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Role of Phospholipid and Protein–Protein Associations in Activation and Stabilization of Soluble Ca^{2+}-ATPase of Sarcoplasmic Reticulum

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ABSTRACT: The effect of increasing concentrations of the nonionic detergent Triton X-100 on catalytic activity, stability, phospholipid content, and aggregational state of solubilized Ca^{2+} ion activated adenosinetriphosphatase (Ca^{2+}-ATPase) of sarcoplasmic reticulum has been investigated. Increasing concentrations of Triton X-100 in the range 0.2–0.6% (w/v) inhibited ATP hydrolysis and *p*-nitrophenyl phosphate hydrolysis in parallel to the extent of 50% and 95%, respectively. Inactivation of *p*-nitrophenyl phosphate hydrolysis by preincubation in excess ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) at 25 °C was monophasic and first order at all concentrations of Triton X-100. The rate constant for inactivation increased sharply in the range 0.1–0.6% Triton X-100. At higher concentrations, the increase was less marked. Protein–protein associations of the solubilized ATPase were assessed by glutaraldehyde cross-linking and by ultracentrifugation in sucrose gradients. Both methods indicated a decrease in these associations in the 0.1–0.5% range. Cross-linking studies established that above 0.5% Triton X-100 the enzyme is >90% monomeric. The amount of phospholipid associated with the ATPase, recovered from sucrose gradients, decreased from about 50 mol of phospholipid/mol of ATPase at 0.1% Triton X-100 to about 3 mol of phospholipid/mol of ATPase at 0.5% and higher concentrations. Monomeric ATPase and aggregated ATPase isolated from equilibrium mixtures of these components had similar phospholipid/protein ratios. The results indicated that with increasing Triton X-100 concentrations, inhibition of catalysis, destabilization, loss of protein–protein associations, and loss of phospholipid occur concurrently. However, it was possible to maintain the monomeric state, reverse the inhibition of ATP and *p*-nitrophenyl phosphate hydrolysis, and restore stability by adding soybean phospholipid to the monomeric enzyme in 2% Triton X-100. This suggests that associated phospholipid, and not protein–protein association, is the principle determinant of the activity and stability of Ca^{2+}-ATPase in Triton X-100 solutions. We propose that mixtures of micelles containing one, two, or more ATPases are in slow equilibrium and each ATPase is equally unstable in excess EGTA. It is evident that while Triton X-100 can substitute for phospholipids in supporting catalytic activity, although at a slower rate especially with *p*-nitrophenyl phosphate as substrate, it cannot substitute for phospholipids in maintaining a stable native enzyme structure, and this suggests a specific phospholipid–ATPase interaction.

Intrinsic membrane proteins differ from soluble proteins in that the surface of at least part of the molecule is hydrophobic and associated with phospholipid. It has become clear that in many cases the catalytic activity of membrane proteins and the maintenance of their stable native structure depend, among other things, on the phospholipid–protein interaction. The

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Ca^{2+} ion activated adenosinetriphosphatase (Ca^{2+}-ATPase)[1] of sarcoplasmic reticulum (SR) is a particularly well-studied intrinsic membrane protein. Its phospholipid requirements appear to be fairly nonspecific. The SR is composed of many lipid species (Meissner & Fleischer, 1971; Waku et al., 1971; McIntosh et al., 1977), and none of these seems to selectively associate with the enzyme (Marai & Kuksis, 1973; Warren et al., 1974a; Le Maire et al., 1978). Delipidation with deoxycholate results in an inactive enzyme, but reactivation of ATPase activity may be achieved by adding back or exchanging with a variety of pure phospholipid species (Warren et al., 1974b; Green, 1975; Knowles et al., 1976), and Ca^{2+} pumping activity can be demonstrated in vesicles of Ca^{2+}-ATPase and pure dioleoylphosphatidylcholine (Warren et al., 1974a) although a requirement for phosphatidylethanolamine has been found by others (Knowles et al., 1976).

Nonionic detergents are able to substitute for natural lipids in supporting catalytic activity. Triton X-100 and dodecyl octaethylene glycol monoether ($C_{12}E_8$) activate lipid-depleted ATPase (Dean & Tanford, 1978; Dean & Suarez, 1981), and solubilization of SR vesicles with these detergents results in a preparation with high ATPase activity (McFarland & Inesi, 1971). It has been demonstrated that at high detergent/protein ratios an active monomeric enzyme is formed (Dean & Tanford, 1978; Le Maire et al., 1978; Silva & Verjovski-Almeida, 1983). The soluble monomer has a number of unique characteristics not exhibited by the native ATPase in SR vesicles. For example, it is unstable, especially in EGTA (Moller et al., 1980; Andersen et al., 1982; Kosk-Kosicka et al., 1983), it has a low reactivity to P_i in EGTA (Kosk-Kosicka et al., 1983), dephosphorylation is Mg^{2+} independent (Yamamoto & Tonomura, 1982), and there is no secondary activation by ATP (Dean & Tanford, 1978; Moller et al., 1980; Kosk-Kosicka et al., 1983). The instability of the monomer has led to confusion as to whether Ca^{2+} binding and Ca^{2+} activation of ATPase activity are altered by solubilization (Watanabe et al., 1981; Verjovski-Almeida & Silva, 1981; Andersen et al., 1982; Silva & Verjovski-Almeida, 1983). A time-dependent change in cooperativity occurs on incubation of the enzyme in detergent (Kosk-Kosicka et al., 1983). It is unclear whether this is the result of inactivation or monomerization. In general, these characteristics of the soluble monomer have been attributed to loss of oligomeric structure. However, Le Maire et al. (1978) have provided evidence that monomerization is accompanied by phospholipid depletion. Despite the fact that the enzyme can tolerate many phospholipid species and that the monomer in nonionic detergents retains high ATPase activity, it cannot be assumed that the unique characteristics of the soluble monomer are due to loss of oligomeric structure and not to detergent substituting for endogenous phospholipid. In this paper, we have addressed this issue by monitoring the effect of Triton X-100 on overall catalytic activity, stability, and protein–protein associations in conjuction with measurement of the endogenous phospholipid content of the soluble Ca^{2+}-ATPAse and the effect of supplementation with exogenous soybean phospholipid. The findings indicate that destabilization and loss of catalytic activity are principally due to substitution of detergent for phospholipid in the immediate environment of the ATPase, rather than interruption of protein–protein associations. We also describe a highly active, phospholipid-stabilized, and monomeric Ca^{2+}-ATPase in Triton X-100 which will be useful for characterizing the properties of the monomeric enzyme.

