Preservation of the native structure and function of Ca^{2+} -ATPase from sarcoplasmic reticulum:

Solubilization and reconstitution by new short-chain phospholipid detergent 1,2-diheptanoyl-*sn*-phosphatidylcholine

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The properties of Ca^{2+} -ATPase purified and reconstituted from rabbit skeletal sarcoplasmic reticulum (SR) has been studied in comparison with the preparations obtained by the commonly used detergent poly(oxyethylene)8-lauryl ether (C_{12}E_8) and the bile salt detergents cholate and deoxycholate. 1,2-Diheptanoyl-*sn*-phosphatidylcholine (DHPC) has been shown to be excellent for solubilizing a wide variety of membrane proteins [Kessi, Poiree, Wehrli, Bachofen, Semenza and Hauser (1994) *Biochemistry* 33, 10825–10836]. The DHPC method consistently gave higher yields of purified Ca^{2+} -ATPase with a greater specific activity than the methods with C_{12}E_8 , cholate, or deoxycholate. DHPC and C_{12}E_8 were superior to cholate and deoxycholate in active enzyme yields and specific activity. DHPC-solubilized Ca^{2+} -ATPase purified on a density gradient retained the E1Ca–E1*Ca conformational transition, whereas the enzyme from the C_{12}E_8 purification did not retain this transition. The

coupling of Ca^{2+} transported to ATP hydrolysed in the DHPC-purified enzyme was maximal and matched the values obtained with native SR, whereas the coupling was much lower for the C_{12}E_8 -purified enzyme. The specific activity of Ca^{2+} -ATPase reconstituted into dioleoylphosphatidylcholine vesicles with DHPC was up to 2-fold greater than that achieved with C_{12}E_8 , and is comparable to that measured in the native SR. Finally, the dissociation of Ca^{2+} -ATPase into monomers by DHPC preserved the ATPase activity, whereas similar dissociation by C_{12}E_8 gave only one-sixth the activity of that obtained with DHPC. These studies show that the Ca^{2+} -ATPase solubilized, purified and reconstituted with DHPC is superior to that obtained with C_{12}E_8 in significant ways, making it a preparation suitable for detailed studies on the mechanism of ion transport and the role of protein–lipid interactions in the function of membrane proteins.

INTRODUCTION

The Ca^{2+} -ATPase from skeletal muscle sarcoplasmic reticulum (SR) is one of the most studied intrinsic membrane proteins because it is a single-chain transmembrane protein with a conveniently measured function that is present in high concentration in the SR membrane. Ca^{2+} -ATPase couples the transport of 2 mol of Ca^{2+} across the SR membrane with the hydrolysis of 1 mol of ATP; a conformational change accompanies the reaction cycle [1–3]. To elucidate fully the structure and function of Ca^{2+} -ATPase, and particularly the role of lipid–protein interactions in the conformational changes that accompany ion translocation, it is necessary to isolate the protein and separate it from other membrane constituents. The most successful approach so far involves the use of detergents for solubilization [3–10] and for reconstitution into defined lipids [11–20].

A major challenge in the solubilization and reconstitution of membrane proteins is the choice of a detergent that preserves the native protein structure and biological function. Ionic detergents such as deoxycholate [3] and cholate [4] and nonionic detergents such as Triton X-100 [8], C_{12}E_9 [7] and poly(oxyethylene)8-lauryl ether (C_{12}E_8) [9,21] have been employed for the solubilization and purification of Ca^{2+} -ATPase from SR. These procedures have produced varied results in terms of active enzyme yield, specific activity and formation of phosphorylated enzyme.

Reconstitution of SR Ca^{2+} -ATPase into defined lipids has been performed with several detergents, including cholate and deoxycholate [13–15], C_{12}E_8 [15,20,22], Triton X-100 [15,23] and octylglucoside [15]. From these studies it is clear that the nature of the detergent used for reconstitution is a key factor in determining the properties of the reconstituted system. Among the detergents used in the above studies, C_{12}E_8 has emerged as one of the best for optimizing the recovery of active enzyme that retains properties most similar to the enzyme in the native SR.

A new detergent method of solubilizing membrane proteins has been described by Kessi et al. [24], using the short-chain phospholipid 1,2-diheptanoyl-*sn*-phosphatidylcholine (DHPC). Those authors showed this method to be successful in solubilizing a variety of membrane proteins, while retaining maximal native activity.

We have applied the method with DHPC to the solubilization, purification and reconstitution of Ca^{2+} -ATPase from rabbit skeletal muscle SR. In the present paper we compare the solubilization and sucrose density gradient purification of Ca^{2+} -ATPase by using DHPC with that prepared by using C_{12}E_8 , cholate and deoxycholate. Further detailed comparison of the DHPC preparation with the C_{12}E_8 preparation was made by tryptophan fluorescence, by CD and by Tween-80 gel-filtration chromatography. Reconstitution of Ca^{2+} -ATPase into dioleoylphosphatidylcholine (DOPC) by using DHPC and C_{12}E_8 was performed and the resulting vesicles were compared. Our results

Abbreviations used: C_{12}E_8 , poly(oxyethylene)8-lauryl ether; DHPC, 1,2-diheptanoyl-*sn*-phosphatidylcholine; DOPC, dioleoylphosphatidylcholine; DTT, D,L-1,4-dithiothreitol; SR, sarcoplasmic reticulum.

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demonstrate that this DHPC method produces a purified and reconstituted Ca^{2+} -ATPase with a higher activity and greater similarity to the properties of the enzyme in native SR vesicles than that obtained with any previously described preparation.

MATERIALS AND METHODS

Materials

DHPC and DOPC were obtained from Avanti Polar Lipids. C_{12}E_8 , NADH, ATP, PK-LDH enzymes, phosphoenolpyruvate, the Ca ionophore A23187, PMSF, sucrose, L-histidine, Trizma base, D,L-1,4-dithiothreitol (DTT), EGTA, Tween-80 and Sepharose CL-6B were from Sigma. Acrylamide, bisacrylamide, *N,N,N',N'*-tetramethylethylenediamine, SDS, ammonium persulphate and SM-2 Bio-Beads were obtained from Bio-Rad. Bio-Beads were washed extensively before use. Ammonium molybdate and MgCl_2 were from Fisher. All detergents were used as supplied. The purity of the detergents was given by the supplier as better than 98 %. All other chemicals were of reagent grade or superior.

