Adenosinetriphosphatase Site Stoichiometry in Sarcoplasmic Reticulum Vesicles and Purified Enzyme†

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ABSTRACT: The stoichiometry of phosphorylation (catalytic) sites in sarcoplasmic reticulum vesicles (SRV) and SR ATPase purified by differential solubilization with deoxycholate was found to be 4.77 ± 0.4 and 6.05 ± 0.18 nmol/mg of protein, respectively, when phosphorylation was carried out under conditions permitting ^{32}P labeling of nearly all sites. Assuming that each site corresponds to a single 115K ATPase chain, the observed site stoichiometry accounts only for 55% and 70% of the total protein. Failure to obtain higher phosphorylation levels was due to the presence of nonspecific protein contaminants in SRV or to the presence of inactive aggregates in the ATPase purified with deoxycholate. This was demonstrated by dissolving SRV and purified ATPase with lithium dodecyl sulfate, subjecting them to molecular sieve HPLC, and collecting the elution fractions for determination of protein, measurement of ^{32}P-labeled sites, and electrophoretic analysis. In fact, in the specific elution peak containing the 115K ATPase chains, phosphorylation levels were 6.62 ± 0.33 and 7.03 ± 0.18 in SRV and purified ATPase, corresponding to 68% and 86% of the protein in the specific elution peak. An alternate purification method was then developed, based on solubilization of SRV with dodecyl octaethylene glycol monoether ($C_{12}E_8$), separation of delipidated ATPase by anion-exchange chromatography, and enzyme reactivation with phosphatidylcholine. This preparation yields 7.3 ± 0.44 nmol of phosphorylation site/mg of protein of the SRV fraction before HPLC. Analysis by HPLC chromatography demonstrates that the ATPase purified by this method does not have the tendency to form inactive aggregates. It is therefore possible to obtain conditions in which the stoichiometric ratio of phosphorylation (catalytic) sites and 115K chains is nearly 1. Therefore, aggregational states that have been detected in the native SR membrane are likely to occur by interaction of nonpolar polypeptide segments within the bilayer, permitting polar segments of individual chains to retain enzyme activity at the membrane–water interface.

An outstanding problem in studies of the sarcoplasmic reticulum (SR) calcium pump is the stoichiometry of phosphorylation (i.e., catalytic) sites and enzyme units in various preparations of SR vesicles and purified ATPase. It is commonly reported that incubation of SR vesicles with ATP in the presence of Ca^{2+} yields a maximal level of 3–4 nmol of phosphorylated enzyme intermediate/mg of protein, even under conditions inhibiting hydrolytic cleavage of the phosphoenzyme [for a review, see Moller et al. (1982)]. This level is less than expected (i.e., ~8 nmol/mg of protein) from the appearance of sodium dodecyl sulfate (SDS) electrophoresis gels in which a band including an M_r ~115 000 ATPase chain accounts for most of the membrane protein.

This discrepancy has been interpreted by many as a real difference between the steady-state level of phosphoenzyme and the number of catalytic sites and explained with "half of the sites" reactivity or more general mechanisms including equivalent steady-state levels of phosphorylated and non-(covalently) phosphorylated intermediates (Eckert et al., 1977; Froehlich & Taylor, 1975). An alternative possibility, however, is the presence of heterogeneous protein or denatured enzyme, which may not be accurately quantitated by SDS gel electrophoresis. This is a very basic and important question since realistic analysis of equilibrium and kinetic measurements requires a correct knowledge of site stoichiometry.

Accordingly, we have performed a series of detailed studies in order to compare site stoichiometry and protein composition in native SR vesicles (SRV) and various preparations of purified SR ATPase. In these studies we have used for the first time molecular sieve high-pressure liquid chromatography (HPLC) for separation and quantitation of solubilized components of the SR membrane. Further characterization was obtained by gel electrophoresis and by functional parameters such as ATPase activity and enzyme phosphorylation with ATP and P_i. Finally, we developed a procedure for ATPase purification by solubilization with dodecyl octaethylene glycol monoether ($C_{12}E_8$) and anion-exchange chromatography. By this procedure we were able to obtain pure and stable ATPase and to account for a site stoichiometry consistent with that of single 115K polypeptide chains.

Materials and Methods

Enzyme Preparations. SRV were prepared as previously described (Eletr & Inesi, 1972). ATPase purification by differential solubilization with deoxycholate was by the method of MacLennan (1970). ATPase purification by solubilization with $C_{12}E_8$ and anion-exchange chromatography was obtained as follows: SRV (50 mg of protein) were dissolved at room temperature in 25 mL of a medium containing 20 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) (pH 7.5), 0.15 mM $CaCl_2$, 50mM NaCl, 20% (v/v) glycerol, and 4 mg of $C_{12}E_8$/mL. Soon after solubilization, this mixture was passed through a 0.45-μm Millipore filter, and the clear filtrate was passed through a 1.5 × 20.0 cm column containing 30 mL of DEAE (DE-52, Whatman)-cellulose preequilibrated with 20 mM Tris-HCl (pH 7.5), 0.15 mM $CaCl_2$, 50 mM NaCl, 20% (v/v) glycerol, and 0.5 mg of $C_{12}E_8$/mL. The column was then washed with approximately 30 mL of preequilibration medium (30–40 mL/h) until the optical absorption (λ = 280 nm) dropped to a low base line. The delipidated ATPase was then eluted with approximately 30 mL of 20 mM Tris-HCl (pH 7.3), 0.15 μM $CaCl_2$, 120 mM NaCl, 20% (v/v) glycerol, and 0.5 mg of $C_{12}E_8$/mL. (The remaining protein can be eluted with the same medium containing 400

