Bile Salt Delipidation, Residual Phospholipids and Reactivation of the Ca^{2+}-ATPase from Sarcoplasmic Reticulum

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1. Delipidation of the Ca^{2+}-ATPase of sarcoplasmic reticulum membranes by gel chromatography employing ionic detergents (cholate, deoxycholate and mixtures of both) in the presence of glycerol has been studied with respect to residual phospholipids and ATPase activities.

2. The extent of delipidation depends on the detergent chosen and on the ionic strength of the elution buffer. Increasing ionic strength favours a more effective removal of phospholipids, down to about 1 phospholipid molecule per ATPase molecule.

3. The residual ATPase activities of the delipidated preparations are negligibly low. Extensive restoration of the Ca^{2+}-dependent ATPase activity has been achieved by oleic acid, a lysolecithin (myristoylglycerophosphocholine) and a lecithin (dimyristoylglycerophosphocholine). The percentage of reactivation by oleate depends linearly on the amount of residual phospholipids and on the detergent employed.

4. After gel filtration through an Ultrogel or Sepharose column containing 1% cholate in the elution buffer the delipidated ATPase is eluted as a reactivatable high molecular aggregate, whereas 1% deoxycholate favours the formation of completely lipid-free monomeric units which cannot be reactivated, however. A high molecular aggregate is also formed in deoxycholate, the ratio of monomer to polymer depending on the solubilizing and elution conditions.

5. The residual lipids are always composed of a mixture of all different lipid classes present in the native sarcoplasmic vesicles, even at high degrees of delipidation. Specific changes with varying extent of delipidation were not detected.

Introduction

The essential role of phospholipids in the functioning of the Ca^{2+}-transport ATPase of sarcoplasmic reticulum vesicles is well established. The effect of modification or removal of membrane phospholipids on the enzymatic properties of the Ca^{2+}-ATPase of sarcoplasmic membranes has been studied by several investigators in recent years (comp. *e.g.* [1–6]). From the 90–100 phospholipid molecules associated with one ATPase unit with a molecular weight of about 100 000, approximately two thirds can be removed without a significant loss of activity [6, 7]. A more extensive removal of phospholipids leads to a drastic decline of ATPase activity which can be reconstituted, however, under certain conditions. The problem of finding conditions under which the membrane phospholipids can be completely removed without an irreversible inactivation of the enzyme has not yet been satisfactorily solved.

In a previous study [1] we had achieved a modification of the sarcoplasmic vesicle phospholipids by phospholipase A_2 digestion and partial removal of the splitting products by bovine serum albumin, but delipidation by this rather mild procedure left at least 15–20% of the original lipid phosphate content. Recent work employing a combination of phospholipases A_2 and C as well as sphingomyelinase, followed by ether extraction of the neutral lipids led to an "apo-ATPase" with only 2 residual phospholipid molecules per ATPase molecule [2]. The other approach, namely delipidation with detergents, is a convenient and highly effective method, provided that the conditions employed permit a reactivation of the enzyme. While high concentrations of deoxycholate inactivate the Ca^{2+}-ATPase irreversibly [8, 9], low ratios of deoxycholate to protein can be used to purify the Ca^{2+}-ATPase from sarcoplasmic vesicles [2, 10, 11]. Cholate is less critical, but also less effective in its delipidating power.

Abbreviation: EGTA, ethyleneglycol bis-(2-aminoethyl)-N,N,N′,N′-tetraacetic acid.
Enzymes: Ca^{2+}-ATPase (EC 3.6.1.3); phospholipase A_2 (EC 3.1.1.4); phospholipase C (EC 3.1.4.3); sphingomyelinase (EC 3.1.4.12).

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In this report the delipidating effect of the bile salts is studied systematically in order to find the common physicochemical denominator of the various methods which have been applied more or less arbitrarily in the past. A coherent concept for the different effectivity of cholate and deoxycholate is presented. Furthermore, we have explored optimal conditions for a simple, one-step preparation of a practically phospholipid-free Ca^{2+}-ATPase with a negligible residual activity and a high degree of restoration of the Ca^{2+}-dependent ATPase activity after readdition of lipids. At first our interest was focussed only on the restitution of ATPase activity. Employing gel chromatography on Ultrogel or Sepharose columns in cholate, deoxycholate or mixtures of both in the presence of glycerol [5] we varied the ionic strength of the elution buffers and analyzed the eluted protein peaks with respect to associated residual phospholipids and ATPase activities. In our case it proved unnecessary to prepare purified ATPase [2], since experiments starting directly from native vesicles gave identical results. The lower molecular weight constituents of sarcoplasmic membrane proteins were sufficiently separated from the ATPase protein on the column as judged by the elution profiles and dodecylsulfate gel electrophoresis [12].