The buffers used were as follows: buffer A, 0.3 M sucrose/20 mM L-histidine/1 mM DTT/5 μM PMSF (pH 8.0); buffer B, 0.3 M sucrose/10 mM L-histidine/0.6 M KCl/1 mM DTT/5 μM PMSF (pH 8.0); buffer C, 0.4 M sucrose/30 mM Mops/1 mM DTT (pH 7.5); buffer D, 50 mM Mops/1.0 M KCl/0.25 M sucrose/1 mM DTT (pH 7.5); buffer E, 50 mM Mops/0.25 M sucrose/1.0 M KCl/1 mM MgCl_2 /0.1 mM Ca^{2+} /1.0 mM DTT/0.025 % sodium azide (pH 7.5); buffer F, 50 mM Mops/80 mM KCl/5 mM MgCl_2 /1.0 mM EGTA/1.1 mM CaCl_2 (pH 7.0); buffer G, 50 mM Mops/1 M KCl/1 mM Mg^{2+} /0.1 mM Ca^{2+} /1 mM DTT/0.025 % sodium azide (pH 7.5).

Membrane preparations

Light SR vesicles were prepared from the fast-twitch skeletal muscle of New Zealand White female rabbits by using a combination of the protocols of Fernandez et al. [8] and East and Lee [3]. The SR preparation was frozen rapidly in liquid nitrogen and stored at 80 °C until use.

Analytical procedures

Protein was determined by bicinchoninic acid assay in accordance with the procedure of Smith et al. [25], with BSA as the standard. SDS/PAGE analysis was performed by the method of Laemmli [26]. Phospholipid was analysed by the procedure of Bartlett [27]. The fatty acid compositions of pure SR vesicles and of purified and reconstituted Ca^{2+} -ATPase preparations were analysed by GLC in accordance with the procedure of Carlson [28], with slight modifications.

Solubilization of SR

SR vesicle solubilization was performed with DHPC and C_{12}E_8 as described in Kessi et al. [24], with modifications. Typically, 200 mM stock solutions of detergents in solubilization buffer (buffer D) were added to freshly thawed SR vesicles to the desired final detergent concentration at a constant SR vesicle protein concentration of 6 mg/ml in the same buffer. Unless stated otherwise, SR vesicles were solubilized at 0 °C by dropwise addition of stock detergent solution with vortex-mixing for 30–60 s. Solubilization continued on ice for 30 min with intermittent mixing. The solubilization mixture was centrifuged at 100000 g_{av} at 4 °C for 25 min (Beckman Optima TLX ultracentrifuge with TL-100.3 rotor). Solubilization of protein, enzyme activity and lipid concentration were determined on ap-

propriate aliquots from the supernatant as described in Kessi et al. [24].

Purification of Ca^{2+} -ATPase on discontinuous sucrose gradient

Purified partially delipidated Ca^{2+} -ATPase was prepared from the detergent-solubilized SR vesicles by using a discontinuous sucrose gradient as described by East and Lee [3], with minor modifications. Optimal detergent concentrations of 20, 10, 10 and 7 mM were used respectively for DHPC, C_{12}E_8 , deoxycholate and cholate, on the basis of the solubilization data of the present study.

Reconstitution of purified Ca^{2+} -ATPase

Ca^{2+} -ATPase was reconstituted into the exogenous lipid DOPC by using a combination of methods from the literature [13,15], with modifications. Typically, 50 mg of DOPC in 1.5–2.0 ml of buffer (buffer E) containing appropriate detergent to give a final lipid:detergent ratio of 1:1.5 (w/w) was vortex-mixed vigorously for approx. 60 s to disperse the lipid and left at room temperature (23 °C) for 60–90 min. The suspension was clarified by sonication for 1–2 min with a Branson model 200 sonifier/cell disrupter. Partially delipidated Ca^{2+} -ATPase purified on a sucrose gradient was solubilized at a ratio of 0.6:1 (w/w) of detergent to Ca^{2+} -ATPase. The Ca^{2+} -ATPase was solubilized by vortex-mixing for 60 s and left at room temperature (23 °C) for 10 min followed by further incubation on ice for an additional 90 min.

The solubilized Ca^{2+} -ATPase was centrifuged at low speed to remove insoluble material and aggregates. The clear sample was then mixed with pre-solubilized exogenous lipid to a desired molar ratio of lipid to Ca^{2+} -ATPase, and vortex-mixed for 30 s and left at room temperature for 15 min. This mixture was incubated at 4 °C for 90 min. At the end of the incubation the detergent was removed by adsorption on Bio-Beads in two batches of 700 mg each for 1 h at 4 °C. The reconstituted vesicles were separated from the Bio-Beads by low-speed centrifugation.

Ca^{2+} -ATPase assay

Ca^{2+} -ATPase activity was measured at 37 °C in buffer (buffer F) by using the enzyme-linked continuous ATPase assay described by Coll and Murphy [7], in the presence of 1 $\mu\text{g}/\text{ml}$ of the Ca^{2+} ionophore A23187. Detergent and sucrose were eliminated from assay aliquots by a minimum of 800-fold dilution into the assay mixture.

Simultaneous measurement of Ca^{2+} uptake and ATP hydrolysis

Simultaneous measurements of Ca^{2+} uptake and ATP hydrolysis were performed with the newly described Fura Red absorbance continuous spectrophotometric assay under conditions described by Karon et al. [29]. All assays were performed at 790 nm ionized free calcium determined from the equation described by Karon et al. [29], with appropriate concentrations of SR protein or purified or reconstituted Ca^{2+} -ATPase.

