH value in the suspension from 8.0 (see ref. 9) to 7.5–7.6 also protects enzyme from the inhibitory action of the detergent. The addition of 0.5 mg deoxycholate per mg protein led to a significant activation of ATPase in P_1 preparation (Table III). After its subsequent centrifugation more than 80 % of the ATPase activity was found in the transparent S_2 supernatant. P_2 precipitate which possesses negligible Ca^{2+}-dependent ATPase activity contains mainly low molecular weight proteins (Fig. 6, gel c).

The subsequent addition of ammonium acetate to S_2 or S_3 supernatant (0.27–0.30 ml/ml) resulted in a significant turbidity of suspensions. Ca^{2+}-dependent ATPase (P_4) with a molecular weight of 100 000–105 000 (Table III, Fig. 6, gel d) could be further precipitated from S_2 or S_3 by simple centrifugation at $1500 \times g$ for 30 min.

Precipitated ATPase dissolves completely even when the concentration of enzyme is as high as 25 mg protein/ml. In a soluble form the purified enzyme can be preserved while kept on ice in a medium containing 1.0 M sucrose. As with ATPase from skeletal muscle [9] the addition of ammonium acetate prevented the quick decrease of enzyme activity. The activity of heart muscle Ca^{2+}-dependent ATPase decreases in this case by 5–20 % in the first 10 days.

According to results of 6 separate isolation experiments specific activity of Ca^{2+}-dependent ATPase (P_4) from pigeon heart muscle is about 12–16 units per mg protein. The specific activity of enzyme isolated from rabbit skeletal muscle (Fig. 6, gel e) varied from 15 to 30 units per mg protein.

Characterization of purified enzyme

A high degree of purity of Ca^{2+}-dependent ATPase preparation, from heart sarcoplasmic reticulum proved by the existence of a single protein band in polyacrylamide gel, (Fig. 6, gel g) is also supported by the data on measurements of other ATPases in a preparation (Table IV). In our preparation ouabain-inhibited ($Na^+ + K^+$)-ATPase and azide-sensitive ATPase (mitochondrial) are absent. Uncoupling agent 2,4-dinitrophenol does not affect the rate of ATPase reaction in the preparation P_4. The value of Mg^{2+}-dependent ATPase activity was about 0.4 % of Ca^{2+}-dependent ATPase. Complete inhibition of ATPase activity could be achieved in the presence of 0.2 mM *p*-chloromercuribenzoate (Table IV).

The activity of purified Ca^{2+}-dependent ATPase is linear in the first 3 min of incubation (Fig. 7A) in the presence, as well as in the absence of ATP-regenerating system. Inhibition of ATPase in the absence of ATP-regenerating system due to an increase of reaction products can be overcome by increasing the ATP concentration or by decreasing the amount of protein in a mixture.

In Fig. 7, B, C and D the data are presented on the effects of pH, Ca^{2+} and the temperature on the activity of Ca^{2+}-dependent ATPases isolated from heart and skeletal muscle sarcoplasmic reticulum. It is evident that the parameters of the enzymes from two types of muscles are similar. pH optimum for both enzymes is in the range

TABLE IV

EFFECT OF VARIOUS AGENTS ON THE ACTIVITY OF HEART Ca^{2+}-DEPENDENT ATPase

Assay conditions were described in the legend to Table III. Sodium azide was omitted from the initial reaction mixture. Experiments 1 and 2 were carried out with different ATPase preparations. The mean values of 5 assays are presented.

Additions	Enzyme activity (units per mg protein)*
Experiment 1	
—	14.2
1 mM sodium azide	14.4
1 mM EGTA	0.05
Experiment 2	
—	12.1
0.1 mM ouabain	12.3
0.1 mM 2,4-dinitrophenol	12.2
0.2 mM *p*-chloromercuribenzoate	0.01

* 1 unit equals the production of 1 μmol P_i per min at 37 °C.