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mM NaCl.) The elution peak containing the delipidated ATPase (very low activity at this stage) was pooled (20–22 mg of protein in 7–8 mL) and diluted in the same elution buffer to 2.0–2.5 mg/mL final protein concentration, and 15 mg of phosphatidylcholine was added. For this purpose an appropriate volume of a chloroform–methanol solution of phosphatidylcholine (Type V-EA, Sigma) was dried in a stream of nitrogen, and the dissociated residue was dispersed in 0.5 mL of the ATPase elution medium by a 2-min immersion in a sonication bath (20 °C). The ATPase eluate supplemented with phosphatidylcholine was incubated for 90 min at room temperature, and the resultant protein was stored in a freezer at −70 °C. Protein concentration in the presence of detergent and glycerol was measured by the Lowry method (1959) modified according to Bensadoun & Weinstein (1976).

Molecular Sieve HPLC. Samples for HPLC were prepared by dissolving 1 mg of protein in 0.25 mL of 1% lithium dodecyl sulfate (LDS), 0.1 M lithium sulfate, and 0.05 M lithium acetate (pH 4.5). Following filtration on a 0.45-μm Millipore filter, 0.10–0.15 mL of the solubilized sample was injected into a Waters HPLC system equipped with a size exclusion column type TSK G 3000 SW (Tojo Soda, purchased from HPLC Technology). The sample was eluted with a 0.4 mL/min flow rate at room temperature by using an elution buffer containing 1% LDS, 0.1 M lithium sulfate, and 0.05 M lithium acetate (pH 4.5). Light absorption of the elution flow was monitored continuously with a Waters 440 UV detector (280 nm). In addition, the protein concentration in fractional elution samples (0.2 mL) was measured by the Lowry method standardized with serum albumin. The protein concentrations obtained for the ATPase elution peak by this method correspond closely to those estimated by UV absorption monitored by the Waters detector, using the conversion factor $A_{280}^{1\%} = 11.5$. Phospholipid phosphorus was measured in fractional elution samples by the method of Bartlett (1959), except that concentrated perchloric acid (PCA) was used instead of 10 N sulfuric acid for the digestion.

(1) Electrophoretic Separations. Electrophoresis was carried out by Laemmli method (Laemmli, 1970) on a 7–15% acrylamide gradient. Gels were stained with the Coomassie blue reagent by using the Fairbanks method (1971). Usually 10–30 μg of protein in 30–50 μL was used for each well of the slab. When necessary, the fractions obtained from the eluate of the HPLC column were concentrated in a minicom CS15 (Amicon) before the electrophoretic run.

(2) Phosphorylation by ATP and P_i. The conditions for enzyme phosphorylations were as described in the tables and figures. The reaction was initiated by addition of [γ-^{32}P]ATP or [^{32}P]P_i and quenched by the addition of ice-cold perchloric acid containing P_i (final concentrations 125 mM and 4 mM, respectively). The protein precipitate was washed 3 times with 4-mL aliquots of ice-cold 125 mM perchloric acid containing 4 mM P_i and once with 4 mL of water. The washed pellets were dissolved in 0.35 mL of 1% LDS, 0.1 M Li_2SO_4, and 0.05 M lithium acetate, pH 4.5. Aliquots were taken for protein determination and liquid scintillation counting. When indicated, a third aliquot was run through a molecular sieve column of an HPLC system. When low amounts of protein were used (0.1 mg or less) in the determination of the phosphoenzyme levels, the reaction media were quenched with 2 volumes of 10% (w/v) trichloroacetic acid containing 4 mM P_i and filtered through a Millipore filter (medium pore size 0.43 μm). The filters were washed 5 times with this solution and counted in a scintillation counter (Knowles & Racker, 1975). In order to measure nonspecific radioactive binding,

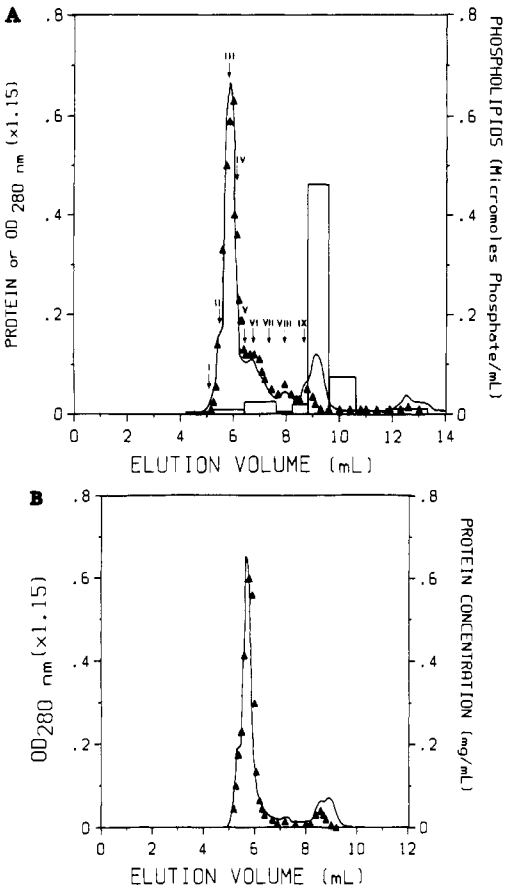

FIGURE 1: HPLC fractionation of SRV and purified ATPase (MacLennan, 1970) solubilized in LDS. SRV (0.60 mg) (A) and ATPase (0.50 mg) purified according to MacLennan (B) were dissolved in a 0.05 M lithium acetate buffer, pH 4.5, containing 1% LDS and 0.1 M Li_2SO_4 injected in a size exclusion column and eluted as described under Materials and Methods. Protein in the eluate (Δ) and phospholipids (bars) were determined as described under Materials and Methods. The continuous line represents UV (280-nm) absorption. The arrows show fractions collected for electrophoresis.

controls were performed in which the enzyme was quenched with perchloric acid before addition of [γ-^{32}P]ATP or [^{32}P]P_i.