Gel-filtration chromatography of detergent-dissociated sucrose-gradient-purified molecular species of Ca^{2+} -ATPase

Detergent-solubilized pure Ca^{2+} -ATPase molecular species were characterized by Sepharose CL-6B chromatography at 4 °C. The column was calibrated with standards from Sigma. A Sepharose CL-6B column (0.9 cm $\times$ 100 cm) was prepared and equilibrated with degassed 50 mM Mops buffer, pH 7.5, containing 100 mM

KCl, 0.1 mM Ca^{2+} , 0.1 mg/ml Tween-80 and 1 mM sodium azide. With DHPC a final detergent concentration of 40 mM was used for solubilization of the sample; with C_{12}E_8 a detergent concentration of 28 mM was used. To each of the solubilization mixtures 4 mg of solid DTT was added and the sample was vortex-mixed, left on ice for 30 min, and centrifuged for 10 min at low speed. The clear supernatant was applied to the Sepharose column, which was then washed with equilibration buffer at a flow rate of 6 ml/h. Fractions of 35 drops per tube were collected at 4 °C, analysed for lipid and protein, and assayed for Ca^{2+} -ATPase activity.

Fluorescence spectroscopy

Fluorescence measurements were made by diluting an aliquot of native SR vesicles or purified enzyme into 2.5 ml of buffer [150 mM Mops/2 mM EGTA (pH 7.2)]. The intrinsic fluorescence was measured with an SLM-Aminco 8300C fluorimeter, with an excitation wavelength of 285 nm. Fluorescence emission spectra of the E1 , $\text{E1}^*\cdot\text{Ca}$ and $\text{E}\cdot\text{Mg}$ states of Ca^{2+} -ATPase in native SR vesicles and in the purified enzyme preparations were recorded at 50 $\mu\text{g}/\text{ml}$ in 2.5 ml of 150 mM Mops/2 mM EGTA (pH 7.2) buffer at room temperature (23 °C). Ca^{2+} and Mg^{2+} transitions were induced by adding aliquots of 1 M stock solutions to give final concentrations of 1 mM CaCl_2 and 10 mM MgCl_2 respectively. The sequential additions were followed at a constant emission wavelength of 333 nm.

CD

CD measurements were made at room temperature with a Jasco 700 spectropolarimeter, with a band width of 2 nm and a scan speed of 2 nm/min. Each sample was scanned four times; the scans were automatically averaged. The possible effects of light-scattering in the CD measurements were minimized by using a CD cell with a narrow path length (0.1 mm) and by using identical concentrations of protein and other experimental parameters for each sample in these comparative studies.

Quasi-elastic light scattering

Quasi-elastic light scattering was performed with the Nicomp particle sizer from the Pacific Scientific (Silver Spring, MD, U.S.A.), with temperature control by Peltier heating and cooling as described previously [30].

RESULTS

Comparison of solubilization of SR membranes by DHPC, C_{12}E_8 , cholate and deoxycholate

Native SR vesicles prepared as described above were solubilized with DHPC, C_{12}E_8 , cholate, and deoxycholate at several concentrations, each ranging from 0 to 50 mM. To compare the SR-solubilizing ability of these detergents, Ca^{2+} -ATPase activity, total protein and phospholipid solubilized as a percentage of the starting amount in native SR were determined in the supernatant of the solubilized membranes after pelleting. Assays were performed after a minimum of 800-fold dilution so that essentially no detergent was present in the assay mixture. The results shown in Figure 1 represent the activity recovered after dilution.

Figure 1(A) shows the Ca^{2+} -ATPase activity recovered from the supernatant of the respective detergent-treated samples. In Figure 1(A) it is shown that significantly more activity is solubilized by the DHPC over a broad range of DHPC con-

Figure 1 Solubilization of SR total Ca^{2+} -ATPase activity by DHPC, cholate, deoxycholate and C_{12}E_8 as percentage of starting SR Ca^{2+} -ATPase activity

Activity was measured after dilution of enzyme aliquots at least 800-fold. (A) Total activity in the supernatant. Symbols: $\circ$, DHPC; $\triangle$, C_{12}E_8 ; $\square$, cholate; $\diamond$, deoxycholate at various detergent concentrations as percentages of starting SR ATPase activity. (B) Total pelleted ATPase activity. Symbols: $\circ$, DHPC; $\triangle$, C_{12}E_8 ; $\square$, cholate; $\diamond$, deoxycholate at different detergent concentrations as percentages of starting SR ATPase activity. (C) Specific activity of solubilized Ca^{2+} -ATPase by DHPC ($\circ$), C_{12}E_8 ($\triangle$), cholate ($\square$) and deoxycholate ($\diamond$) in detergent mixed micelles form. Specific activity is defined as units of ATPase activity/min per mg of protein at 37 °C under standard assay conditions as described in the Materials and methods section.

trations than by the C_{12}E_8 or bile salt detergents. Figure 1(B) shows the activity associated with the pelleted fractions of each detergent-treated sample. Analysis of the total Ca^{2+} -ATPase activity recovered (supernatant plus pelleted activity) after detergent treatment, again shows that DHPC treatment results in greater recovery of the total activity than treatment with any of the other three detergents. The loss in activity due to irreversible inactivation was far less in DHPC-solubilized samples than in those solubilized by C_{12}E_8 or the bile salt detergents over a wide range of detergent concentrations.

The specific activities of these preparations are shown in Figure 1(C) as a function of detergent concentration. At very low detergent concentrations (less than 5 mM), the C_{12}E_8 - and cholate-solubilized enzyme had a higher specific activity than that solubilized by DHPC. However, above 5 mM detergent the specific activity of the DHPC-solubilized Ca^{2+} -ATPase was significantly greater than that solubilized by any of the other three detergents. In addition, the deoxycholate-, cholate- and C_{12}E_8 -solubilized enzymes rapidly lost activity with increasing

Figure 2 Solubilization of SR total protein (supernatant) by DHPC, C₁₂E₈, cholate and deoxycholate at various concentrations of each detergent as percentages of starting SR protein: ○, DHPC; △, C₁₂E₈; □, cholate; ◇, deoxycholate

detergent concentration; the optimum range for these detergents is therefore very narrow. In contrast, the optimum detergent concentration range for DHPC is relatively broad. The maximum specific activity of these preparations is 16 units, obtained with 20 mM DHPC.