[1] Abbreviations: Ca^{2+}-ATPase, Ca^{2+} ion activated adenosinetriphosphatase; SR, sarcoplasmic reticulum; Mes, 2-(*N*-morpholino)-ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; $C_{12}E_8$, dodecyl octaethylene glycol monoether; pNPP, *p*-nitrophenyl phosphate; SDS, sodium dodecyl sulfate; MOPS, 3-(*N*-morpholino)propanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; Cl_3CCOOH, trichloroacetic acid; PAGE, polyacrylamide gel electrophoresis; PCh, phosphatidylcholine.

Materials and Methods

Materials. Triton X-100 and soybean phosphatidylcholine (type 11-2) were purchased from Sigma Chemical Co. The former was purified by the method of Askani & Latravas (1980). Dodecyl octaethylene glycol monoether was obtained from Nikkol, through Kouyoh Trading Co., Tokyo, Japan. Marker proteins for gel electrophoresis and Bio-Beads SM-2 were from Bio-Rad. The Ca^{2+} ionophore A23187 was a product of Calbiochem.

SR vesicles were prepared from rabbit back and hind limb white muscle by the method of Eletr & Inesi (1972). These were stored at 0 °C as a suspension in 10 mM imidazole, pH 7.4, and 0.3 M sucrose and used within 4 days.

Catalytic Activity. SR vesicles (0.4 mg of protein/mL) were preincubated in 50 mM MOPS, pH 7.0, 100 mM KCl, 5 mM $MgCl_2$, 50 μM $CaCl_2$, and various Triton X-100 concentrations for 15 min at 25 °C. Additional $CaCl_2$ was then added to give final concentrations of 0.1–0.2 mM, depending on the Triton X-100 concentration. Preliminary experiments established the optimum $CaCl_2$ concentration for maximum activity. ATPase activity was initiated by adding 5 mM [γ-^{32}P]ATP. Aliquots were taken at timed intervals and quenched with equal volumes of ice-cold perchloric acid (PCA). P_i concentration was determined by the molybdic acid procedure (de Meis & Carvalho, 1974). *p*-Nitrophenyl phosphate (pNPP) hydrolysis was measured by monitoring *p*-nitrophenol release at 405 nm in the same medium except pNPP (5 mM) replaced ATP. The Ca^{2+}-dependent component for each activity was obtained by subtracting the activity measured in the presence of 0.5 mM EGTA and without additional $CaCl_2$ from that obtained in the presence of $CaCl_2$. At concentrations of Triton X-100 less than 0.05%, 50 μM A23187 was included in the medium.

Inactivation. SR vesicles (0.4 mg of protein/mL) were preincubated in 50 mM MOPS, pH 7.0, 100 mM KCl, 5 mM $MgCl_2$, 50 μM $CaCl_2$, and various Triton X-100 concentrations for 15 min at 25 °C. Inactivation was initiated by adding 1 mM EGTA and stopped at timed intervals with 1.1 mM $CaCl_2$. pNPP (5 mM) *plus* sufficient Tris base to maintain the pH at 7.0 were added immediately, and *p*-nitrophenol release was monitored at 405 nm and at 25 °C. In parallel experiments, but without addition of $CaCl_2$, Ca^{2+}-independent activity was measured at the same timed intervals. The activity shown in the figures represents the Ca^{2+}-stimulated component.

Sucrose Gradient Centrifugation. Solutions of Ca^{2+}-ATPase were obtained by preincubation of SR vesicles (0.4 or 0.8 mg of protein/mL) in 50 mM MOPS, pH 7.0, 100 mM KCl, 5 mM $MgCl_2$, 50 μM $CaCl_2$, and various Triton X-100 concentrations for 15 min at 25 °C, cooled on ice, and then centrifuged at 100000*g* for 20 min at 0 °C. Aliquots of the supernatants (1–2 mL) were applied to the top of ice-cold linear gradients of 15–50% (w/v) sucrose in 50 mM MOPS, pH 7.0, 100 mM KCl, 5 mM $MgCl_2$, and 50 μM $CaCl_2$. Samples were centrifuged in a Beckman SW 40.1 rotor at 40 000 rpm for 24 h at 0 °C. The contents were recovered through a puncture in the bottom of the tube, and 1-mL fractions were collected. Fractions were assayed for Triton X-100, protein, and phospholipid.