Materials and Methods

Sarcoplasmic reticulum vesicles from rabbit skeletal muscle were prepared according to Hasselbach and Makinose [13] as modified by de Meis and Hasselbach [14].

Sodium cholate, sodium deoxycholate and dithioerythritol were obtained from Merck (Darmstadt, Bundesrepublik Deutschland). L-α-Myristoyl-glycerophosphocholine was from Calbiochem (Lahn-Gießen, Bundesrepublik Deutschland) and L-α-dimyristoylglycerophosphocholine from Serva (Heidelberg, Bundesrepublik Deutschland).

Preparation of delipidated Ca^{2+}-ATPase by gel chromatography

a) *With 1% sodium cholate.* Native sarcoplasmic reticulum vesicles (about 50–80 mg protein) were suspended in a solution containing 20% glycerol, 50 mM Tris-Cl (pH depending on the elution buffer used) and sodium cholate (1 mg per mg protein). 3 ml (about 35–50 mg protein) of the slightly turbid suspension were applied to a column (2.5 × 90 cm) of Ultrogel AcA 34 (LKB, Bromma, Sweden; fractionation range 20 000–350 000) or in some cases Sepharose 6B, after adding solid sucrose to increase the density above that of the elution buffer. Bromphenolblue was usually added as a total volume marker. In one case asolectin (Associated Concentrates, Woodside. N.Y., USA; 0.2 µmol/mg protein), marked with "fluram" (fluorescamine, Hoffmann-La Roche, Basel, Switzerland; 0.5 µmol per µmol asolectin), was included to indicate the position of the bulk of phospholipids. The columns were preequilibrated with 20% glycerol, 50 mM Tris-Cl, pH 7.2–8.1 (measured at room temperature; comp. Table I), 1% sodium cholate, usually 1 mM dithioerythritol. In some experiments salts were added (KCl or NaCl showing no differences in the concentration range 0.1–0.6 M used). When high NaCl concentrations were employed, solid NaCl was added to the solubilizing mixture, resulting in an optically clear solution. The linear flow rate was about 3 cm · h^{-1} with the Ultrogel columns and 4 cm · h^{-1} with Sepharose 6 B; temperature 4 °C. The yield of delipidated ATPase was usually between 40 and 55% based on native vesicle protein.

From time to time the columns were calibrated with dextrane blue, indicating the void volume, and with bovine serum albumin (molecular weight 67 000). High molecular weight standard proteins could not be successfully applied, since they were dissociated under the conditions.

In most column runs an aliquot of the detergent solubilized vesicle suspension ready to apply to the column was put aside as a control for comparison of the ATPase activities. This control sample was diluted with elution buffer to a protein concentration approximately corresponding to that of the delipidated product (between 0.5 and 1 mg protein/ml eluate) and kept at 4 °C for the time the column took to run (about 20–30 h). To remove the bulk of the detergent about 5–6 ml of the eluted material as well as of the corresponding control were dialyzed as described under ATPase activities.

Two column runs were performed starting with prepurified ATPase that was prepared from native sarcoplasmic vesicles employing a low concentration of deoxycholate according to [2]. There was no difference in the results compared to starting with na-

tive vesicles. The purified ATPase had about 1 μmol phospholipids per mg protein.

b) *With 1% sodium deoxycholate.* As in a), except that the elution buffer contained 1% sodium deoxycholate instead of sodium cholate. High salt concentrations could not be used due to a gel like solidification of the deoxycholate mixture. The pH was 8.1 (50 mM Tris-Cl). The vesicles were solubilized in either cholate (slightly turbid) or deoxycholate (optically clear solution), employing 1 mg detergent per mg protein.

c) *With a mixture of cholate and deoxycholate.* As in a), except that the elution buffer contained 0.8% sodium cholate and 0.2% sodium deoxycholate as detergent. The pH was chosen and salts were added according to Table I. The vesicles were solubilized with cholate (1 : 1, w/w).

ATPase activities

ATPase activities were measured as described in [1], usually at room temperature (20–23 °C) and/or 37 °C. Before determining activity most of the detergent was generally removed by a 2 step dialysis, maintaining a glycerol level. (Solution A: 20% glycerol, 30 mM Tris-Cl pH 8.1, 0.1 M KCl; 4–6 h at room temperature. Solution B: 10% glycerol, 30 mM Tris-Cl pH 7.5, 0.05 M KCl; about 15 h at 4 °C). About 1.5–2.5 ml dialysate, corresponding to 1 mg protein, was used for each 10 ml assay. Some ATPase activities were also determined directly from column fractions (1% cholate). The cholate concentration in the assay was then 0.1%, the protein concentration between 0.05 and 0.1 mg/ml. For comparison controls using sarcoplasmic vesicles in the same medium as for gel filtration were measured parallel to the delipidated column products. Glycerol and different salt concentrations as used for the columns had no effect on the ATPase activity of control vesicles.