Figure 2 shows the amount of total protein solubilized by these detergents as a function of detergent concentration. As shown in the figure, the maximum solubilization of total protein was obtained with deoxycholate, with an optimum detergent range around 15 mM. However, DHPC, cholate and C₁₂E₈ showed more than 80% of the starting protein solubilization at total detergent concentrations of 10–20 mM, corresponding to a detergent-to-protein ratio of 1:2 by weight.

Purification of detergent-solubilized Ca²⁺-ATPase

The detergent-solubilized preparations were further purified by discontinuous sucrose density gradient centrifugation. The optimum concentration determined from the data of Figure 1 was chosen for each detergent. Figure 3 shows a representative example of the results from the use of 20 mM DHPC, 10 mM C₁₂E₈, 7 mM cholate or 10 mM deoxycholate. The data are presented as recovered protein in Figure 3(A) and as recovered Ca²⁺-ATPase activity in Figure 3(B), as percentages of the starting material of the preparation. Cholate, deoxycholate and C₁₂E₈ showed one peak each, whereas the DHPC preparation gave two peaks. The specific activity of the major peak from the DHPC solubilization was significantly greater than that obtained from the preparation solubilized with C₁₂E₈ or bile salt detergents. The distribution of the activity between the two peaks from DHPC varied from preparation to preparation; in many cases over 90% of the recovered activity was in the lower-density peak. The total activity recovered from the gradient was consistently at least 20% higher from the DHPC-solubilized preparations than from those solubilized by C₁₂E₈ or the bile salt detergents. The total activities recovered and the specific activities of the two bile salt detergent preparations were significantly lower than the DHPC or C₁₂E₈ preparations.

The most active fractions from these preparations were pooled and purified further by pelleting and extensive dialysis as described in the Materials and methods section. Ca²⁺-ATPase preparations seemed homogeneous from all four detergent preparations when tested on SDS/polyacrylamide gels

Figure 3 Discontinuous sucrose density gradient purification profile of SR solubilized with 20 mM DHPC, 10 mM C₁₂E₈, 7 mM cholate or 10 mM deoxycholate

(A) Total protein recovered: ○, DHPC; △, C₁₂E₈; □, cholate; ◇, deoxycholate. (B) Total ATPase activity: ○, DHPC; △, C₁₂E₈; □, cholate; ◇, deoxycholate. (C) Specific activity: ○, DHPC; △, C₁₂E₈; □, cholate; ◇, deoxycholate. Activity was measured after at least 800-fold dilution.

containing both 7.5% and 12% (w/v) gel. The purity of the enzyme was more than 95% in these preparations from these detergents. The enzyme appeared as a major band of molecular mass 110 kDa with trace amounts of higher aggregates and components at a molecular mass of approx. 45 kDa in all the samples. However, the samples from cholate, deoxycholate and C₁₂E₈ preparations showed significantly greater proportions of larger aggregates than the DHPC sample on the SDS gels.

Table 1 summarizes the results from the sucrose gradients shown in Figure 3, after pelleting and dialysis, showing the yields, as well as the amount of lipid per enzyme molecule. The two peaks from the DHPC preparation were pooled separately. The total activity obtained from the DHPC preparation is more than 25% greater than that obtained from C₁₂E₈, and 58% and 137% more than those obtained with cholate and deoxycholate preparations respectively. Similar results were obtained in multiple preparations. These data demonstrate that the DHPC procedure results in a significantly greater quantity of high-specific-activity Ca²⁺-ATPase than the other detergents. On the basis of the results shown in Table 1, further characterization of these preparations as well as reconstitution studies were limited to DHPC and C₁₂E₈.

Table 1 Summary of sucrose density gradient purification of SR Ca^{2+} -ATPase solubilized by cholate, deoxycholate, DHPC or C_{12}E_8 at optimal solubilization

DHPC1 represents the pool of fractions 3–5 (29.8–33.8% sucrose); DHPC2 represents the pool of fractions 9–10 (48.6–58% sucrose).

Sample	Detergent (mM)	Activity (units)	Protein (mg)	Specific activity (units/mg)	Activity recovered (%)	Lipid/protein (mol/mol)
SR	0	173	11.2	15.4	100	107
Cholate	7	58	6.3	9.2	33.5	81
Deoxycholate	10	39	6.4	6.1	22.5	71
DHPC1	20	65	3.3	19.7	37.6	74
DHPC2	20	27	2.1	13.0	15.7	51
Total DHPC.....		92	5.4	17.1*	53.3	64.62*
C_{12}E_8	10	74	4.4	16.9	42.8	77

* Weighted average.

Figure 4 Tryptophan fluorescence

(A) Emission fluorescence spectra of the ^3E , $\text{E} \cdot \text{Ca}_2$ transition for native SR vesicles. Fluorescence spectra were measured with $\lambda_{\text{ex}} = 285 \text{ nm}$ and $\lambda_{\text{em}} = 333 \text{ nm}$, and bandwidths of 4 and 16 nm respectively for excitation and emission. (B) Fluorescence changes on the addition of CaCl_2 and MgCl_2 to native SR vesicles (NSRV) and to sucrose-gradient-purified Ca^{2+} -ATPase preparations of 10 mM C_{12}E_8 - and 20 mM DHPC-solubilized SR vesicle preparations. Experimental conditions were the same as for Figure 3(A). Two additions of buffer corresponding to 2% dilutions each were made to calibrate the basal fluorescence signal. Subsequent additions led to final concentrations of 10 mM Mg^{2+} and 1 mM Ca^{2+} respectively.

