Effects of Nonsolubilizing and Solubilizing Concentrations of Triton X-100 on Ca^{2+} Binding and Ca^{2+}-ATPase Activity of Sarcoplasmic Reticulum†

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ABSTRACT: The effect of low concentrations of Triton X-100, below that required for solubilization, on the properties of the Ca^{2+}-ATPase of sarcoplasmic reticulum has been investigated. The changes observed have been compared with the changes produced on solubilization of the vesicles at higher concentrations of detergent. In the range 0.02–0.05% (w/v) Triton X-100, concentrations which did not solubilize the vesicles but completely inhibit ATP-mediated Ca^{2+} accumulation, 8–16 mol of detergent/mol of ATPase was associated with the vesicles. This amount of Triton X-100 altered equilibrium Ca^{2+} binding and Ca^{2+} activation of *p*-nitrophenyl phosphate and of ATP hydrolysis in a manner which lowered the apparent Ca^{2+} cooperativity (n_H = 1 or less), and which increased the $K_{0.5(Ca)}$ value 20-fold. These changes in Ca^{2+} binding and activation parameters were associated with a 90% lower Ca^{2+}-induced change in fluorescence of fluorescein isothiocyanate modified enzyme. The rates of *p*-nitrophenyl phosphate and of ATP hydrolysis, at saturating Ca^{2+} concentrations, were about half that of detergent-free vesicles. The rate constant for phosphoenzyme hydrolysis in the absence of Ca^{2+}, calculated from medium $P_i \rightleftharpoons HOH$ exchange and phosphoenzyme measurements, was lowered from 38 to 11 s^{-1}. The steady-state level of phosphoenzyme formed from P_i in the absence of Ca^{2+} was slightly increased up to 0.02% Triton X-100 and then decreased about half at 0.05%. The synthesis of ATP in single turnover type experiments was not affected by detergent binding. $P_i \rightleftharpoons$ ATP exchange was inhibited 65%. Solubilization of the vesicles [0.2% (w/v) Triton X-100 and 5 mg of Triton X-100/mg of protein] completely or partially reversed these changes except for the $K_{0.5(Ca)}$ and n_H values for ATPase activity, the rate of *p*-nitrophenyl phosphate hydrolysis at saturating Ca^{2+} concentrations, and the phosphoenzyme levels formed from P_i. It is proposed that intercalation of Triton X-100 into sarcoplasmic reticulum vesicles interacts with the ATPase, producing a functionally modified enzyme with low affinity for Ca^{2+} and no cooperativity between Ca^{2+} binding sites and which is partially inhibited in several catalytic properties. The inhibition, at least in part, is accounted for by the lower rate constant for phosphoenzyme hydrolysis. A large increase in the fluidity of the environment of the ATPase in the detergent micelle may account for the restoration of the Ca^{2+}-induced conformational change and catalytic properties of the solubilized ATPase. The relatively large increase in $K_{0.5(Ca)}$ for ATPase activity following solubilization can be largely ascribed to a loss of ATP enhancement of Ca^{2+} binding affinity.

Sarcoplasmic reticulum (SR)[1] vesicles isolated from mammalian skeletal muscle are composed largely of the Ca^{2+}-ATPase together with several other proteins present in smaller amounts. The ATPase is an integral membrane protein surrounded by lipid, mainly phospholipids and smaller amounts of cholesterol (Waku et al., 1971; Owens et al., 1972; Meissner & Fleischer, 1971; McIntosh et al., 1977). There are about 75–90 phospholipid molecules per molecule of ATPase. The purified Ca^{2+}-ATPase is capable of transporting Ca^{2+} in reconstituted vesicles (Racker, 1972; Meissner & Flescher, 1973; Warren et al., 1974; Knowles & Racker, 1975) although the absence of proteolipid, which may affect transport (Racker & Eytan, 1975), was not rigorously demonstrated. The activity of the Ca^{2+}-ATPase appears also to be modulated by some of the other protein components of the membrane. A 53 000-dalton glycoprotein stimulates ATPase activity (Chiesi & Carafoli, 1982), a 60 000-dalton protein which is phosphorylated in a calmodulin-dependent manner affects Ca^{2+} accumulation (Campbell & MacLennan, 1982), and an endogenous kinase and phosphatase, which phosphorylates and dephosphorylates the Ca^{2+}-ATPase, also influences its activity (Varsanyi & Heilmeyer, 1981; Heilmeyer et al., 1982). In addition to these possible protein–protein interactions, the extent to which Ca^{2+} transport is dependent on or modulated by protein–protein association of ATPases is at present not resolved [for reviews, see Ikemoto, (1982) and Moller et al. (1982)]. One approach to the latter problem has been to solubilize the enzyme and attempt to dissociate it into its minimal functional unit. Nonionic detergents, such as Triton X-100 and dodecyl octaethylene glycol monoether ($C_{12}E_8$), solubilize the sarcoplasmic reticulum membrane, with retention of high Ca^{2+}-stimulated ATPase activity. Substitution of most of the phospholipids with such detergents does not appear to be detrimental to activity (Dean & Tanford, 1978). It is possible under appropriate conditions to obtain a monomer of solubilized ATPase which also retains ATPase activity (Dean & Tanford, 1978; Moller et al., 1980).

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[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; FITC, fluorescein isothiocyanate; pNPP, *p*-nitrophenyl phosphate; MOPS, 3-(*N*-morpholino)propanesulfonic acid; ANS, 8-anilino-1-naphthalenesulfonic acid; $P^{18}O_4$, inorganic phosphate with all four oxygen atoms substituted with ^{18}O; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; pNPPase, *p*-nitrophenylphosphatase; SDS, sodium dodecyl sulfate.