Reactivation with lipids. Reactivation with oleic acid was achieved by incubation of the delipidated ATPase in the assay medium (containing all components except ATP) with 50 mM sodium oleate in 20% ethanol (1 μmol oleic acid per mg ATPase) for 10 min at room temperature. The reaction was then started by addition of ATP. Myristoylglycerophosphocholine was solubilized in water and added to the assay (*e.g.* 1 mg ≈ 2 μmol per mg ATPase, incubation 20 min). For reactivation with dimyristoylglycerophosphocholine the assay was incubated with 1 mg (≈ 1.5 μmol, "solubilized" in water by prolonged ultrasonication) per mg protein for a total of 20 min (10 min sonication followed by 10 min stirring at 37 °C). In some experiments the reactivating phospholipids were directly added to the delipidated ATPase eluted from the column, before dialysis. Phospholipid analysis before and after dialysis showed that a relatively small percentage of the C_{14} phospholipids was lost during dialysis. The Ca^{2+}-dependent ATPase activities of delipidated preparations reactivated this way were in the same range as those measured after addition of the equivalent amount of phospholipid to the assay directly, as described above.

Protein concentrations

For sarcoplasmic vesicles the Biuret method, standardized by Kjeldahl, was used. Protein concentrations of solutions containing cholate, deoxycholate and/or glycerol were determined spectrophotometrically from the extinction at 280 nm in 0.1–1% sodium dodecylsulfate and 0.1 M Tris-Cl, pH 7.5, using $E^{1\%}_{1\,cm} = 12.0$ for ATPase and $E^{1\%}_{1\,cm} = 10.0$ for sarcoplasmic reticulum vesicles [15].

Phospholipids

Membrane lipids were extracted by the method of Folch [16], using 0.1 M KCl for the upper phase. Analysis of the residual phospholipids was usually carried out directly from the column eluate as well as after dialysis (for conditions see ATPase activities), giving essentially identical results. When column fractions containing glycerol and detergents were extracted the lower phase was washed once or twice with an "upper phase mixture" [16] additionally containing 0.2 M Tris-Cl, pH 8.1 and 0.05 M KCl. Lipid phosphorus was measured after ashing with $HClO_4$ according to Böttcher [17] or the nanomol method of Chalvardjian [18].

Thin-layer chromatography

Phospholipid separation on thin-layer plates (Silicagel G) was performed as described in [1]. For qualitative purposes the spots were detected by iodine vapour. A ninhydrin spray [19] was used to identify phosphatidylethanolamine and phosphatidylserine. For the quantitative determination of the phospholipid composition an aliquot of the lipid ex-

tract, corresponding to about 80–120 nmol phospholipid, was applied to the thin-layer plate as a streak. The fractions were coloured by either iodine vapour or rhodamin 6G [20], scraped off the plate and subjected to another Folch assay [16], whereby the silicagel remained in the interphase. Aliquots corresponding to an estimate of between 2 and 8 nmol phosphate were ashed and measured according to [18]. For controls a lipid extract of native vesicles and a solution of dimyristoylphosphocholine were treated the same way. The overall recoveries of phospholipids from the plates were between 50 and 80% with delipidated preparations and around 80–90% with the controls. Preparations with very low residual phospholipid (≦0.01 μmol/mg protein, especially after deoxycholate treatment) were difficult to evaluate accurately because of relatively high blank values.

Results

Gel chromatography

When native sarcoplasmic vesicles were solubilized in a mixture of glycerol, Tris-Cl buffer and bile salt detergent and applied to a gel chromatography column the properties of the eluted protein fractions depended primarily on the detergent used for solubilization and secondly on the composition of the elution buffer. A typical gel chromatogram employing 1% sodium cholate is shown in Fig. 1. The major part of the ATPase protein (peak I) was eluted as a slightly turbid suspension together with the void volume on Ultrogel columns (fractionation range 20 000 – 350 000) as well as on Sepharose 6 B which has an exclusion limit of about 4×10^6 with globular proteins. In some column runs peak I was not symmetrical, indicating that the high molecular aggregates were not homogeneous. There were only traces of a probably monomolecular ATPase (peak II? around tube number 44) and hardly any aggregates of molecular weights $2-4 \times 10^5$ detectable. In the sodium dodecylsulfate gel electrophoresis controls of the eluted ATPase no significant amounts of proteins below a molecular weight 100 000 were detected. Peak III should then contain the main bulk of the small extrinsic proteins which was not further checked. In the case of cholate the addition of salts did not exert an appreciable effect on the elution pattern, although analysis of the eluted material showed characteristic changes in the degree of delipidation, as will be described below.