Conformational characterization of purified Ca^{2+} -ATPase

Binding of Ca^{2+} ions to the high-affinity Ca^{2+} -binding site of Ca^{2+} -ATPase results in an increase in the intrinsic tryptophan fluorescence intensity [31,32]. Figure 4(A) shows an approx. 6% increase in the fluorescence intensity of SR vesicles in the presence of Ca^{2+} . Ca^{2+} binding to the DHPC-solubilized Ca^{2+} -ATPase purified on a sucrose gradient resulted in a net $5.3 \pm 0.1\%$ (mean $\pm$ S.E.M.) increase in tryptophan fluorescence, similar to that observed for SR vesicles; in contrast, the C_{12}E_8 purified

Figure 5 CD spectra of C_{12}E_8 - and DHPC-solubilized and sucrose-gradient-purified SR Ca^{2+} -ATPase: path length 0.1 mm; protein concentration 0.1 mg/ml

The spectra are averages of four accumulations. Samples were prepared with (1) 20 mM DHPC; (2) 10 mM C_{12}E_8 ; (3) 20 mM C_{12}E_8 ; (4) 40 mM DHPC. Solid lines, buffer; broken lines, buffer plus Ca^{2+} .

enzyme showed a marginal increase of $2 \pm 0.1\%$ (results not shown). The effect of Ca^{2+} and Mg^{2+} binding on the conformational transitions was monitored by following the fluorescence changes at constant wavelength on the sequential addition of Ca^{2+} and Mg^{2+} . In Figure 4(B) are shown the successive responses of the two sucrose-gradient-purified Ca^{2+} -ATPase preparations to the addition of Ca^{2+} and Mg^{2+} , compared with native SR vesicles. The SR showed typical Mg^{2+} - and additive Ca^{2+} -induced transitions when MgCl_2 was added to a final concentration of 10 mM followed by CaCl_2 to a final concentration of 1 mM. The DHPC-purified enzyme showed a final Ca^{2+} -induced transition comparable to that of the native SR, but did not exhibit the Mg^{2+} -induced change. The C_{12}E_8 -purified enzyme did not exhibit either the Ca^{2+} - or Mg^{2+} -induced conformational transitions. This shows that the DHPC-purified enzyme retained more native characteristics than the C_{12}E_8 preparation.

The CD spectra of the C_{12}E_8 - and DHPC-solubilized and gradient-purified Ca^{2+} -ATPase preparations are shown in Figure 5. All the Ca^{2+} -ATPase preparations showed a positive maximum at 192 nm and a double-lobed negative peak with a minimum at

Figure 6 Gel-filtration chromatography pattern on Sepharose CL-6B of DHPC- and $C_{12}E_8$ -dissociated purified Ca^{2+} -ATPase

Sucrose-density-gradient purified Ca^{2+} -ATPase (5 mg) obtained by solubilization of SR with 20 mM DHPC or 10 mM $C_{12}E_8$ was subjected to complete dissociation by 40 mM DHPC or 28 mM $C_{12}E_8$ and chromatographed on Sepharose CL-6B as described in the Materials and methods section. Fractions were assayed for Ca^{2+} -ATPase activity ($\circ$); protein ($\bullet$) and total phospholipid ($\triangle$). (A) DHPC dissociation profile; (B) $C_{12}E_8$ dissociation profile. Molecular masses of peaks were estimated by standard molecular mass markers (MW-GF-1000 kit from Sigma), calibrated on the same column as indicated above. Peak labels: V_0 , void volume; V_t , total volume; O_{gm} , trimeric-tetrameric Ca^{2+} -ATPase; M, monomeric Ca^{2+} -ATPase; PL, phospholipid.

223 nm characteristic of the high α -helix content of the protein. None of the spectra showed any significant effect of added Ca^{2+} . However, a comparison of the CD spectra from the four preparations measured under identical conditions showed significant differences in the shape and amplitude of the ellipticity at all characteristic wavelengths. These results, together with the specific activity of the respective preparations, indicate that the increased negative ellipticity at 223 nm is well correlated with decreased specific activity. Although great care was taken to minimize light-scattering effects and to ensure that the compared samples were identical, quantitative analyses of the spectra were not justified.

Dissociation of Ca^{2+} -ATPase into active monomers

To determine the ability of DHPC to dissociate Ca^{2+} -ATPase to form active monomer, the sucrose-gradient-purified Ca^{2+} -ATPase preparations from DHPC and $C_{12}E_8$ were re-solubilized with detergents and analysed by gel-filtration chromatography as described in the Materials and methods section [10].

Figures 6(A) and 6(B) show the Sepharose CL-6B elution profile of the DHPC- and $C_{12}E_8$ -dissociated Ca^{2+} -ATPase respectively. The profiles of both show two protein peaks, a minor peak immediately after the void volume corresponding to larger aggregates, followed by a broad major peak between the void

Figure 7 Sucrose density gradient centrifugation of DOPC vesicles reconstituted with purified Ca^{2+} -ATPase preparations obtained from SR solubilization with $C_{12}E_8$ at 15 mM and DHPC at 20 mM

(A) Total protein as a percentage of starting amount: $\circ$, DHPC; $\triangle$, $C_{12}E_8$. (B) ATPase as a percentage of starting activity: $\circ$, DHPC; $\triangle$, $C_{12}E_8$. Specific activity: $\bullet$, DHPC; $\blacktriangle$, $C_{12}E_8$. (C) Total phospholipid recovered: $\circ$, DHPC; $\triangle$, $C_{12}E_8$.

volume and the total volume. The activity profile was overlapping with the main protein peak in both samples. This peak corresponds to the monomeric molecular mass of 108 ± 10 kDa based on the column calibration curve. At the leading edge of this major peak is the shoulder peak corresponding to dimeric and higher oligomeric species. Comparison of Figures 6(A) and 6(B) shows that the activity in the peak of the DHPC-solubilized preparation after 800-fold dilution into the assay mixture is 6–8-fold greater than that of the $C_{12}E_8$ -solubilized protein. The elution of the phospholipid is at a different position in the two profiles; the lipid-detergent micelles are smaller and better separated from the protein peak in the DHPC-solubilized preparation than in the $C_{12}E_8$ -solubilized preparation. These results demonstrate that DHPC is able to dissociate the Ca^{2+} -ATPase into monomers and small oligomers while retaining Ca^{2+} -ATPase activity. In comparison, the $C_{12}E_8$ solubilization resulted in monomers and small oligomers similar to those produced by DHPC solubilization, but they had less than 15% of the activity of the DHPC-solubilized material.

Reconstitution of DHPC- and $C_{12}E_8$ -purified Ca^{2+} -ATPase into DOPC

The DHPC- and $C_{12}E_8$ -solubilized and sucrose-gradient-purified Ca^{2+} -ATPase preparations were reconstituted into exogenous

Table 2 Quasi-elastic light scattering particle size analysis of native SR vesicles, sucrose-gradient-purified and reconstituted Ca²⁺-ATPase preparations of C₁₂E₈ and DHPC

Each result is the mean $\pm$ S.E.M. of four preparations. Results obtained are from the best-fitting volume weight Nicomp distribution analysis.