ATPase Activity. Enzyme activity was assessed by measuring P_i cleavage from ATP in media containing 20 mM Tris-HCl (pH 7.5), 0.1 mM $CaCl_2$, 80 mM KCl, 10 mM $MgCl_2$, and 10–15 μg of protein/mL. When indicated, $C_{12}E_8$ (1 mg/mL) was also present. The reaction was started by addition of 2 mM ATP and quenched with the molybdovanadate reagent (Lin & Morales, 1977) after a 1-min incubation at 35 °C.

Results

Analysis of SR Preparations by HPLC and Electrophoresis. The elution profile of SR vesicles solubilized in SDS and passed through an HPLC molecular sieve column is shown in Figure 1A. It should be noted that most of the membrane phospholipid, expressed in the figure as organic phosphorus, exits the column with the eighth to tenth milliliter of elution volume.

Protein elution is expressed in Figure 1 in terms of optical density at 280 nm and concentration obtained by the Lowry method. The two measurements show the same pattern, except at the 9-mL elution volume in which light scattering by phospholipid and detergent micelles interferes with continuous UV monitoring. It is apparent in Figure 1A that the protein elution profile is complex and, even though complete resolution of various peaks is not obtained, the contribution of different

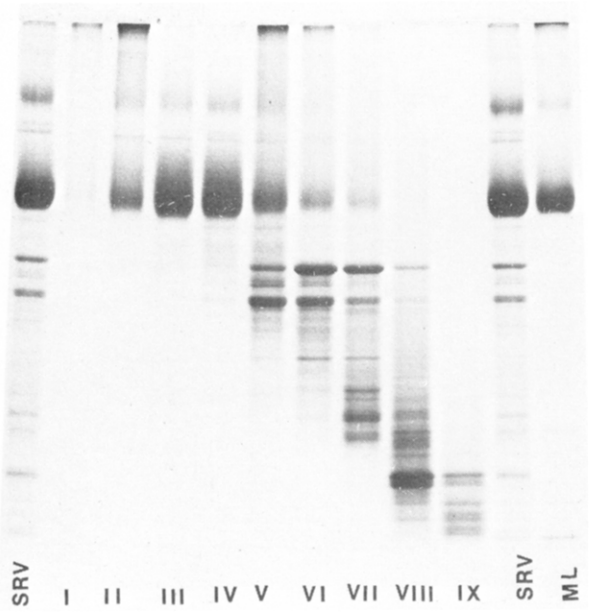

FIGURE 2: SDS gel electrophoresis of SRV and SRV fractions (I–IX in Figure 1A) eluted from molecular sieve HPLC. For comparison, the pattern of purified ATPase (MacLennan, 1970) is also shown (ML).

protein components can be clearly recognized.

The composition of various fractions indicated by arrows in Figure 1A was then analyzed by SDS gel electrophoresis (Figure 2). It was found first of all that the fraction eluted with the dead volume is composed of high M_r protein that does not enter the gels (sample I in Figures 1A and 2). Therefore, this component would not be detected if the vesicles were analyzed only by SDS gel electrophoresis.

The fraction eluted with the sixth milliliter of the elution volume (samples III and IV in Figures 1A and 2) appears to be highly enriched with M_r 115K chains; the faint bands of higher M_r present in these samples are likely due to slight aggregation produced during handling of the sample for SDS gel electrophoresis (see below). In addition, two protein contaminants of high M_r are still present in trace amounts. Calsequestrin and other minor components of the SR membrane (MacLennan, 1975) are contained in the fraction eluted with the seventh milliliter of the elution volume (samples V–VII in Figures 1A and 2). Proteolipid and other small proteins are eluted last (samples VIII and IX in Figures 1A and 2).

It is of interest that even samples of highly purified ATPase, prepared according to the method of MacLennan (1970) and yielding essentially one band on electrophoresis gels (ML in Figure 2), show a small amount of a low molecular weight protein and significant asymmetry of the HPLC elution profile due to the presence of high M_r material (Figures 1b and 2). It is likely that such high M_r material is the result of ATPase aggregation during the purification procedure or storage. In fact, it is shown in Figure 3 that an increasing fraction of the M_r 115 000 band can be aggregated simply by exposing the ATPase preparation to 45 °C temperature for short periods of time. This aggregation is somewhat unusual inasmuch as it is not prevented by strong detergents such as LDS and SDS, reducing agents such as mercaptoethanol and dithioerythritol, or agents interfering with hydrogen-bond formation such as urea or guanidine. Significant aggregation of ATPase chains occurs even when the preparation is stored at 0 °C. It is of interest that the native membrane of SR vesicles undergoes much less aggregation than the purified ATPase, when exposed

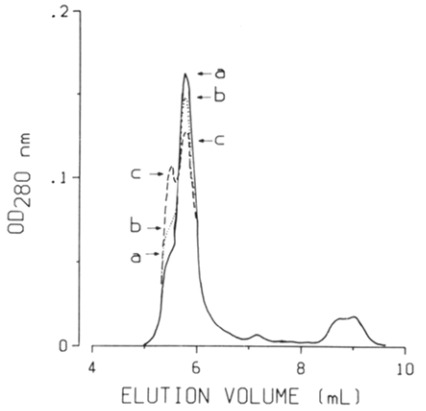

FIGURE 3: Heat aggregation of purified ATPase. Samples containing 100 μg of ATPase purified according to MacLennan (1970) were heated for 5 or 15 min at 45 °C and then dissolved in 0.05 M lithium acetate buffer, pH 4.5, containing 1% LDS and 0.1 M L_iSO_4. Aliquots (60 μg) were run through size exclusion HPLC as described under Materials and Methods. (a) ATPase before heating, (b) heated for 5 min, and (c) heated for 15 min.