Cross-Linking and Gel Electrophoresis. SR vesicles (0.4 or 0.8 mg of protein/mL) were preincubated in 50 mM MOPS, pH 7.0, 20 mM KCl, 5 mM $MgCl_2$, 50 μM $CaCl_2$,

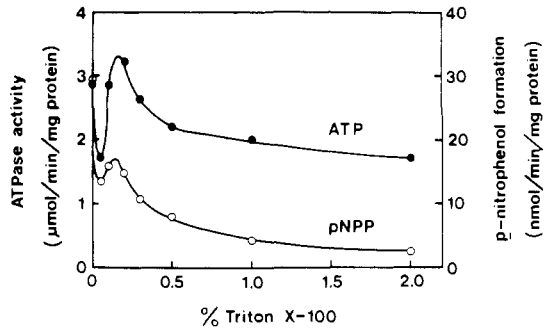

FIGURE 1: Effect of Triton X-100 concentration on ATP and pNPP hydrolysis. SR vesicles (0.4 mg of protein/mL) were preincubated for 15 min at 25 °C in 50 mM MOPS, pH 7.0, 100 mM KCl, 5 mM $MgCl_2$, 50 μM $CaCl_2$, and the Triton X-100 concentrations shown. Additional $CaCl_2$ (up to 0.2 mM) was added to the soluble enzyme, and the hydrolysis activity was initiated by adding ATP or pNPP in a concentrated form to minimize dilution. A23187 (50 μM) was included in the medium at concentrations of detergent <0.05%.

and various Triton X-100 concentrations for 15 min at 25 °C. The mixtures were centrifuged at 100000*g* for 30 min at 0 °C. An initial protein concentration of 0.8 mg/mL was used at a Triton X-100 concentration of 0.12% which yielded a supernatant of approximately 0.4 mg of protein/mL. In all other cases, 0.4 mg of protein/mL was used. Cross-linking was initiated by adding 40 mM glutaraldehyde. The reaction was quenched with 100 mM hydrazine. Aliquots were heated at 100 °C for 5 min in an equal volume of solubilization buffer, containing 3.5% SDS and 0.9% β-mercaptoethanol. Aliquots containing 5–10 μg of protein were taken for SDS electrophoresis, which was carried out by using the discontinuous buffer system of Laemmli (1970) in a 1.5-mm-thick gradient gel (4–20% acrylamide and 0.1% SDS). Approximate molecular weight assignments were made by comparison with protein standards.

Other Procedures. Soybean phosphatidylcholine was introduced into detergent-solubilized preparations by sonication in a bath-type sonicator, Model G 1125 PLT (Laboratory Supplies Co., Inc., Hicksville, NY), for 2–10 min at room temperature.

Triton X-100 was removed from solutions by passage through columns (0.5 × 30 cm) of Bio-Bead SM-2 equilibrated with 50 mM MOPS, pH 7.0, 20 mM KCl, 5 mM $MgCl_2$, and 50 μM $CaCl_2$ (Peterson et al., 1978).

Analytical Procedures. Protein concentrations were estimated by the Lowry procedure using bovine serum albumin as standard. Interference from Triton X-100 was eliminated, when necessary, by including 0.5% SDS in the alkaline reagent (Dulley & Grieve, 1975).

Phospholipids were extracted with chloroform/methanol mixtures and quantitated by the Bartlett method (Bartlett, 1959) as modified by Kates (1972). Triton X-100 concentrations in chloroform/methanol extracts were measured spectrophotometrically at 275 nm by using an extinction coefficient of 2.16 cm^2 mg^{-1} which was determined experimentally from standard Triton X-100 solutions.

RESULTS

Effects of Triton X-100 on ATP and pNPP Hydrolysis. The effects of Triton X-100 concentration on ATP and *p*-nitrophenyl phosphate (pNPP) hydrolysis are shown in Figure 1. It has been established from previous studies that solubilization occurs in the 0.04–0.08% range (McIntosh & Davidson, 1984). It was also shown that hydrolysis of both substrates is inhibited by low concentrations of Triton X-100, below that at which solubilization occurs. The inhibition is similar with both substrates, but the activation upon solubilization is less with pNPP as substrate. Above 0.2% Triton X-100, there is an abrupt and parallel inhibition of both activities, resulting in about 50% and 95% inhibition of ATP and pNPP hydrolysis, respectively. Triton X-100 is not unique in inhibiting catalysis at higher concentrations, and both $C_{12}E_8$ and lubrol WX have this characteristic (Le Maire et al., 1976).

Stability of Soluble Ca^{2+}-ATPase. Despite the lower activity with pNPP as substrate, we have found it a more convenient assay for assessing the stability of the enzyme. The kinetics of inactivation of phosphatase activity at various concentrations of Triton X-100 and excess EGTA are shown in Figure 2. In preliminary experiments, we found that micromolar concentrations of Ca^{2+} protected completely against inactivation in the time scale of the experiments, but no reversal occurred. Similar results have been found for $C_{12}E_8$ (Moller et al., 1980; Kosk-Kosicka et al., 1983). Sucrose (30%) and glycerol (4 mM) had no effect on inactivation in EGTA (results not shown), contrary to what has been observed for the Ca-liganded form of the soluble enzyme (Dean &