Sample	Vesicle size (nm)
SR vesicles	250 $\pm$ 44
10 mM C ₁₂ E ₈ -solubilized sucrose gradient enzyme	173 $\pm$ 27
20 mM DHPC-solubilized sucrose gradient enzyme	206 $\pm$ 20
DOPC-C ₁₂ E ₈ reconstituted vesicles	162 $\pm$ 3
DOPC-DHPC reconstituted vesicles	248 $\pm$ 44

DOPC, with the procedure described in the Materials and methods section. DOPC was chosen because it has been previously shown to maximize the recovery of activity when reconstituted [13,33]. To maximize the replacement of endogenous lipid, a high lipid-to-Ca²⁺-ATPase ratio of 1500:1 was used. The reconstituted mixtures were purified on a discontinuous sucrose gradient to separate any protein-free liposomes from the reconstituted liposomes. The sucrose gradient profiles from the DHPC and C₁₂E₈ procedures are shown in Figure 7. Figure 7(A) shows the protein recovery profiles. There were no protein-free liposomes in either of the samples, although

some aggregates were found in both the samples at higher sucrose density. Figure 7(B) shows the activity recovery and the specific activity of both the preparations. The total activity recovered for the DHPC-reconstituted vesicles was 49.2 % of the starting activity, compared with 18 % of the starting activity for C₁₂E₈. Figure 7(C) shows that the amount of lipid was greater in the C₁₂E₈ preparations than in the DHPC preparation in the active peak of the gradient.

The size distributions of the reconstituted liposomes were measured by quasi-elastic light scattering; the results are shown in Table 2, along with the data for SR vesicles and the sucrose-gradient-purified preparations from each detergent. The results show a particle size from 240 to 300 nm for the most active reconstituted vesicles of the DHPC preparation, compared with the most active liposome size of 160–170 nm for C₁₂E₈ reconstituted vesicles. The results of Table 2 show that all of the preparations from each detergent have a similar vesicle size, with the various forms of C₁₂E₈ preparation generally being slightly smaller than those from DHPC.

The Ca²⁺ transport rate and ATPase activity of the reconstituted preparations, in comparison with the sucrose-gradient-purified preparations and with SR, measured by the simultaneous assay described in the Materials and methods section, are shown in Table 3. The coupling efficiencies of the DHPC- and C₁₂E₈-reconstituted vesicles were similar and close to that of the native SR vesicles. The sucrose-gradient-purified preparations, which are vesicular in nature, had lower coupling

Table 3 Ca²⁺ uptake and ATPase rates of SR vesicles, DHPC- and C₁₂E₈-purified and reconstituted enzyme at 790 nM free ionized Ca and 25 °C

Ca²⁺ uptake and ATPase rates were measured simultaneously as described in the Materials and methods section at 790 nM free ionized Ca at 25 °C. The coupling ratio is the ratio of Ca uptake to ATPase rate (measured by the simultaneous assay in both cases). Values given as means $\pm$ S.E.M.

Sample	Simultaneous Ca ²⁺ uptake (μ mol/min per mg)	Simultaneous ATPase rate (μ mol/min per mg)	Coupling ratio (μ mol Ca ²⁺ / μ mol ATP per min per mg)
SR vesicles	4 $\pm$ 0.1	2.05 $\pm$ 0.04	1.95 $\pm$ 0.01
DHPC-purified enzyme	4 $\pm$ 0.1	2.45 $\pm$ 0.20	1.60 $\pm$ 0.09
C ₁₂ E ₈ -purified enzyme	0.8 $\pm$ 0.1	1.90 $\pm$ 0.05	0.42 $\pm$ 0.04
DOPC-DHPC reconstituted vesicles	7.46 $\pm$ 0.4	4.30 $\pm$ 0.30	1.74 $\pm$ 0.04
DOPC-C ₁₂ E ₈ reconstituted vesicles	6.45 $\pm$ 0.4	3.6 $\pm$ 0.25	1.80 $\pm$ 0.08

Table 4 Fatty acid composition of SR vesicles, and purified and reconstituted Ca²⁺-ATPase

Results are shown as mol% of each fatty acid determined on the basis of moles of fatty acid in each sample, and represent the averages of duplicates. Abbreviations: SRVs, SR vesicles; DHPC-Enz, sucrose-gradient-purified Ca²⁺-ATPase with DHPC as the detergent; C₁₂E₈-Enz, sucrose-gradient-purified Ca²⁺-ATPase with C₁₂E₈ as the detergent; DOPC-DHPC, sucrose-gradient-purified Ca²⁺-ATPase reconstituted into DOPC with DHPC as the detergent for both purification and reconstitution; DOPC-C₁₂E₈, sucrose-gradient-purified Ca²⁺-ATPase reconstituted into DOPC with C₁₂E₈ as the detergent for both purification and reconstitution.

Fatty acid	Fatty acid proportion (mol%)				
	SRVs	DHPC-Enz	C ₁₂ E ₈ -Enz	DOPC-DHPC	DOPC-C ₁₂ E ₈
C _{15:0}	0.9 $\pm$ 0.1	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
C _{16:0}	32 $\pm$ 1.3	32 $\pm$ 0.1	33 $\pm$ 1.3	3.9 $\pm$ 1.2	4 $\pm$ 1.3
C _{16:1}	2 $\pm$ 0.7	0.0 $\pm$ 0.0	2 $\pm$ 0.1	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
C _{18:0}	9 $\pm$ 0.4	20 $\pm$ 0.1	12 $\pm$ 0.6	4.4 $\pm$ 0.1	5.7 $\pm$ 0.2
C _{18:1}	16 $\pm$ 1.4	16 $\pm$ 0.1	16 $\pm$ 1.4	87 $\pm$ 1.0	87 $\pm$ 1.1
C _{18:2}	20 $\pm$ 1.3	20 $\pm$ 0.6	21 $\pm$ 0.9	1.3 $\pm$ 0.4	1.3 $\pm$ 0.3
C _{18:3}	1.1 $\pm$ 0.1	0.0 $\pm$ 0.0	2.3 $\pm$ 0.2	1.3 $\pm$ 0.1	1.3 $\pm$ 0.1
C _{20:3}	4 $\pm$ 2.0	0.0 $\pm$ 0.0	0.7 $\pm$ 0.1	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
C _{20:4}	10 $\pm$ 0.9	9.0 $\pm$ 0.3	12 $\pm$ 0.4	0.7 $\pm$ 0.0	0.7 $\pm$ 0.0
C _{22:6}	1.8 $\pm$ 0.2	3.0 $\pm$ 0.8	1.0 $\pm$ 0.4	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
C _{24:1}	3.2 $\pm$ 0.2	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	1.4 $\pm$ 0.1	0.0 $\pm$ 0.0

ratios, with the $C_{12}E_8$ having a much lower coupling ratio than the DHPC preparation.