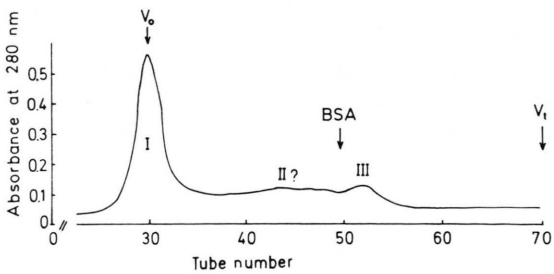

Fig. 1. Typical elution profile of cholate-solubilized sarcoplasmic vesicles in 1% sodium cholate. The sample was solubilized in a mixture of 20% glycerol, 50 mM Tris-Cl (pH as in the elution buffer) and sodium cholate (1 mg per mg protein) and was applied to a column (90 × 2.5 cm, Ultrogel AcA 34) preequilibrated with 20% glycerol, 50 mM Tris-Cl (pH varying from 7.2 to 8.1, comp. Table I), 1% sodium cholate, usually 1 mM dithioerythritol, with or without salts (0.1 M KCl or 0.5 M NaCl). Peak I = delipidated ATPase (aggregate), II = eventual traces of monomeric delipidated ATPase, III = mainly calsequestrin. The arrows indicate positions of markers for the void volume (v_o), total volume (v_t) and a standard protein (BSA = bovine serum albumin, molecular weight 67 000).

In contrast, 1% deoxycholate in the elution buffer led to varying elution profiles, depending on the ionic strength and the original solubilizing conditions. Fig. 2 demonstrates that a high percentage of a monomeric ATPase, corresponding to a molecular weight of about 100 000, was formed after solubilization employing a relatively high deoxycholate/protein ration (1 mg per mg protein) which, however, produced an irreversibly inactive enzyme. The cholate solubilized preparations (peak I), on the other hand, could be reactivated to some extent, even after being eluted with 1% deoxycholate. Both ATPase fractions (peaks I and II) were optically clear and contained very little residual phospholipid (comp. Table I). Even on Sepharose 6 B the elution pattern did not show significant amounts of dimers, trimers or tetramers.

Employing a mixture of sodium cholate (0.8%) and sodium deoxycholate (0.2%) in the elution buffer and using cholate as the solubilizing agent the delipidated ATPase was eluted mainly as the high molecular aggregate as well on Ultrogel as on Sepharose 6 B. The elution pattern resembled case (——) in Fig. 2. Different salt concentrations had effects only on the degree of delipidation.

In order to demonstrate the position of the bulk of membrane phospholipids removed during elution, a small amount of asolectin, marked with low concentrations of fluorescamine, was added to the solubiliz-

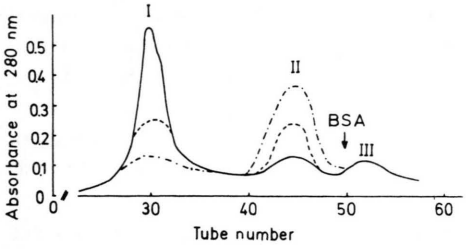

Fig. 2. Elution profiles of detergent-solubilized sarcoplasmic vesicles in 1% sodium deoxycholate. As in Fig. 1, except that 1% sodium deoxycholate and always pH 8.1 was used for elution. Peaks are numbered as in Fig. 1. Salt concentrations and solubilizing conditions as indicated below. (——) vesicles solubilized in cholate (1 : 1, w/w), no salt added. (- - -) vesicles solubilized in cholate (1 : 1, w/w), 0.1 M KCl in the elution buffer. (· - · - ·) Vesicles solubilized in deoxycholate (1 : 1, w/w), with or without 0.1 M KCl.

ing mixture before applying it to a column (1% cholate). Two yellow, fluorescent zones developed, the first one giving rise to a broad peak of low intensity overlapping with peak III, which is beyond the ATPase region. The main phospholipid peak was eluted together with the total volume.

Whenever the delipidated ATPase was in contact with bile salt detergent in appreciable amounts, a level of 20% glycerol was maintained [5]. Dithioerythritol was usually added, although several experiments without this reagent did not give divergent results, neither regarding the extent of delipidation nor ATPase activities.