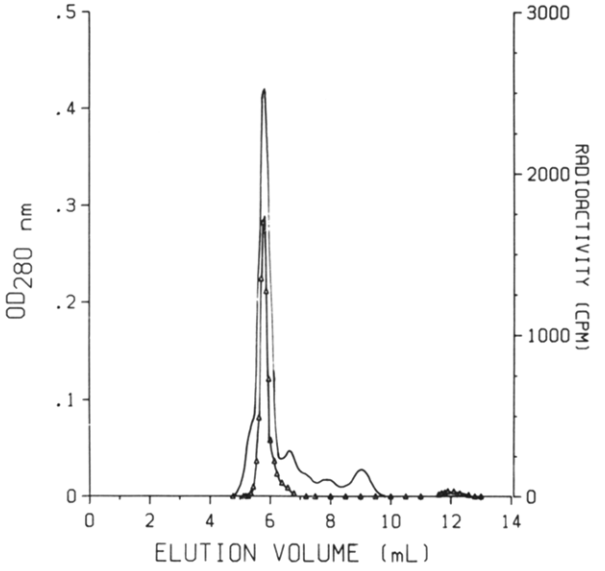

FIGURE 4: HPLC fractionation of SRV phosphorylated with ATP and solubilized in LDS. SRV (0.8 mg/mL) was phosphorylated at 25 °C in a reaction medium containing 30 mM Tris-HCl buffer, pH 7.5, 5 mM $MgCl_2$, 10 mM $CaCl_2$, 80 mM KCl, and 0.1 mM [γ-^{32}P]ATP. The reaction was initiated by addition of ATP and stopped after 10 s by adding perchloric acid. An aliquot of the washed pellet containing 0.4 mg of protein was solubilized and fractionated by HPLC as described under Materials and Methods. Radioactivity (Δ) and protein concentration (—) are displayed in the figure.

to the same conditions (not shown).

Stoichiometry of Phosphorylation Sites in SRV and in ATPase Purified by Differential Solubilization with Deoxycholate. The stoichiometry of ATPase catalytic sites was determined under conditions permitting maximal levels of phosphorylation. When ATP was used as the [γ-^{32}P]-phosphoryl donor (Yamamoto & Tonomura, 1967; Makinose, 1969), 10 mM Ca^{2+} (deMeis & Inesi, 1982) and 80 mM KCl were added to the medium to achieve high steady-state levels of phosphoenzyme. On the other hand, when phosphorylation was obtained by enzyme equilibration with [^{32}P]P_i in the absence of Ca^{2+} (Masuda & de Meis, 1973), dimethyl sulfoxide was added to the medium in order to achieve high equilibrium levels of phosphenzyme in the presence of relatively low concentrations of P_i (DeMeis et al., 1980).

Following appropriate incubations, acid quenching, and washing, the phosphorylated preparations were dissolved in

Table I: Phosphorylation Levels in SRV, Purified ATPase, and 115 K HPLC Elution Fractions[a]

sample	EP_t (μmol of P/mg of protein)	EP_e (μmol of P/mg of protein)	EP_t/EP_e
SRV + ATP	3.91 ± 0.18	5.83 ± 0.22	0.67
SRV + P_i	4.47 ± 0.40	6.62 ± 0.33	0.68
ATPase + ATP	5.03 ± 0.30	6.30 ± 0.27	0.80
ATPase + P_i	6.05 ± 0.18	7.03 ± 0.18	0.86

[a] SRV (1.0 mg/mL) was phosphorylated with either [γ-^{32}P]ATP or [^{32}P]P_i. Phosphorylation by ATP was carried out at 25 °C for 10 s in a reaction medium containing 30 mM Tris-HCl buffer, pH 7.5, 5 mM $MgCl_2$, 10 mM $CaCl_2$, 80 mM KCl, and 0.1 mM [γ-^{32}P]ATP. Phosphorylation by P_i was carried out in a reaction medium containing 30 mM Mes–Tris buffer, pH 6.0, 40% Me_2SO, 20 mM $MgCl_2$, 1 mM EGTA, and 1 mM [^{32}P]P_i–Tris for 10 min at 25 °C. The reaction was initiated by adding either ATP or P_i and quenched with perchloric acid. ATPase purified according to MacLennan (1972) was phosphorylated under the same conditions described for SRV. EP in the total sample (EP_t) before HPLC was determined as described under Materials and Methods. Aliquots of the phosphorylated proteins were also fractionated by size exclusion HPLC, and the protein concentration and radioactivity were determined in the 115K elution peak (EP_e). The values are the mean ± SD of four to six experiments.