The fatty acid compositions of native SR vesicles compared with the two detergent Ca^{2+} -ATPase preparations before and after reconstitution into DOPC are given in Table 4. The results show that the sucrose-gradient-purified enzymes from both detergents have fatty acid compositions similar to the SR. The reconstituted vesicles in DOPC showed similar fatty acid compositions from both detergents; at least 87% was represented by oleic acid, with some traces of stearate and palmitate.

DISCUSSION

We have shown that the Ca^{2+} -ATPase purified and reconstituted with DHPC is superior in significant ways to that obtained with $C_{12}E_8$, making it a preparation that is suitable for detailed studies on the mechanism of ion transport and the role of protein-lipid interactions in the function of membrane proteins. The DHPC method resulted in a consistently higher yield of enzyme with a greater specific activity than the methods with $C_{12}E_8$, cholate or deoxycholate. Further comparison of the DHPC preparation with the $C_{12}E_8$ preparation showed that the Ca^{2+} -induced conformational change as observed in SR vesicles by tryptophan fluorescence was retained in the DHPC-purified enzyme but not in that from $C_{12}E_8$. The ratio of Ca^{2+} transported to ATP hydrolysed was also much lower for the $C_{12}E_8$ -purified enzyme, whereas for the DHPC preparation it was similar to that of native SR. More importantly, the specific activity of Ca^{2+} -ATPase in reconstituted vesicles was up to 2-fold greater than that achieved with $C_{12}E_8$. Lastly, the dissociation of Ca^{2+} -ATPase into monomers by DHPC followed by Tween-80 gel filtration preserved the ATPase activity, whereas the similar dissociation with $C_{12}E_8$ resulted in only approx. one-sixth of the activity of that obtained with DHPC. These points are discussed in detail below.

Efficacy of DHPC, $C_{12}E_8$, cholate and deoxycholate in SR solubilization and purification

The present comparative studies of the results from solubilization and purification of SR membrane Ca^{2+} -ATPase demonstrate the inherent variations exhibited by various detergent groups when interacting with SR. The four detergents employed in this comparative study, DHPC, $C_{12}E_8$, cholate and deoxycholate, represent well-differentiated groups of soluble amphiphiles, i.e. the phospholipid, non-ionic and bile salt derivatives. The DHPC solubilization consistently provides greater yields, with specific activities significantly greater over a wide concentration range of the detergent than that achieved by $C_{12}E_8$ or the two bile salt detergents. The yield of total units of activity at the optimal solubilization concentration of each of the three other detergents is 30–40% lower than that with DHPC. Combining the relative yields through solubilization and sucrose gradient purification, the total yield of activity units with DHPC was 25%, 59% and 137% more than that obtained with $C_{12}E_8$, cholate and deoxycholate respectively.

Conformational properties of purified Ca^{2+} -ATPase

Tryptophan fluorescence was used to determine the extent to which the native conformational properties that have been demonstrated in native SR vesicles are preserved in the sucrose-gradient-purified preparations from the two best detergents of this study. Many studies have shown that changes in tryptophan fluorescence parallel Ca^{2+} binding to the ATPase, and it is

believed that the change in tryptophan fluorescence is indicative of the $E1Ca$ to $E1^*Ca_2$ transition of the protein [31,32,35,36]. The DHPC-solubilized enzyme showed a Ca^{2+} -induced transition in tryptophan fluorescence comparable to that in native SR, although it failed to show the Mg^{2+} transition. The $C_{12}E_8$ -solubilized enzyme failed to show either the Ca^{2+} or the Mg^{2+} transition. This indicates that the DHPC-solubilized and purified enzyme retains the ability to undergo the Ca^{2+} -induced conformational transition, whereas the $C_{12}E_8$ purified protein has lost this ability. This is consistent with the low coupling ratio for the $C_{12}E_8$ preparation compared with the DHPC preparation (Table 3). The reason for this difference in the properties is probably related to the mechanism of solubilization of the two detergents: in the DHPC preparation it is likely that the immediate surroundings of the protein might retain more endogenous lipids than the protein treated with $C_{12}E_8$, even though both preparations have approximately the same lipid-to-protein ratio. As discussed by de Foresta et al. [21], $C_{12}E_8$ interacts directly with the protein, replacing lipids in contact with the protein; it is possible that the sucrose-gradient-purified $C_{12}E_8$ -solubilized Ca^{2+} -ATPase retains significant $C_{12}E_8$ in the interfacial region of the protein.

The CD spectra of DHPC- and $C_{12}E_8$ -solubilized and sucrose-density-gradient purified preparations from two concentrations of each detergent were measured to detect any secondary structural changes. In general the CD spectra were similar to those observed previously for SR vesicles [37,38] and for delipidated Ca^{2+} -ATPase in $C_{12}E_8$ [6]. Our results show an increase in the negative maximum at 223 nm as the concentration of detergent used to solubilize the protein is increased. This increase in negative ellipticity seems to be correlated with the decrease in specific activity of these preparations. No difference in the spectra was observed for any of the preparations in the presence of Ca^{2+} ; this is in agreement with earlier observations [37,38]. This suggests that conformational effects of ligand binding do not cause any significant secondary structure rearrangements. The differences in the CD spectra for the sucrose-gradient-purified enzymes from the two detergent methods seems to be correlated with the differences in specific activity of the preparations. This suggests that the lower specific activities are related to some denaturation of the protein.