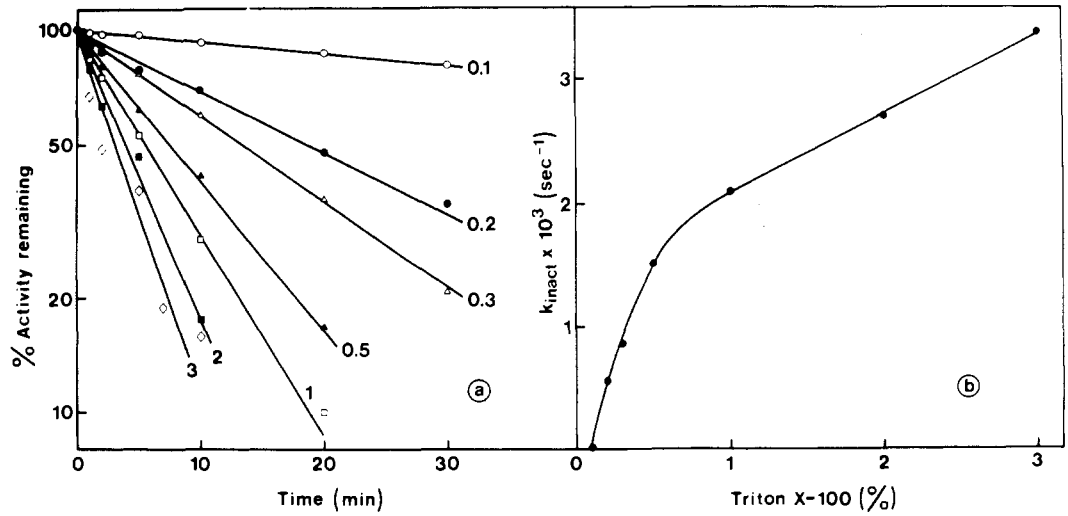

FIGURE 2: Effect of Triton X-100 concentration on the stability of soluble ATPase. The enzyme was first preincubated with Triton X-100 as in Figure 1. Inactivation at 25 °C was initiated by adding 1.0 mM EGTA and stopped with 1.1 mM $CaCl_2$ at the timed intervals shown. Steady-state pNPP hydrolytic activity was assayed immediately. (a) Semilogarithmic plot of the time dependence of inactivation. The percent Triton X-100 is shown alongside each curve. (b) First-order rate constant of inactivation obtained from (a) plotted against Triton X-100 concentration.

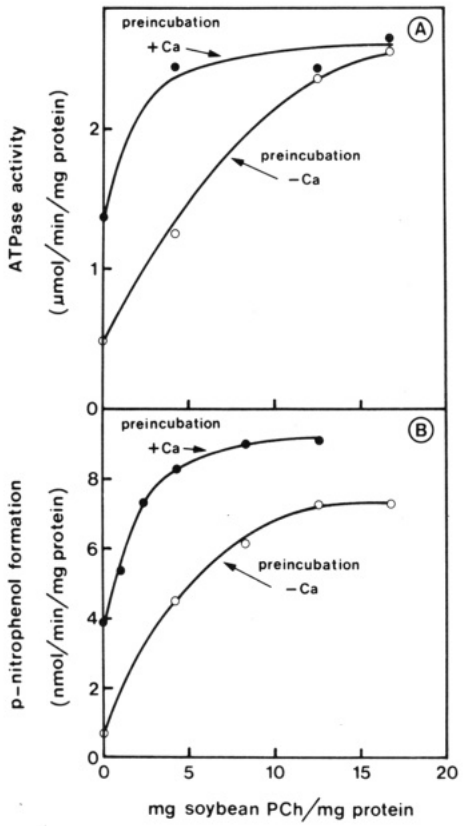

FIGURE 3: Effect of adding exogenous soybean PCh to ATPase solubilized in 2.0% Triton X-100. Preincubation in 2.0% Triton X-100 was as in Figure 1. Soybean PCh, in the amounts shown, was added and the mixture sonicated to clarity at 25 °C. The preparation was incubated at 25 °C for 15 min without (●) or with (○) 5 mM EGTA and then assayed for ATP hydrolysis (A) or pNPP hydrolysis (B) immediately. For simplicity, the preparation preincubated with 5 mM EGTA is labeled "−Ca".

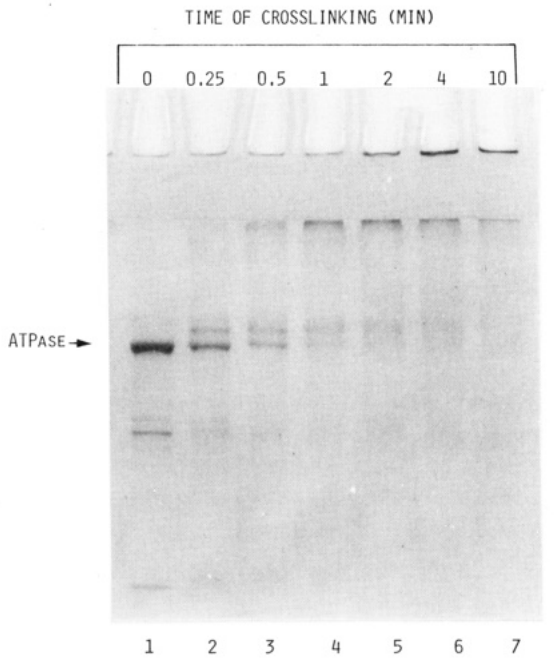

FIGURE 4: Time dependence of cross-linking with glutaraldehyde. SR vesicles (0.8 mg of protein/mL) were preincubated at 25 °C for 15 min in 50 mM MOPS, pH 7.0, 20 mM KCl, 5 mM $MgCl_2$, 50 μM $CaCl_2$, and 0.12% Triton X-100. The suspension was centrifuged at 100000*g* for 30 min at 0 °C. To the supernatant (0.4 mg of protein/mL) was added 40 mM glutaraldehyde and the reaction quenched at the times shown with 100 mM hydrazine. Aliquots were incubated with an SDS solution and then subjected to PAGE. Lower concentrations of KCl were used in the cross-linking experiments to avoid the necessity of dialyzing the samples prior to mixing with SDS. In some experiments, not shown, 80 mM KCl has been used successfully with similar results.