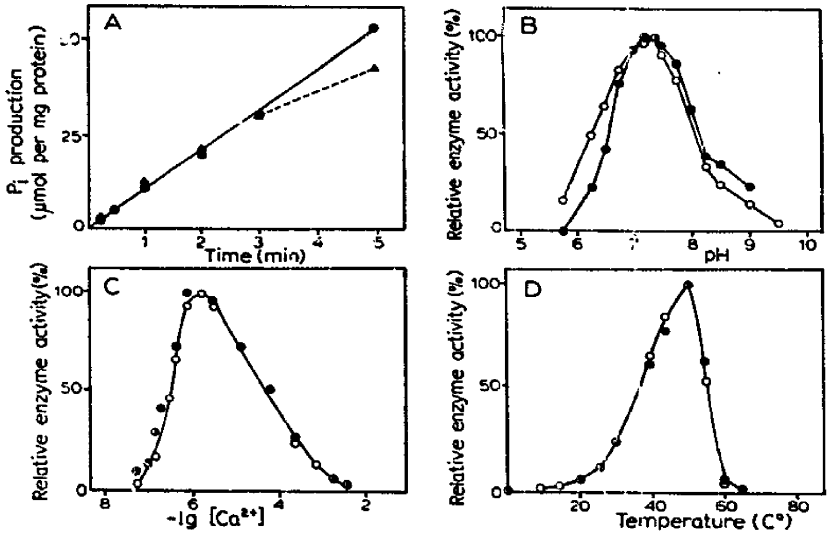

Fig. 7. Properties of Ca^{2+}-dependent ATPases isolated from pigeon heart and rabbit skeletal muscles. For composition of the incubation medium and conditions of assay see legend to Table III. A. The progress curves for the P_i production by heart Ca^{2+}-dependent ATPase. The reaction was initiated by the addition of 30 μg protein. ○—●, in the presence of 10 mM phosphoenolpyruvate and 0.6 I.U. per ml of pyruvate kinase; ▲---▲, in the absence of ATP-regenerating system. B. pH curves for ATPases. Reaction was initiated by the addition of 18 μg (skeletal muscle, ○—○) and 30 μg (heart muscle, ●—●) of protein. Incubation time was 3 min. C. ATPase activity vs. Ca^{2+}. 10 μg skeletal ATPase protein (○—○) or 24 μg of heart ATPase protein (●—●) was added to the assay mixture. 2 mM EGTA and various amounts of $CaCl_2$ were present in the mixtures, ATP and $MgCl_2$ concentrations were 3 and 4 mM, respectively. Concentration of free Ca^{2+} was calculated using K_b for EGTA with Ca^{2+} equal to $4.78 \cdot 10^6 \cdot M^{-1}$ at pH 7.0 [32]. D. Temperature dependence of ATPase activity. pH was equal to 7.15 at each temperature value. ○—○, skeletal ATPase (10 μg protein); ●—●, heart ATPase (24 μg protein).

7.2–7.4. Dependence of ATPase activities on Ca^{2+} reveals a sharp peak at a concentration of free Ca^{2+} close to 10^{-6} M.

The values of activated energy calculated from data given in Fig. 7D are also similar (27.5 and 28.3 kcal/mol for heart and skeletal ATPase, respectively). Preliminary experiments have shown that the significant differences in phospholipid composition observed in crude preparations of heart and skeletal microsomes do not exist in the purified preparations and in Ca^{2+}-dependent ATPases from these sources.

DISCUSSION

In addition to the main purpose, i.e. purification of sarcoplasmic reticulum and Ca^{2+}-dependent ATPase from heart muscle, in the course of this study it was necessary to solve a number of complicated methodological problems met by everybody dealing with heart sarcoplasmic reticulum. This concerns, first of all, the storage of enzyme preparations. We have found that quick freezing of hearts in liquid nitrogen and their storage at −70 °C for up to 2 months preserves completely the activity of Ca^{2+}-dependent ATPase and only slightly affects the ability of microsomes to accumulate Ca^{2+} [26, 27].

Important factors for the preservation of high heart "calcium pump" activity are the quick preliminary purification of microsomes in salt medium and the optimal regime of homogenization (see also refs. 5 and 13). In addition the presence of 40 % sucrose [33] or 1 M KCl in suspension and the freezing and storage of preparations at −70 °C prevents the loss of heart calcium pump activity at various stages of purification.

The results of several experiments led to the conclusion that for the isolation of homogeneous and highly active Ca^{2+}-dependent ATPase from heart muscle it is necessary to have a purified preparation of sarcoplasmic reticulum or calcium-oxalate preparation. All attempts to purify Ca^{2+}-dependent ATPase by direct treatment of crude microsomal heart preparation with detergents have failed. After the treatment of crude microsomes with cholate, deoxycholate or Triton X-100 ATPase activity always decreased significantly. This could be partially prevented by the addition to the medium of 3–5 mM $CaCl_2$. However, this procedure led to the degradation of Ca^{2+}-dependent ATPase molecule into two or more components [26, 27]. Endogenous proteases activated by calcium also lay obstacles for the isolation of the homogeneous enzyme from heart muscle [10, 12, 26, 27] by fractionation on Sepharose 4B column.

Calcium-oxalate preparation isolated from heart sarcoplasmic reticulum possesses a high Ca^{2+}-dependent ATPase activity. This is achieved by the partial loading of microsomes with calcium oxalate before centrifugation only by 15–20 % from maximal saturation. It was therefore possible to avoid the inhibition of enzyme activity (Fig. 3) apparently observed in experiments of Carsten and Reedy [14]. It is noteworthy that calcium-oxalate preparation obtained by this procedure was also able to accumulate up to 10 μmol Ca^{2+} per mg protein.

Analysis of the values of the calcium-oxalate capacity, the rates of ATPase reaction and the protein composition reveals the similarity of the properties of purified heart sarcoplasmic reticulum and reticulum from skeletal muscle. The basic components of the calcium pump system in two types of muscles are Ca^{2+}-dependent

ATPase (M_{100}) and the protein M_{55}. At the same time it cannot be completely ruled out that in the heart sarcoplasmic reticulum some low molecular weight protein components may be present which are absent in skeletal sarcoplasmic reticulum (compare gels e and f, Fig. 4). In this respect it is of interest to check the presence in purified heart "calcium pump" of protein kinase regulating the rate of calcium transport in crude preparations of heart microsomes [34–36].

The specific activity of purified Ca^{2+}-dependent ATPase from heart sarcoplasmic reticulum is about 2-fold lower than the activity of analogous enzyme isolated by MacLennan from skeletal muscle [9]. There are reasons to believe that specific activity of Ca^{2+}-dependent ATPase from heart sarcoplasmic reticulum can be increased after certain changes in homogenization procedure and by the inhibition or removal of contaminating lysosomal enzymes. The same is true, however, for skeletal muscle vesicles since one may expect the following improvement in their isolation procedure and as a result a significant increase in the ATPase activity. Thus, the activity of a purified enzyme can hardly be regarded as a decisive argument in comparing the functions of various relaxing systems.

On the basis of data obtained, the differences in relaxing systems in two types of muscles could more likely be explained by differences in the content of sarcoplasmic reticulum in cells as well as by the possible existence of special systems regulating the function of "calcium pumps".

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