Dissociation of Ca^{2+} -ATPase by $C_{12}E_8$ and DHPC

A central question concerning the function of SR Ca^{2+} -ATPase is the molecular basis of the activity and transporting unit. Several reports of the solubilization and delipidation of SR Ca^{2+} -ATPase with various detergents have been published [6,9,10] in which many of the resulting enzyme-detergent micelles contained protein aggregates with low activity. In many of these studies, by gel filtration, molecular species corresponding to monomers or dimers with some Ca^{2+} -ATPase activity have been obtained. However, the activity obtained in these studies is significantly lower than those obtained with DHPC solubilization in the present study. With the method of Moller et al. [10] we found only negligible activity in the monomer-dimer peak with the $C_{12}E_8$ -solubilized ATPase, but 6–8-fold greater activity with the Ca^{2+} -ATPase solubilized with DHPC. Moller et al. [10] found no activity by using $C_{12}E_8$, but their enzyme had been solubilized with deoxycholate before delipidation with $C_{12}E_8$. Our results show that solubilization with DHPC results in highly active monomers. This activity might be due to the presence of some residual endogenous lipids that are retained in DHPC. This result supports the finding that DHPC is a more benign detergent

for the solubilization of Ca^{2+} -ATPase than the others, including C_{12}E_8 .

Reconstitution into DOPC

In the present investigation we compared the results of reconstitution into DOPC with DHPC and C_{12}E_8 by following the currently accepted C_{12}E_8 procedure [15]. Our results showed that the DHPC method resulted in reconstituted vesicles with a 3-fold greater percentage of the starting activity than the parallel C_{12}E_8 method. The amount of protein reconstituted was similar for the two methods, but the specific activity of the DHPC-reconstituted Ca^{2+} -ATPase was 2–4-fold greater than that of the C_{12}E_8 -reconstituted protein in the gradient fractions. The reconstituted vesicles from both procedures had similar coupling ratios, but the low specific activity of the C_{12}E_8 -prepared vesicles suggests that a considerable portion of the reconstituted protein was inactive. The combined advantages of DHPC over C_{12}E_8 for treatments from solubilization through reconstitution represent a 3-fold greater yield of material. In addition, the range of detergent concentration over which enzyme with high specific activity was recovered in the solubilization step is considerably broader for DHPC than for C_{12}E_8 . These factors alone make the DHPC preparation superior to the C_{12}E_8 preparation for obtaining material for further studies. This is particularly important for large-scale preparations necessary for biophysical studies such as calorimetry or NMR studies.

Mechanism of action of DHPC

Both non-ionic detergents, such as Triton X-100 and C_{12}E_8 , and ionic detergents, such as cholate and deoxycholate, have been used for the purification and reconstitution of Ca^{2+} -ATPase from SR. In general, non-ionic detergents are effective for solubilizing membrane proteins because they usually do not perturb the proteins; however, they can produce aggregation and they are often difficult to remove because of low critical micelle concentrations [6,39,40]. The ionic detergents such as cholate and deoxycholate have a more bulky chemical structure and the negative charges give rise to more complex micellar behaviour; these detergents can also cause protein denaturation. It has also been shown that the molecular geometry of a particular detergent is an important factor that determines its mechanism of action [4]. The two detergents that are most widely used for SR Ca^{2+} -ATPase study are C_{12}E_8 and cholate.

Gabriel and Roberts [41] first demonstrated the ability of short-chain phospholipids to disperse bilayers of long-chain phospholipids. It was subsequently shown by Kessi et al. [24] that DHPC acts as an effective solubilizing agent for biological membranes. These authors showed that, for a variety of membrane proteins, DHPC was very effective in solubilization, purification and reconstitution, while preserving the native characteristics of the membrane proteins in terms of function and structure. They suggested that this efficacy is due to a mechanism of solubilization that involves disruption of the lipid portion of the membrane rather than by direct interactions with the membrane proteins. This conclusion was based on the low concentrations of this detergent required for dispersing the membranes, and the fairly wide range of detergent concentrations that could be used without disturbing the activity of the membrane protein. Because it is a natural phospholipid it has no particular interactions with the membrane protein interface, and is probably preferentially excluded from this domain by the chain length incompatibility. This is in contrast with the probable mechanism of solubilization of C_{12}E_8 , which has been shown to

displace lipids from the interfacial region of Ca^{2+} -ATPase [21]. Our results in comparing the Ca^{2+} -ATPase prepared with the use of solubilization and reconstitution by C_{12}E_8 and by DHPC support this mechanism of solubilization for DHPC. In the detergent-solubilized and sucrose-gradient-purified preparations, the DHPC enzyme retains its ability to undergo the Ca^{2+} -induced conformational change, as well as remaining close to the maximal Ca^{2+} -to-ATP coupling ratio, whereas the C_{12}E_8 preparation has lost these native properties. This is consistent with the suggestion of de Foresta et al. [21] that the C_{12}E_8 displaces some lipids from the protein–lipid interface. The retention of these native properties by the DHPC preparation is consistent with a mechanism of solubilization of Ca^{2+} -ATPase by DHPC in which the detergent either does not interact directly with the protein or, because it is itself a phospholipid, can replace these lipids without affecting the protein conformation and activity. The question could be raised as to whether this might make it difficult to prepare reconstituted vesicles in which the endogenous lipids have been completely replaced by defined exogenous lipids. However, because DHPC is itself a phospholipid, it seems likely that it would contribute to diluting the endogenous lipids during solubilization and allow replacement by exogenous lipids. The results of the fatty acid analysis of the DOPC-reconstituted vesicles showed that more than 87% of the fatty acids were oleic acid in both preparations.

Conclusion

In conclusion, we have demonstrated that the Ca^{2+} -ATPase solubilized, purified and reconstituted with the new short-chain phospholipid detergent DHPC is superior to any previously described reconstituted Ca^{2+} -ATPase system in terms of specific activity, yields and retained native properties. This provides a model membrane protein–lipid system that can be used for detailed studies of the mechanism of ion transport and lipid–protein interactions with many techniques, including those requiring relatively large amounts of material.

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