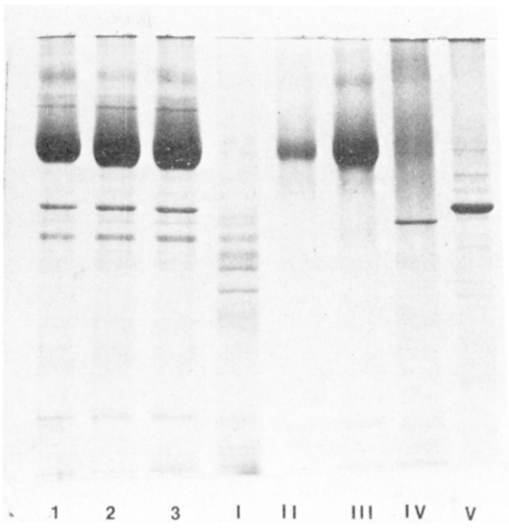

FIGURE 5: SDS gel electrophoresis of SRV and samples obtained during purification by solubilization in $C_{12}E_8$ and anion-exchange chromatography. SRV (1); SRV solubilized in $C_{12}E_8$ before (2) and after (3) Millipore filtration; fraction obtained by elution from the anion-exchange column by discontinuous NaCl gradients (fractions I–V in Figure 7).

LDS at pH 4.5 and passed through the HPLC size exclusion column. It is shown in Figure 4 that the [^{32}P]phosphate eluted with a symmetric peak corresponding to the 115K ATPase chains, and no radioactivity was found associated with smaller as well as with higher molecular weight components. Therefore, the high molecular weight components are inactive, even if they are the result of ATPase aggregation. Only trace amounts of [^{32}P]P_i were found in the elution volume (12th milliliter in Figure 4), indicating that negligible hydrolysis of the phosphoenzyme occurred within the few minutes required for HPLC at acid pH. Identical patterns of [^{32}P]phosphate elution were obtained with SRV and purified ATPase, independent of whether phosphorylation was obtained with ATP or P_i.

With regard to the phosphorylation levels, we obtained 3.91 ± 0.18 and 5.03 ± 0.30 nmol of phosphoenzyme/mg of SRV or purified ATPase (MacLennan, 1970), respectively (Table I). These levels are consistent with the highest values reported in the literature for these preparations [for a review, see Moller et al. (1982)] and reflect an enrichment of the concentration of active sites as a consequence of the purification procedure. As expected, the phosphoenzyme levels are higher when the measurements of [^{32}P]phosphate are made on the 115K ATPase peak eluted from the HPLC column. In this case, 5.83 ± 0.22 and 6.30 ± 0.27 nmol of phosphoenzyme/mg of protein are obtained from the specific elution peak of solubilized vesicles or purified ATPase (MacLennan, 1970), respectively (Table I). Therefore, the 115K peak contains approximately the same concentration of phosphorylation sites, independent of whether the preparation undergoing phosphorylation was SRV or purified ATPase.

It should be noted that the phosphoenzyme levels obtained with P_i are consistently 10–15% higher than those obtained with ATP (Table I). This difference is due to the steady-state character of the ATP experiments and to distribution of the enzyme in different states. On the other hand, incubation with P_i yields equilibrium levels of phosphoenzyme that are nearly identical with the maximal number of phosphorylation sites, owing to the high equilibrium constant for the phosphorylation reaction under our experimental conditions (de Meis et al., 1982). Therefore, the maximal numbers of phosphorylation sites are 4.47 ± 0.40 and 6.05 ± 0.18 nmol/mg of protein in SRV and purified ATPase (MacLennan, 1970) prepared in our laboratory. Assuming an M_r of 115K for the ATPase chains, these numbers correspond to 52% and 71% of the SRV and the purified ATPase, respectively. On the other hand, the phosphoenzyme levels of 6.62 ± 0.33 and 7.03 ± 0.18 found in the specific peak of the HPLC eluates correspond to 77% and 82% of the specific peak of ATPase chains eluted from solubilized SRV or purified ATPase, respectively. Therefore, the phosphoenzyme levels measured in the specific elution peak approximate closely the number of 115K ATPase chains present in the peak. Furthermore, it is clear that the common failure to obtain phosphoenzyme levels matching the expected stoichiometry of active sites (~8 nmol/mg of ATPase) in preparations that are not subjected to HPLC is due to the presence of nonspecific protein components in SRV or to aggregation of inactive ATPase chains when Ca^{2+} ATPase is purified by fractional solubilization with deoxycholate.

ATPase Purification by Solubilization in Nonionic Detergent and Anion-Exchange Chromatography. Owing to the tendency of ionic detergents to produce partial denaturation, we attempted to establish a purification procedure based on enzyme solubilization with nonionic detergents that are considered to be milder agents on SR ATPase. Specifically, we have selected $C_{12}E_8$ since the effects of SRV solubilization with this nonionic detergent have been studied in detail by Dean & Tanford (1978), by Moller et al. (1980), and in our own laboratory (Kosk-Kosicka et al., 1983). In fact, we performed our own experiments to establish optimal conditions for SRV solubilization with $C_{12}E_8$ and found that no loss of activity occurs within a 20-h period, if 100 μM Ca^{2+} and 50 mM Na^+ are present in the solubilization medium (pH 7.4). Glycerol and sucrose have hardly any protective effect during the solubilization (however, see below).