Extent of delipidation

The analysis of the delipidated ATPase preparations for residual phospholipids revealed a significant dependence of the amount of phospholipids still associated with the aggregated ATPase protein (peak I of Figs. 1 and 2) on the ionic strength of the elution buffer, as demonstrated in Table I. A series of experiments with 1% sodium cholate and 50 mM Tris-Cl buffer of different pH showed that even small differences in the ionic strength accompanying the pH changes from 8.1 to 7.2 were reflected in characteristic changes in the residual phospholipid content. While 1% cholate in 50 mM Tris-Cl, pH 8.1 ($I \approx 0.02$ at 4 °C) produced only an unsatisfactory delipidation, the residual phospholipids comprising more than 0.1 μmol/mg ATPase (average of 6 experiments), pH 7.5 and 7.2 ($I \approx 0.035$ and 0.04, respectively) gave average values of 0.074 and 0.066 μmol residual phospholipids per mg ATPase (6 and 11 experiments, respectively; comp. Table I). In order to

Table I. Dependence of the amount of residual phospholipids on the column elution conditions. Delipidation of the sarcoplasmic vesicles and phospholipid determination were performed as described in Materials and Methods. Apart from the components varied in the table the elution buffer contained 20% glycerol and 1 mM dithioerythritol. Mean values are given ± S.E. (*n* in parentheses)

Composition of elution buffer			Residual phospholipids		
50 mM Tris · Cl pH	salt	ionic strength [a]	1% cholate [μmol · mg^{-1} protein]	0.8% cholate + 0.2% deoxycholate	1% deoxycholate
8.1	–	0.02	0.11 ± 0.01 (5)	0.05	0.027 ± 0.007 (3) [b] 0.006 ± 0.002 (2) [c]
7.5	–	0.035	0.074 ± 0.006 (6)	0.024	–
8.1 [d]	–	0.035	0.072	–	–
7.2	–	0.04	0.066 ± 0.003 (11)	–	–
8.1	0.1 M KCl	0.12	0.035	0.025; 0.020	0.012 ± 0.001 (4) [b] 0.003 ± 0.001 (4) [c]
7.5	0.1 M KCl	0.135	0.028	0.017	–
7.5	0.4 M NaCl	0.44	–	0.008	–
7.8	0.5 M NaCl	0.53	–	0.010	–
7.5	0.5 M NaCl	0.54	0.018 ± 0.002 (3)	–	–
8.1	0.6 M NaCl	0.62	–	0.010; 0.007	–
7.5	0.6 M NaCl	0.64	–	0.11	–

[a] Calculated for 4 °C, without consideration of the detergent.
[b] Aggregated ATPase.
[c] Monomeric ATPase.
[d] 100 mM Tris.Cl.

determine whether this effect was simply due to the change of ionic strength or rather to the pH change as such, an analogous column run was performed with 100 mM Tris-Cl, pH 8.1 ($I = 0.035$). The resulting phospholipid value of 0.072 µmol per mg ATPase indicated that the ionic strength of the medium must be a main factor determining the extent of delipidation, apart from the detergent chosen. If this was true, addition of salts should be expected to further decrease the number of phospholipid molecules still associated with the ATPase. This was, in fact, the case, as 0.5 M NaCl (together with 1% cholate) brought about a delipidation to less than 0.02 µmol phospholipids/mg ATPase. Presumably this salt effect has to do with the lowering of the critical micellar concentration of cholate with increasing ionic strength [21, 22].

With 1% deoxycholate in the elution buffer delipidation was far more effective even at low ionic strength (Table I). The residual phospholipids of the aggregated ATPase ranged from 2–3 molecules down to less than 1 molecule per ATPase unit, independent of the solubilizing conditions. The 100 000 peak was even delipidated to about 0.003 µmol residual phospholipids per mg ATPase. Probably a few phospholipid molecules are always enclosed in the protein aggregates and complete dissociation of the complexes is a prerequisite for the removal of the last traces of phospholipid molecules.

As the ATPase activities could not or only to a very low extent be restored after this practically complete delipidation with deoxycholate, a mixture of cholate and deoxycholate seemed a reasonable compromise between rather extensive delipidation and satisfactory reactivatability of the enzyme. As expected the delipidating ability of a mixture of 0.8% cholate and 0.2% deoxycholate according to Table I was found in between the two detergents alone, displaying a similar dependence on ionic strength. The residual phospholipid values ranged from about 0.05 µmol (at low I) to around 0.01 µmol (with 0.4–0.6 M NaCl) per mg ATPase protein.