Tanford, 1978; Moller et al., 1980).

As can be seen in Figure 2, inactivation was monophasic and first order in the range 0.1–3% Triton X-100. The effect of increasing Triton X-100 concentration on the rate constant of inactivation, k_{inact}, is shown in Figure 2b. It is evident there are two phases of destabilization, one occurring in the 0.1–0.5% range which closely parallels the inhibition of catalytic activity (Figure 1) and another above 0.5% which results in further but less marked destabilization.

The effect of soybean phosphatidylcholine (PCh) on the activity and stability of the Ca^{2+}-ATPase in 2% Triton X-100 is shown in Figure 3. Increasing concentrations of the lipid activated ATP and pNPP hydrolysis to a plateau level of approximately 80% and 50%, respectively, of the maximal activity of the soluble enzyme (see Figure 1). Similar results have been obtained by Green (1975) using a variety of pure phospholipids. Soybean PCh also substantially stabilized the soluble enzyme (Figure 3). Preincubation of soluble enzyme without added phospholipid in excess EGTA for 15 min resulted in 60–80% inactivation depending on the preparation. The optimally phospholipid supplemented preparation showed complete protection against inactivation if ATPase activity was used as the marker of catalytic activity (Figure 3A) and somewhat less stabilization (25% inactivation) if pNPP hydrolysis was the marker (Figure 3B).

Cross-Linking of Soluble Ca^{2+}-ATPase. The time course of glutaraldehyde cross-linking of the supernatant fraction of 50% solubilized SR vesicles is shown in Figure 4. From the pattern at time zero (lane 1), it is evident that the preparation consisted of approximately 90% ATPase (M_r 100000), 5% calsequestrin (M_r 50000), and 5% remaining proteins. Within 15 s, the ATPase band decreased appreciably, the calsequestrin band disappeared, and a new band at M_r 120000 appeared. Longer incubation times (lanes 3–7) resulted in the disappearance of the ATPase band and the appearance of bands at the top of the gel, and then later higher molecular weight species that did not even enter the stacking gel. The new M_r 120000 band disappeared at a slightly slower rate. Dimers and trimers of the ATPase which could have been resolved on the gel were not observed. Negligible amounts of monomeric ATPase (M_r 100000 band) remained after 4 min, and we considered that this time of cross-linking was most suitable for determining the aggregational status of the solubilized ATPase.

The effects of increasing Triton X-100 concentration on protein–protein associations of the SR proteins are shown in Figure 5. In the absence of detergent, all proteins cross-linked to high molecular weight aggregates that did not enter the gel (lane 3). Lane 4 again demonstrates that the proteins of the supernatant fraction of 50% solubilized SR vesicles exhibit protein–protein associations, although less so than in the native membrane (lane 3). Protein–protein associations were considerably less at 0.2% Triton X-100 (lane 5). Principal components of the gel were the monomeric ATPase, the M_r 120000 complex, the dimeric ATPase, and aggregates which entered the stacking gel, but not the running gel. At 0.5% Triton X-100 (lane 6), the monomer M_r 120000 component predominate; smaller amounts of dimer and possibly tetramer were also present. Higher concentrations of detergent (lanes 7 and 8) diminished the amount of the M_r 120000 component and increased that of the monomer without any change in the dimer band. This suggests that the M_r 120000 component

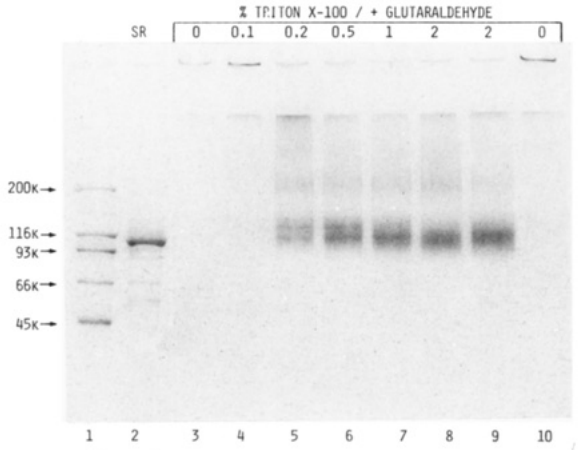

FIGURE 5: Effect of Triton X-100 concentration on cross-linking with glutaraldehyde. SR vesicles (0.8 mg of protein/mL in lane 4 and 0.4 mg/mL for the remaining lanes) were preincubated as in Figure 4 at the Triton X-100 concentrations shown except for lane 10 which was 2.0%. The mixtures were then centrifuged (100000*g* for 30 min at 0 °C). To two of the preparations dissolved in 2.0% Triton X-100 was added soybean PCh (16.7 mg/mL) (lanes 9 and 10), and one of these was passed through a Bio-Bead column (lane 10). All samples (0.4 mg of protein/mL) were then cross-linked with 40 mM glutaraldehyde for 4 min. The Triton X-100 concentration indicated in the figure refers to the concentration at the time of cross-linking.