For the purification procedure we solubilized SRV with a 2:1 (w/w) $C_{12}E_8$ to protein ratio, at pH 7.4 and in the presence of 100 μM $CaCl_2$ and 50 mM NaCl. During the preliminary purification studies, we prepared a 2-mL sample containing 4.0 mg of protein, which was then passed through a 0.45-μm Millipore filter. Approximately 10% of the original protein was retained on the filter. The material before solubilization yields an electrophoretic pattern identical with that of the

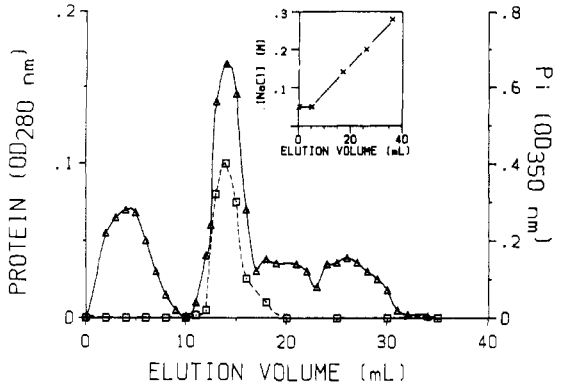

FIGURE 6: Elution of SRV solubilized in $C_{12}E_8$ from an anion-exchange column: continuous NaCl gradient. SRV (3 mg of protein) were solubilized in 1.5 mL of Tris-HCl, pH 7.5, 4 mg of $C_{12}E_8$/mL, 0.15 mM $CaCl_2$, 50 mM NaCl, and 20% glycerol. The sample was then filtered (0.45-μm Millipore) and added to a 2-mL DEAE-cellulose column equilibrated with 20 mM Tris-HCl, pH 7.3, 0.5 mg of $C_{12}E_8$/mL, 0.15 mM $CaCl_2$, and 50 mM NaCl. Elution was obtained with a continuous 0.05–0.30 M NaCl gradient. OD_{280} (△), ATPase activity (□), and NaCl concentration (×) are shown in the figure.

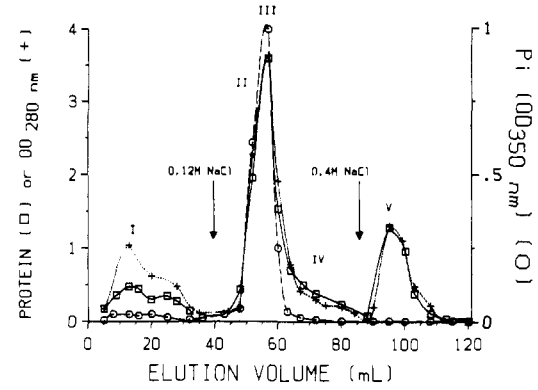

FIGURE 7: Purification of SR ATPase by $C_{12}E_8$ solubilization and anion-exchange chromatography. The purification procedure was as described under Materials and Methods, and the figure shows the elution by discontinuous NaCl gradients. Protein concentration by the Lowry method (□); protein concentration by UV (280 nm) absorption (+); ATPase activity (○). I–V indicate samples analyzed by electrophoresis (Figure 5).

solubilized and filtered protein (Figure 5). Therefore, the incomplete solubilization was probably the result of the detergent:protein ratio rather than a selective detergent effect.

The filtered sample (3–4 mg of protein in approximately 2 mL) was then placed on a 1.3 × 3.0 cm anion-exchange column (DEAE-cellulose) that was previously equilibrated with a medium containing 30 mM Tris-HCl (pH 7.5), 0.15 μM $CaCl_2$, 20% glycerol, 50 mM NaCl, and 0.5 mg of $C_{12}E_8$/mL. When elution was obtained with a continuous 0.1–0.3 M NaCl gradient, the SR phospholipid exited the column with the void volume together with a small amount of inactive protein. The delipidated enzyme appeared then as a single peak (Figure 6) between 100 mM and 150 mM NaCl. This enzyme was nearly inactive upon elution but regained Ca^{2+}–Mg^{2+}-dependent ATPase activity in reaction media supplemented with 1 mg of $C_{12}E_8$/mL. The ability of the delipidated enzyme to regain activity was maintained for a longer time in the presence of glycerol, confirming previous reports on the protective effect of glycerol on the delipidated enzyme (Dean & Tanford, 1978). Other minor protein peaks devoid of enzyme activity were eluted from the column at various NaCl concentrations (Figure 6).

As an alternative to the continuous gradient, we found it advantageous to use discontinous NaCl gradients in the elution of solubilized preparations from the anion-exchange columns, in order to obtain concentrated samples of purified ATPase in a very short time. In these elutions, a small amount of nonenzymatically active protein (together with SR phospholipid) exited the column before the NaCl concentration of the eluate was increased (Figure 7, from 1 to 10 mL). Electrophoretic analysis showed that this first fraction contained 45K and smaller M_r components (Figure 5). The SR ATPase was then obtained as a single peak (Figure 7) upon increasing the NaCl concentration in the elution medium from 50 to 120 mM. While most of this peak contained an electrophoretically pure (Figure 5) fraction of 115K chains, its asymmetrical tail contained a glycoprotein fraction migrating on electrophoretic gels between the 45K and the calsequestrin bands (Figure 5). The remaining SR protein components were finally eluted by increasing the NaCl concentration in the elution fluid from 0.120 to 0.4 M (Figure 7). These components were identified as calsequestrin and large aggregates that do not enter the electrophoretic gel (Figure 5). The entire procedure including solubilization and anion-exchange elution of an essentially pure fraction of 115K ATPase chains required less than 2 h time.

Table II: Hydrolytic Activity and Phosphoenzyme Levels in ATPase Purified by Solubilization in $C_{12}E_8$ and Anion-Exchange Chromatography[a]

sample	activity (μmol mg^{-1} min^{-1})		EP (nmol/mg of protein)	
	$-C_{12}E_8$	$+C_{12}E_8$	with ATP	with P_i
SRV in $C_{12}E_8$	5.3 ± 0.30	7.0 ± 0.68	4.01 ± 0.26 (n = 12)	4.61 ± 0.29 (n = 6)
purified ATPase	3.4 ± 0.15	13.8 ± 0.9		
purified ATPase + PC	11.7 ± 1.1		6.15 ± 0.4 (n = 13)	7.3 ± 0.44 (n = 5)

[a] ATPase activity was measured at 35 °C in media containing 30 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 0.06 mM $CaCl_2$, 2 mM ATP, and 1 mg/mL $C_{12}E_8$ when indicated. The SRV values were obtained from the same preparations used for purification by anion-exchange chromatography.