ATPase activities and reactivations

The residual ATPase activities of the delipidated ATPase preparations were negligibly low. The total activities, measured in presence of 10^{-4} M Ca^{2+}-ions, did not exceed 0.04 µmol $P_i \cdot mg^{-1}$ protein $\cdot min^{-1}$ at room temperature (20–23 °C) and 0.06 µmol $P_i \cdot mg^{-1}$ protein $\cdot min^{-1}$ at 37 °C, below a residual phospholipid content of 0.1 µmol per mg protein. No marked dependence on the extent of delipidation was observed in this range. Generally the ATPase activities were measured after removal of the bulk of detergent from the column eluate by dialysis, whereby the dialysis buffers still contained glycerol. In order to look for a possible correlation between ATPase activity and residual phospholipid content it was necessary to use samples dialyzed under identical conditions for all determinations. Care had to be taken to avoid prolonged dialysis, since in the course of time (several days) part of the phospholipids was lost into the dialysis buffer. In addition, control vesicles that were detergent-solubilized, but not delipidated, were dialyzed and measured together with the delipidated preparations. These control activities were rather high in the cases of cholate (around 1 µmol $P_i \cdot mg^{-1}$ protein $\cdot min^{-1}$ at 23 °C), but close to 0 with vesicles solubilized with deoxycholate (1 : 1) and kept in 1% deoxycholate at column conditions. Therefore a series with different mixtures of cholate and deoxycholate was started (Table II). It can be seen that the ATPase activity is not significantly reduced, as long as the deoxycholate concentration is kept low. Since conditions under which the ATPase activity of control vesicles is already low cannot be expected to permit the reactivation of a delipidated preparation, a mixture of 0.8% cholate with not more than 0.2% deoxcholate was chosen for elution of the columns.

The restoration of ATPase activity was investigated with myristoylglycerophosphocholine, dimyristoylglycerophosphocholine and oleic acid. In most experiments oleic acid was chosen as the test lipid for the reactivatability of a preparation, since it had proved to be very effective in the case of phospholipase A_2/albumin [1, 23, 24,]. While myristoylglycerophosphocholine and oleic acid produced comparable reactivations at room temperature, at 37 °C the lysolecithin was far more effective (Table III), if compared at their optimal lipid/protein ratios (1 µmol oleic acid/mg protein [23] and 2 µmol myristoylglycerophosphocholine/mg protein, Table III and [2]). About 60–70% reactivation were achieved with myristoylglycerophosphocholine. The reactivating ability of dimyristoylglycerophosphocholine was in between that of oleic acid and the lysolecithin at higher temperature. There was no pronounced difference whether the C_{14} phospholipids were added

Table II. ATPase activities of detergent solubilized sarcoplasmic vesicles. The vesicles were suspended in a solution containing 20% glycerol, 50 mM Tris.Cl pH 8.1 and sodium cholate (1 mg/mg protein). Aliquots of 0.4 ml were diluted to 10 ml with a mixture of 20% glycerol, 50 mM Tris.Cl pH 8.1 and varying amounts of cholate/deoxycholate as listed in the table. 1 ml of each resulting solution (protein concentration 0.64 mg/ml) was used for direct ATPase activity measurements (diluted 1 : 10 in the assay); another 4 ml were dialyzed and a volume of dialysate corresponding to 0.1 mg protein per ml ATPase assay was used. The ATP splitting medium contained 5 mM ATP, 5 mM $MgCl_2$, 20 mM histidine pH 7.0, 40 mM KCl and 0.1 mM $CaCl_2$ (23 °C). Without detergent treatment the vesicle preparation had a total ATPase activity of 1.12 ± 0.04 μmol $P_i \cdot mg^{-1}$ protein $\cdot min^{-1}$ ($n = 17$). Mean values are given ± S.E. (n).

Concn. of detergent in the mixture added		ATPase activity (total)			
		directly measured		after dialysis	
cholate [%]	deoxycholate [%]	1 experiment	mean [μmol $P_i \cdot mg^{-1} \cdot min^{-1}$]	1 experiment	mean
1	–	0.65	0.52 ± 0.04 (8)	0.74	0.96 ± 0.05 (20)
0.8	0.2	0.41	0.58 ± 0.06 (7)	0.61	0.80 ± 0.04 (11)
0.5	0.5	0.33		0.26	
0.2	0.8	0.18		0.12	
–	1	0.17		0.07	0.17 ± 0.06 (4)
–	1[a]	0.0		0.0	
–	–	1.46[b]		0.78	

[a] In this case the vesicles were solubilized with deoxycholate (1 mg/mg protein) instead of cholate.
[b] Concentration of cholate (from initial solubilization) during ATPase activity measurement 0.0065%, which is far below the critical micellar concentration of cholate.

Table III. Reactivation of delipidated ATPase by lipids. Delipidation of sarcoplasmic vesicles and readdition of lipids to the ATPase assay were performed as described in Materials and Methods. The ATPase activities were measured after dialysis. The composition of the ATP splitting medium was 5 mM ATP, 5 mM $MgCl_2$, 20 mM histidine pH 7.0, 40 mM KCl, 0.1 mM $CaCl_2$ and 0.1 mg protein per ml assay. Temperature as indicated.