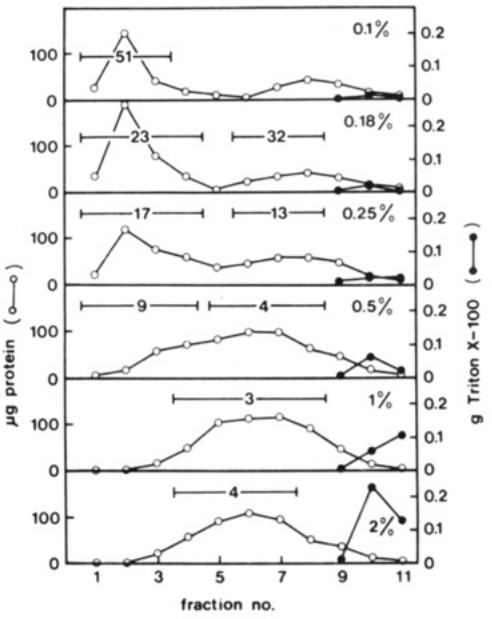

FIGURE 6: Effect of Triton X-100 concentration on the sedimentation pattern and associated phospholipid of soluble ATPase. SR vesicles (0.4 mg of protein/mL) were preincubated as in Figure 1 at the concentrations (in percent) of Triton X-100 shown in the right-hand side of each frame. The mixtures were centrifuged at 100000*g* for 30 min at 0 °C. The supernatants were applied to buffered sucrose gradients not containing detergent and centrifuged. Each fraction (numbered from the bottom of the tube) was assayed for protein and Triton X-100. Triton X-100 in fractions 1–8 at all concentrations of detergent was not detectable, and the data points have not been plotted. The peak fractions indicated were pooled for phospholipid determination, and the phospholipid/ATPase (molar) ratio is shown in the horizontal bars. The percent ATPase in each pool was estimated from Coomassie blue stained gels obtained by SDS–PAGE.

is made up of monomeric ATPase and a smaller protein/proteolipid and should therefore be considered as part of the monomeric ATPase population. In this case, at 0.5% Triton X-100 and higher concentrations, the preparation is principally monomeric.

Addition of soybean PCh to the SR vesicles, solubilized in 2.0% Triton X-100, did not enhance protein–protein associations, and the preparation remained essentially monomeric (Figure 5, lane 9). This is an important finding in light of the stabilization and activation of the enzyme by the exogenous lipid detailed above. Removal of the detergent by passage down a Bio-Bead SM-2 column yielded a turbid suspension, which readily cross-linked to high molecular weight aggregates, similar to the native vesicles (lane 10). This indicates that reconstitution led to extensive reassociation of the ATPase. It also demonstrates that the lack of cross-linking in the presence of detergent and soybean PCh was not due to inhibition of the glutaraldehyde–protein reaction by exogenous lipid. The broad protein bands are also indicative of a glutaraldehyde–protein reaction.

We consistently observed the presence of a small amount of cross-linked ATPase dimer (2–10% of the total Coomassie Blue stained bands) at high detergent concentrations (0.5–2.0%) (Figure 5, lanes 6–8). This could be due either to random collisions of monomeric ATPases or to the presence of dimeric ATPAse, or perhaps both possibilities contributed. We attempted to distinguish between these by examining the time dependence of cross-linking of ATPases in 0.5% Triton X-100 and comparing it to that of phosphorylase *b* and of albumin at the same protein concentration. The former is either a dimer or a tetramer, depending on its phosphorylation state, and albumin is monomeric. We found that there was a small time-dependent increase in the proportion of cross-linked ATPase dimers over 30 min, which approximately parallels the small increase in dimers observed for albumin (results not shown but submitted with the manuscript for refereeing purposes). Phosphorylase is about 95% and 100% cross-linked at 4 and 30 min, respectively. Although these results do not rule out a small percentage of dimeric ATPase in the preparation, they do suggest that random collisions of monomers account for most of the cross-linked dimers. The facile cross-linking of phosphorylase and not of albumin confirms the validity of the method for monitoring protein–protein associations. It is concluded from the cross-linking studies that there is a pronounced change in the extent of protein–protein interactions between ATPases at 0.15–0.4% Triton X-100 and that above 0.5% Triton X-100 the preparation is greater than 90% monomeric and this fraction is not changed by the addition of stabilizing concentrations of soybean PCh.

We have also determined that during the course of EGTA inactivation of monomeric ATPase at 0.3% Triton X-100 the preparation becomes increasingly susceptible to cross-linking (results not shown but submitted with the manuscript for refereeing purposes). The positions of the cross-linked bands in the gel correspond to an aggregate of three to five ATPases. Larger complexes were not formed. There was a correlation between the amount of inactivated species and the extent of cross-linking.

Ultracentrifugation of Solubilized Ca^{2+}-ATPase in Sucrose Gradients. The effect of increasing Triton X-100 concentration on the size and proportion of protein/phospholipid/detergent particles was assessed by ultracentrifugation in sucrose gradients. An additional objective was to separate protein/phospholipid/detergent particles from pure detergent and detergent/phospholipid micelles in order to determine the protein/phospholipid ratio in the former. The sucrose gradients did not contain Triton X-100, as inclusion of 0.1% Triton X-100 resulted in all recovered protein fractions being devoid of phospholipid in contrast to the situation when it was omitted. Inclusion of Triton X-100 presumably caused the ATPase to be stripped of phospholipid during sedimentation. The results