It is noteworthy that we never noticed a 115K fraction that could be distinguished from the ATPase chains. Nor did we find any fraction possessing Mg^{2+}-dependent ATPase activity without requirement for Ca^{2+}. In fact, the low Mg^{2+}-ATPase associated with SRV disappeared immediately after the initial solubilization.

An advantageous outcome of our detailed evaluation of experimental variables in small-scale solubilization and chromatography work was the development of a large-scale procedure for purification of SR ATPase by solubilization with $C_{12}E_8$ and anion-exchange chromatography. The procedure yields 20–25 mg of pure ATPase from a starting sample of 50 mg of SRV protein. We found that a critical factor to maintain the enzyme stable is immediate addition of phosphatidylcholine to the eluted protein to yield a ratio of 2.5 mol of phosphatidylcholine/mol of $C_{12}E_8$. The reactivated ATPase can be stored at −70 °C for more than 1 month without loss of activity. This large-scale procedure is described in detail under Materials and Methods. Its advantages are rapidity and lack of enzyme aggregation.

Enzyme Activity and Site Stoichiometry in ATPase Purified by Solubilization in $C_{12}E_8$ and Anion-Exchange Chromatography. ATPase activity and phosphoenzyme levels obtained with SRV and enzyme purified by anion-exchange chromatography are given in Table II. It should be noted that some enhancement of ATPase activity is obtained by simple solubilization of SRV with $C_{12}E_8$, probably due to removal of kinetic constraints related to the membrane as-

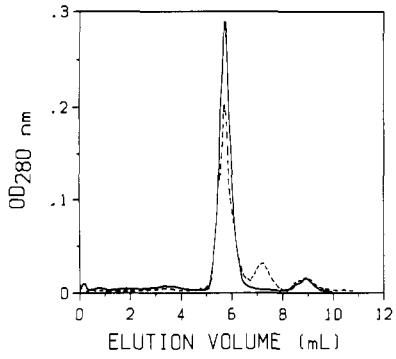

FIGURE 8: Molecular sieve HPLC of ATPase purified by $C_{12}E_8$ solubilization and anion-exchange chromatography. ATPase was purified as described under Materials and Methods and stored for 25 days at 4 °C (---) or −70 °C (—). Solubilization in LDS and molecular sieve HPLC were carried out as for the experiments shown in Figure 1.

sembly of the enzyme (Kosk-Kosicka et al., 1983). Following purification on anion-exchange chromatography, the delipidated ATPase chains eluted from the column sustain a much lower hydrolytic activity but can be reactivated by addition of higher concentration of $C_{12}E_8$ or phosphatidylcholine. In fact, we found that the enzyme reactivated with $C_{12}C_8$ became unstable upon dilution in this reaction medium and underwent denaturation as indicated by a progressively lower reaction velocity. On the contrary, the enzyme reactivated with phosphatidylcholine was very stable and sustained high and constant velocity of ATPase activity. It should be noted in this regard that we established through preliminary experimentation an optimal phosphatidylcholine:$C_{12}E_8$ ratio that must be maintained in order to obtain active and stable enzyme. Typically, the delipidated ATPase fraction was eluted from the anion-exchange column at a concentration of 0.5–4.0 mg of protein/mL. This was immediately diluted with medium to 2.0–2.5 mg/mL, and phosphatidylcholine was added to yield an approximative ratio of 2.5 mol of phosphatidylcholine/mol of $C_{12}E_8$ (which was already present in the elution medium at a concentration of 0.5 mg/mL); at higher protein concentration the reactivation yield of active enzyme was lower.

The reactivated enzyme sustained a constant ATPase velocity (37 °C) of 11.0 ± 1.1 or 19.5 ± 0.5 μmol min^{-1} (mg of protein)$^{-1}$, in the presence of 2 mM ATP and 10 mM $MgCl_2$ or 10 mM ATP and 10 mM $MgCl_2$, respectively. Following 1-month storage at −70 °C this preparation retained unchanged enzyme activity and did not show any denatured aggregates on molecular exclusion HPLC (Figure 8). On the other hand, prolonged storage at 4 °C led to progressive loss of enzyme activity, and the parallel appearance of smaller protein fragments (Figure 8) was probably due to slow proteolysis of the 115K chains.

With respect to the stoichiometry of phosphorylation sites, we obtained maximal levels of 6.15 ± 0.4 and 7.3 ± 0.44 nmol/mg of protein in the purified protein, when the substrate was ATP and P_i, respectively. These levels represent an enrichment of approximately 60% over the levels obtained with SRV. Assuming one phosphorylation site per 115K polypeptide chain and that the phosphorylation levels obtained with P_i closely match the total number of available sites, we find then that we can account for 85% of the expected phosphorylation sites in the enzyme purified by solubilization with $C_{12}E_8$ and anion-exchange chromatography. This is a satisfactory achievement, considering that such high levels were found in other preparations only after resolution of the 115K peak by molecular sieve HPLC.

The purified ATPase reactivated with phosphatidylcholine did not display any *net* calcium uptake with ATP hydrolysis.