Lipid added	Concn. of lipid	ATPase activity (total)			
		1. experiment		2. experiment	
	[μmol $\cdot mg^{-1}$ protein]	23 °C	37 °C [μmol $P_i \cdot mg^{-1}$ protein $\cdot min^{-1}$]	23 °C	37 °C
–	–[a]	0.007	0.013	0.005	0.032
Oleic acid	1.0	0.30	0.41	0.47	–
Myristoylglycero-phosphocholine	0.6	–	–	–	0.6
	0.8	0.17	0.75	–	1.1
	1.0	–	–	–	1.6
	1.4	–	–	–	2.1
	2.0	0.50	1.75	–	2.2
Dimyristoylglycero-phosphocholine	1.5	–	–	0.09	0.83
Control[b]		0.7	3.0	–	–

[a] The residual phospholipid contents of these preparations was about 0.07 μmol per mg ATPase (from cholate).
[b] The same preparation of sarcoplasmic vesicles as used for delipidation, cholate-treated, but not delipidated, containing 0.67 μmol native phospholipids per mg protein.

directly to the column eluate before dialysis or simply to the ATPase assay.

In contrast to the residual ATPase activities the activities restored by oleic acid exhibited an approximately linear decrease with increasing degree of delipidation, as demonstrated in Fig. 3. Surprisingly the almost lipid-free preparations obtained by gel chromatography with cholate/deoxycholate (0.8/0.2%) were reactivated by oleic acid to a somewhat higher extent (20 – 50%) than the preparations from

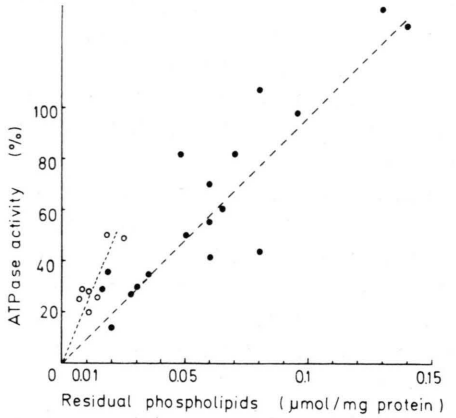

Fig. 3. Dependence of ATPase activity, reactivated with oleic acid, on residual phospholipids. Delipidation by gel chromatography, dialysis and phospholipid determination were performed as described in Materials and Methods. For the ATPase activity assay comp. Table III. Oleic acid (1 μmol per mg ATPase) was added to the assay 10 min before starting the reaction with ATP. The activities were measured at 20–23 °C in presence of 10^{-4} M $CaCl_2$ and refer to the aggregated ATPase peak. The circles represent individual experiments with many different vesicle preparations. 100% corresponds to the activity of the respective control vesicles (detergent treated, but not delipidated), after dialysis under the same conditions (comp. Table II). (●) 1% cholate in the elution buffer, (o) 0.8% cholate + 0.2% deoxycholate in the elution buffer

columns with cholate alone (15 – 35%, below 3 – 4 residual phospholipid molecules per ATPase molecule). The percent values given refer to the individual corresponding control vesicles. 100% reactivation was reached at around 0.08 – 0.1 μmol residual phospholipid content (with cholate) and even exceeded at higher phosphate values. It seems that the phospholipids cannot be completely removed by bile salts without producing an extensive inactivation of the enzyme.

The basal activities, measured in the presence of EGTA and oleate, were between 0.02 and 0.04 μmol $P_i \cdot mg^{-1}$ protein $\cdot min^{-1}$ in the whole series of delipidations. In the presence of 0.1% cholate the total ATPase activity of sarcoplasmic vesicles was depressed (comp. Table II, directly measured values), whereas delipidated preparations, reactivated by oleate, were in some cases by a factor of 2 more active with the cholate than after detergent removal. Most likely in those instances the enzyme was partly inactivated during the about 20 h of dialysis, since subsequent addition of cholate to a dialyzed fraction did not produce significant activation.