of a representative experiment are shown in Figure 6. It should be noted that material, not solubilized, was removed by centrifugation prior to gradient centrifugation. Insoluble material made up 30% of the total at 0.1% Triton X-100, and at 0.18% and higher concentrations, it was less than 5%. At all concentrations of Triton X-100, the detergent remained at the top of the gradient, and none could be detected with the proteinaceous material. Despite this, the ATPase remained dissolved in the sense that there were no turbid regions in the gradient after centrifugation. This phenomenon has been observed by others (Moller et al., 1980). At 0.1% Triton X-100, the protein profile of the sedimentation pattern (Figure 6, top panel) shows that 62% of the protein banded close to the bottom of the tube (fractions 1–3) and 34% banded close to the top (fractions 7–10). SDS–PAGE indicated that the latter fractions were composed principally of non-ATPase protein. At 0.18% and 0.25% Triton X-100, the proportion of proteinaceous material, banding near the bottom of the tube, decreased while there was an increase in the middle of the gradient (fractions 4–7). A shoulder also appeared near the bottom of the tube in fractions 3 and 4. According to the cross-linking results, these new species are likely to be monomeric and dimeric ATPase, respectively. In this case, at 0.5% Triton X-100, the preparation consists of approximately 65% monomer and 35% dimer. Above this concentration, namely, 1.0% and 2.0% Triton X-100, the protein profiles indicated greater homogeneity and presumably close to complete monomerization. Thus, both cross-linking and ultracentrifugation indicate a marked change in the aggregational state of the enzyme around 0.2% and substantial monomerization above 0.5% Triton X-100.

Phospholipid Content of Solubilized Ca^{2+}-ATPase. The total phospholipid content of several pooled fractions, recovered from the sucrose gradients, is also shown in Figure 6. Phospholipid associated with the ATPase diminished as the concentration of detergent was increased. Solubilization (0.1% Triton X-100) removed about 36% of the phospholipid of SR vesicles, presumably into phospholipid/Triton X-100 micelles, which remained at the top of the gradient. The phospholipid/ATPase molar ratio was 23 and 32 for the aggregated and monomeric species, respectively, at 0.18% Triton X-100. Higher concentrations diminished the amount of phospholipid associated with the ATPase until 3–4 mol of phospholipid/mol of ATPase remained. The results also indicate that aggregated and monomeric ATPase in equilibrium mixtures contain similar amounts of phospholipid when expressed on a molar basis.

At high concentrations of Triton X-100, three to four phospholipid molecules remain associated with the ATPase. It has been shown in a number of investigations (Warren et al., 1974a; Green, 1975; Dean & Tanford, 1978; Moller et al., 1980) that delipidation by detergents does not result in the selective enrichment of any phospholipid class extractable into chloroform/methanol, and therefore, we have not identified the few remaining phospholipids. However, it has been found that SR vesicles contain about 1 mol of phosphatidylinositol/mol of ATPase, tightly associated with the ATPase protein, and that phosphorylation of this phospholipid alters the properties of the ATPase (Varsanyi et al., 1983). This phosphatidylinositol was only extracted in acidified chloroform/methanol mixtures, and hence, its selective enrichment could have been overlooked. Following the procedure of Varsanyi et al. (1983), we found 0.58 μg of phosphate/mg of SR protein following Cl_3CCOOH precipitation, washes with H_2O and then chloroform/methanol, and finally extraction into acidified chloroform/methanol. This is equivalent to 1.1 or 2.3 mol of phosphatidylinositol/mol of ATPase, depending on whether the inositol portion is taken as phosphorylated or not, respectively, and hence confirms their findings. Following the same procedure, Ca^{2+}-ATPase, solubilized in 2% Triton X-100 and recovered from sucrose gradients, contained negligible amounts (<0.05 mol of phosphatidylinositol/mol of ATPase) of phospholipid, and it is concluded that under these conditions phosphatidylinositol did not selectively remain associated with the enzyme.

Discussion

Nonionic detergents, and in particular Triton X-100, have been widely used to solubilize and purify membrane proteins because they are among the least perturbing of the detergents (Helenius & Simons, 1975; Tanford & Reynolds, 1976). Their success has been attributed to the micellar state closely mimicking the dimensions and form of the natural phospholipid bilayer (Tanford & Reynolds, 1976). In this way, the hydrophobic requirements of the membrane-embedded section of the protein are satisfied. However, it has been shown that the hydrophilic or poly(ethoxyethylene) portion of nonionic detergents is an important determinant of the activity of lipid-depleted and detergent-activated Ca^{2+}-ATPase (Melgunov & Akimova, 1980; Dean & Suarez, 1981). It is likely that in the natural membrane, interaction between phospholipids and membrane proteins involves not only hydrophobic associations, which are nonspecific, but also interaction between the protein and phospholipid head groups. Such an interaction is implicit in the concept of relatively immobile annular or boundary phospholipid encircling a membrane protein. It is manifest in extreme cases in an enzyme exhibiting an absolute requirement for a particular phospholipid class, such as the requirement of D-β-hydroxybutyrate apodehydrogenase for phosphatidylcholine (Fleischer et al., 1966). In other cases, there is an order of selectivity for particular lipid classes, as the preference of myelin proteolipids for fatty acids (Brophy et al., 1984) or the preference of (Na^+,K^+)-ATPases for negatively charged phospholipids (de Caldentey & Wheeler, 1979; Zachowski & Devaux, 1983). Our results indicate that substitution of phospholipid for Triton X-100 in the immediate vicinity of the Ca^{2+}-ATPase causes pronounced destabilization and alteration in catalytic properties of the enzyme. We demonstrate that this is a direct result of phospholipid substitution and not of loss of ATPase–ATPase associations. This suggests a specific phospholipid requirement for maximum stabilization of the enzyme. A similar requirement is suggested for the (Na^+,K^+)-ATPase (Brotherus et al., 1983) and is in keeping with immobilization of annular lipids in the Ca^{2+}-ATPase (Hesketh et al., 1976; Lentz et al., 1983; Silvius et al., 1984) and (Na^+,K^+)-ATPase (Brotherus et al., 1980). It is compatible with the demonstration of specific amphiphile binding sites on the Ca^{2+}-ATPase and phospholipids exhibiting a much higher affinity than nonionic detergents for the sites (Dean & Suarez, 1981). The specificity presumably arises from interaction with the polar portion of the phospholipids.