Discussion

In early studies of SRV it became apparent that the Ca^{2+}-dependent ATPase is the prominent protein component of the SR membrane. The principal evidence for this structural feature was obtained from SDS electrophoretic gels in which the ATPase migrates as a 115K band. Depending on the purity and type of SRV preparations as well as on the electrophoresis and staining techniques, the 115K band has been estimated to account for a variable percentage of the total protein. This variability makes it very difficult to interpret functional studies of active site titration that, in most cases, yield phosphorylation levels of ~4 nmol/mg of protein. Assuming that each 115K has one phosphorylation (catalytic) site, these levels correspond to only 47% of the total protein of SRV. The rare high levels reported in the literature may be related to exceptionally pure SRV preparation or, more likely, to incorrect specific activity of the radiolabeled substrates used in the phosphorylation reaction, which must be checked each time by HPLC to ensure correct stoichiometry (Barrabin & deMeis, 1983). The outstanding question then is whether the lower number of phosphorylation sites, with respect to the expected number of 115K ATPase chains, is due to (1) an enzyme mechanism permitting steady-state and equilibrium levels that are significantly lower than the total number of sites, (2) a protein assembly that allows only one phosphorylation site out of two 115K chains, or (3) an incorrect estimate of the 115K chain stoichiometry based on densitometry of electrophoretic gels. We have previously ruled out possibility 1, demonstrating that it is possible to phosphorylate nearly all catalytic sites, especially by enzyme equilibration with P_i under suitable conditions (Inesi et al., 1982). We have then dealt with possibilities 2 and 3, as reported in this paper.

In our present work, a technical turning point was the use of molecular sieve HPLC for separation of protein components solubilized with LDS. In this case, collection of the elution fractions renders possible protein determination by conventional solution methods rather than gel densitometry as in electrophoretic analysis. On the basis of the profile of HPLC protein elution we then realized that the specific peak of 115K chains accounts for a smaller percentage of total SRV protein than indicated by our electrophoretic gels. Furthermore, determination of radioactivity in the eluted fractions of SRV phosphorylated with radiolabeled substrates prior to solubilization yields phosphoenzyme levels accounting for nearly 80% of the 115K chain stoichiometry in this specific elution peak. We concluded that densitometry of electrophoretic gels is not quantitatively reliable and that the nonphosphorylated protein present in SRV is mostly due to nonspecific protein components. A prominent phosphorylase contamination was found by Pickart & Jencks (1982) in their preparation of SR vesicles; however, we do not find significant amounts of phosphorylase in our preparation of SR vesicles (Inesi et al., 1982).

One of the most important developments in the study of SR membrane was the purification of ATPase by differential solubilization with deoxycholate and precipitation with ammonium acetate (MacLennan, 1970). This method yields pure enzyme of high hydrolytic activity and has led the way to partial determination of the primary sequence (Allen et al., 1980; Keip et al., 1980; Tong, 1980). However, we found that in ATPase purified in our laboratory by differential solubilization with DOC and precipitation with ammonium acetate, the maximal phosphorylation levels account only for 56% of the protein assuming one phosphorylation site per 115K chain and that the entire preparation contains only 115K chains.

This is in fact an improvement over the ~45% obtained with SRV (Table I), but it is still short of the expected stoichiometry. The explanation was found in the elution patterns of molecular sieve HPLC showing the presence of large aggregates that do not undergo phosphorylation (in addition to the 115K peak, which is phosphorylated with a satisfactory stoichiometry). These aggregates are most likely due to polymerization of the 115K chains, since they increase with storage of the preparation as noted in the early work of MacLennan (1970).

We then turned to a purification method based on solubilization with nonionic detergent $C_{12}E_8$. This detergent was first used by Dean & Tanford (1978) on SR ATPase first purified by sequential treatment with deoxycholate and then treated with $C_{12}E_8$ to delipidate and solubilize the enzyme. $C_{12}E_8$ was also used by Andersen et al. (1980), who obtained different degrees of enzyme delipidation by anion-exchange chromatography. Finally Mitchinson et al. (1982) used low $C_{12}E_8$ concentrations for elution of a seemingly active ATPase still retaining membrane lipids.

During our work we have now established a preparative method for purification of the enzyme by $C_{12}E_8$ solubilization and anion-exchange chromatography, checking all requirements in each step of the procedure. Under our conditions, the enzyme is eluted from the anion-exchange column in a delipidated form of low activity and must be reactivated by addition of phospholipid. The advantage of this method is the short time of preparation, its reproducibility, and a yield of pure and active enzyme with no tendency to form inactive aggregates. The functional state of the enzyme is reflected by a high hydrolytic activity and, most importantly, by phosphorylation levels in satisfactory agreement with the stoichiometry of the 115K chains (Tables I and II).

This method should prove very useful for preparation of pure enzyme to be used in structural and functional studies and for lipid exchange. Specifically, this preparation as well as the results obtained with molecular sieve HPLC has proven to us unequivocally that each ~115K ATPase chain participates with one phosphorylation site to enzyme catalysis. Under optimal conditions, the phosphorylation level accounts for 85% of the protein, 15% short of 100%. This discrepancy may be due to inaccurate calculations related to uncertainties on a precise M_r for the ATPase chains and on the ATPase protein standardization with bovine serum albumin. Our phosphorylation measurements are in agreement with the catalytic site titration performed by Mitchinson et al. (1982) using fluorescein isothiocyanate. Therefore, as previously suggested, chain aggregation found in the native membrane (Scales & Inesi, 1976) must reflect a state in which a number of chains are related by close interaction within the lipid bilayer but yet retain their individual and functional catalytic site on the outer surface of the membrane.

Registry No. ATPase, 9000-83-3.

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