Composition of the residual phospholipids

The thin-layer chromatographic results show that even at high degrees of delipidation the residual lipids are still composed of a mixture of all the different types of phospholipid and neutral lipid classes present in the native sarcoplasmic vesicles. Several lipid extracts of ATPase preparations were analyzed quantitatively by determining the phosphate contents of individual thin-layer fractions. For preparations containing between 0.01 and 0.1 μmol residual phospholipid per mg ATPase protein the following approximate percentage distribution was found: phosphatidylcholine 30 – 40%, sphingomyelin around 20%, phosphatidylinositol 5 – 8%, phosphatidylserine 3%, phosphatidylethanolamine 15 – 20% (unidentified 10 – 30%). Although no significant changes in the phospholipid distributions with varying extent of delipidation in the given range could be detected, the phosphatidylcholine content was considerably lower and the sphingomyelin content higher as compared to native vesicles (see Table I of ref. [1]). In the region of phosphatidylethanolamine the results were difficult to evaluate, since 2 phosphate-containing zones appeared. The compound with the lower R_f value was identified as phosphatidylethanolamine by comparison with a commercial sample and the ninhydrin-positive reaction. The second compound migrated together with or close to cholic acid and contained varying amounts of lipid phosphorus (between about 10 and even 30% of the total phosphate). An accumulation of cardiolipin appeared improbable, since commercial cardiolipin (from Serva, Heidelberg, Bundesrepublik Deutschland) behaved differently. No major attempts were made to identify the unknown compound.

Discussion

The reactivation experiments with oleic acid have shown that the reactivatability of a delipidated ATPase preparation depends linearly on the amount of residual phospholipids. As long as at least 10 phospholipid molecules per ATPase molecule are present, the ATPase activity can be completely restored. At higher degrees of lipid depletion the indicated linear decline is observed, resulting in a complete loss of reactivatability when delipidation is complete. This observation is in agreement with the

results of Hardwicke and Green [8], wo did not make use of the protective effect of glycerol, however [25–27, 5]. Balancing the influence of the detergent as such against that of delipidation our finding of a linear relationship between reconstituted activity and residual phospholipid content excludes specific effects of the bile salts. The simplest explanation for the observed behaviour would be a loss of activity as a consequence of delipidation. In accordance with this conclusion the enzymatic delipidation procedures [1, 2], in which the lipids were not removed by cholate or deoxycholate, have led to a similarly reduced reactivatability with increasing delipidation. ATPase preparations delipidated to the same low residual phospholipid content of 0.02 µmol phospholipid per mg protein by two different methods, namely enzymatically [2] and by gel chromatography with a cholate/deoxycholate mixture (Fig. 3), were reactivated to practically the same extent (about 50%). On the other hand, it cannot be excluded that the complete removal of lipids might lead to an irreversible inactivation in the presence of cholate or deoxycholate. If delipidation is performed by means of a compound, usually a non-ionic detergent, which is able to support ATPase activity, apparently the natural lipids can be extensively or virtually completely removed without inactivation [5, 26, 27]. Yet this kind of experiment does not allow to determine the limiting number of hydrocarbon residues required for stabilizing an active structure. The lipid chains removed are constantly replaced by others, so that the minimal hydrophobic contacts required for stability are maintained.

Bile salts in combination with high ionic strength have been applied to the sarcoplasmic reticulum ATPase before in a more empirical manner, either in order to remove the accessorial proteins or to somewhat reduce the lipid content [10, 11, 28]. The delipidation method described in this report, namely gel chromatography in cholate and/or deoxycholate, systematically varying the ionic strength, permits to adjust the residual phospholipid content to a desired level. While cholate is best suitable for investigating the range between 0.02 and 0.15 µmol residual phospholipid per mg protein, a 4 : 1 mixture of cholate/deoxycholate works between 0.01 and 0.05 µmol phospholipid per mg protein. Deoxycholate alone produces extensive delipidation even at low ionic strength, but also a high degree of inactivation. The most plausible explanation for the different detergent and ionic strength effects would be found in the properties of the bile salt molecules themselves. The chemical difference between the closely related molecules of cholate and deoxycholate being only one OH-group expresses itself in the higher critical micellar concentration and lower micellar size of the more polar cholate species [29]. Both critical micellar concentration values are further decreased in the presence of salts [21] by a factor up to 4, from 0.001 to 0.5 M NaCl [22]. It is interesting to note that cholate + 0.5 M NaCl has a similar critical micellar concentration as deoxycholate at low ionic strength (at least at room temperature, pH about 8–8.5), both conditions leading to rather the same residual phospholipid content. This is plausible, since a lower critical micellar concentration must also increase the tendency of interactions between the detergent and the hydrophobic areas of the lipid and protein.

In addition, the presence of salt has an effect on the elution behaviour of the delipidated ATPase and thus on the aggregation of the solubilized protein. Increasing ionic strength favours the monomeric form. This could be another consequence of the dependence of the critical micellar concentration on the ionic strength. However, one cannot exclude that salt may have a direct effect on the ATPase, affecting the interactions of the hydrophilic parts of the ATPase protein.

The detergents employed have been shown to destroy the basal ATPase from the plasma membrane [30]. Therefore the low basal activity of our detergent-delipidated ATPase must be of mitochondrial origin [31].

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