Increasing concentrations of Triton X-100 caused a parallel replacement of phospholipid by detergent, monomerization, inhibition of catalytic activity, and destabilization. The concomitant loss of phospholipid and monomerization have also been obtained with $C_{12}E_8$ (Le Maire et al., 1978). It was only by first monomerizing and then adding back phospholipid that we could distinguish the effects of the two processes on catalytic activity and stabilization. As long as detergent was present and the complexes maintained in a soluble state, the ATPase polypeptides remained in separate micelles. Removal of detergent caused the ATPases to become susceptible to

cross-linking, despite the presence of about a 16-fold excess of phospholipid over that of the original SR membrane. Inactivated enzyme, even in high Triton X-100 concentrations, rapidly aggregated to complexes of three to five ATPases. This result agrees with that of Green (1975), who estimated from electron micrographs that the inactivated protein was in similar sized complexes. Addition of soybean PCh reversed the inhibition of ATP hydrolysis almost completely, but for pNPP hydrolysis, the reversal was about 50%. This may be because of monomerization or alternatively because soybean PCh is not as effective as endogenous phospholipids. In either case, substantial reversal of inhibition can be attributed to phospholipid displacing Triton X-100 from around the enzyme. The stabilization obtained with soybean PCh was approximately equivalent to the most stable soluble preparation obtained at low Triton X-100/protein ratios.

Our cross-linking results did not provide any evidence for a preferred oligomeric structure of the Ca^{2+}-ATPase. The increased cross-linking at relatively low detergent/protein ratios could be the result of random collisons of ATPase polypeptides in large soluble ATPase/phospholipid/detergent complexes, which contain a number of ATPases. A cross-linking pattern similar to that obtained with the supernatant of 50% solubilized vesicles has been found with untreated vesicles by using a variety of cross-linking agents (Chyn & Martonosi, 1977). Our results contrast with those of Silva & Verjovski-Almeida (1982) who, using gel filtration, found that in $C_{12}E_8$ solutions at relatively low detergent/protein ratios the dimer predominated. We obtain the same cross-linking results using Triton X-100 or $C_{12}E_8$. Hence, this discrepancy is difficult to understand. Recently, two-dimensional crystalline assays of ATPase polypeptides have been observed on incubation of the vesicles with vanadate (Dux & Martonosi, 1983a,b). The unit of the crystal lattice is an ATPase dimer, indicating that at least in the vanadate-liganded conformation there is a preferred oligomeric state of two polypeptides. Low, nonsolubilizing concentrations of Triton X-100 destroy the preformed crystals. We (McIntosh & Davidson, 1984) and others (Ikemoto et al., 1981; Champeil et al., 1982; Ludi & Hasselbach, 1982, 1983; Andersen et al., 1981, 1983) have found that intercalation of small amounts of detergent into the vesicles alters the kinetic and physical characteristics of the enzyme. Possibly, oligomeric interactions are disrupted at this stage, in the same way that the vanadate-induced crystals break up. Thus, it may be difficult to obtain or demonstrate specific oligomeric interactions with the soluble enzyme.

Inactivation of catalytic activity in EGTA at all concentrations of detergent yielded monophasic and apparent first-order kinetics. This could pertain if either the ATPases in the different sized complexes in a mixture are equally unstable or the monomer is more unstable than the dimer/oligomer and the different forms are in rapid equilibrium in comparison to the rate of inactivation. However, we show that the principle determinant of stability in detergent solutions is phospholipid, and the different sized complexes in a mixture have the same phospholipid/ATPase molar ratio. Hence, the first alternative, namely, that at all detergent concentrations there is a homogeneous population of ATPases with regard to stability, is correct. A rapid equilibrium between the different sized complexes is unlikely since we have separated them by sedimentation ultracentrifugation. Thus, when a complex divides, the phospholipid is distributed about equally between ATPases, and the reverse is a simple fusion process. The finding that mixtures of different sized soluble complexes at equilibrium have complexes with approximately the same phospholipid/ATPase molar ratio further suggests that a specific quaternary structure may not exist with the soluble enzyme since, on a molar basis, the monomer could be expected to be surrounded by more phospholipid than the dimer and the latter more than the trimer and so on. It should be pointed out that if a specific oligomeric structure does not exist in detergent solutions, our results do not eliminate the possibility that the putative oligomer in the native membrane is stabilized by protein–protein interactions in addition to phospholipids.

This study has shown that phospholipids play an important role in stabilizing the Ca^{2+}-ATPase which cannot be performed by Triton X-100 and, likely, other nonionic detergents. This suggests that the enzyme requires a specific association with the head groups of phospholipids for maintenance of a stable native structure. Any study seeking to determine the effects of ATPase–ATPase associations by comparing the characteristics of the native enzyme in SR vesicles with soluble monomeric ATPase needs to ensure that any differences are not due to detergent substituting for phospholipid. The phospholipid-stabilized monomeric preparation, which we describe, overcomes this difficulty and is more suitable for investigating the characteristics of the unassociated ATPase.

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