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The nonperturbing effects of these detergents on enzymatic activity make them attractive agents for altering the lipid environment of the ATPase and possibly even disrupting protein–protein associations in sarcoplasmic reticulum vesicles. Sufficient detergent could associate with the membranes before solubilization occurs to hinder protein–protein contact, either via changes in lipid fluidity, by a dilution effect, or through more direct interaction with the proteins. For example, nonsolubilizing concentrations of $C_{12}E_8$ increase the mobility of a proportion of spin-labeled fatty acids, covalently attached to the ATPase (Anderson et al., 1981), and eliminate the heterogeneity of Ca^{2+} binding sites (Ikemoto et al., 1981).

We report here that nonsolubilizing concentrations of Triton X-100 modify the catalytic properties of the Ca^{2+}-ATPase and intermediate reactions, including Ca^{2+} binding, and that these effects are largely reversed following complete solubilization at higher detergent concentrations.

Materials and Methods

SR vesicles were obtained from rabbit back and hind limb white muscle by the method of Eletr & Inesi (1972). They 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 or were lyophilized, stored at −60 °C, and reconstituted with water. Protein concentrations were determined by the Lowry method.

Reagents. Triton X-100 and fluorescein isothiocyanate (FITC) were obtained from Sigma. Triton X-100 was purified by the method of Askani & Catravas (1980). $H^{18}OH$ came from Norsk Hydro (New York, NY) and enriched $P^{18}O_4$ was made essentially as described by Hackney et al. (1980). The Ca^{2+} ionophore A23187 was a product of Calbiochem.

Turbidity Measurements. Turbidity and fluorescent measurements were obtained with an Aminco-Bowman spectrofluorometer equipped with a continuously stirred cuvette maintained at 25 °C. Changes in turbidity were monitored by measuring 90° light scattering at 600 nm.

Triton X-100 Binding. The amount of Triton X-100 associated with SR vesicles was measured spectrophotometrically at various concentrations of detergent. SR vesicles (0.4 mg of protein/mL) in a medium (5 mL) of 150 mM MOPS, pH 7.0, 80 mM KCl, 5 mM $MgCl_2$, and various Triton X-100 concentrations were incubated for 5 min at 25 °C and centrifuged at 100000*g* at 25 °C for 30 min. The supernatant was discarded, the tube and pellet were carefully washed, and the pellet was resuspended in 0.5 mL of medium without the detergent. An aliquot was removed for protein determination and the remainder diluted with an equal volume of 1 N perchloric acid. The protein precipitate was pelleted, and the amount of Triton X-100 was determined in the supernatant by its absorption at 275 nm. A standard curve of Triton X-100 concentration was found to be linear up to 8 ng/mL.

Ca^{2+} Transport. The Ca^{2+} accumulating ability of vesicles was determined by the Millipore filtration method, using $^{45}CaCl_2$ and potassium oxalate (Martonosi & Ferretos, 1964).

p-Nitrophenyl Phosphate Hydrolysis. *p*-Nitrophenyl phosphate (pNPP) hydrolysis was monitored spectrophotometrically at 405 nm. The extinction coefficient of *p*-nitrophenol was determined to be 7.8 $cm^2/\mu mol$ at pH 7.0.

ATPase Activity. ATP hydrolysis was determined from the rate of $[^{32}P]P_i$ release from $[\gamma\text{-}^{32}P]ATP$ by using the molybdic acid procedure (de Meis & Carvalho, 1974).

Phosphoenzyme Formation from P_i and ATP Synthesis. Phosphoenzyme formation was measured as described by Punzengruber et al. (1978). ATP was formed by diluting aliquots of phosphorylating medium and enzyme (see legend to Figure 4) 10-fold into 20 mM Tris-Cl, pH 7.0, 5 mM ADP, and 10 mM $CaCl_2$ and keeping Triton X-100 at the same concentrations as in the phosphorylating medium. At low concentrations of the detergent, (<0.1%), 100 μM A23187 was included in both media. The reaction was quenched after 1 min with an equal volume (5 mL) of ice-cold 1 N perchloric acid, containing 1.5 mL of chloroform and 5 μmol of ATP as carrier. The mixture was vortexed for 1 min and centrifuged, and the protein *plus* organic layer, containing the majority of the detergent, was discarded. The chloroform, dissolved in the aqueous phase, was removed by bubbling nitrogen gas through the solution at 0 °C for 30 min. The ATP was purified on a 0.7 × 3 cm Celite/charcoal column followed by anion-exchange chromatography (AG 1×4) (Hackney et al., 1980). Results were corrected for the recovery of ATP that ranged from 50 to 60%. Blank reaction mixtures lacked added $CaCl_2$.

$P_i \rightleftharpoons$ ATP Exchange. $P_i \rightleftharpoons$ ATP exchange was measured as described by Carvalho et al. (1976). The reaction was measured over 2 min. In preliminary experiments, the linearity of the reaction was established over 5 min. The reaction was started by the addition of a mixture of $[^{32}P]P_i$, ATP, and ADP and stopped by a 5-fold dilution into a solution containing 1 N perchloric acid and 100 mM phosphoric acid. The ATP was processed as described above.

$P_i \rightleftharpoons$ HOH Exchange. Medium $P_i \rightleftharpoons$ HOH exchange was measured as previously described for $P^{18}O_4$ (McIntosh & Boyer, 1983).

Ca^{2+} Binding. Equilibrium Ca^{2+} binding was performed by monitoring the Ca^{2+}-induced changes in fluorescence of 8-anilino-1-naphthalenesulfonic acid, λ_{ex} = 370 nm and λ_{em} = 480 nm (Arav et al., 1983). Measurements were made with an Aminco Bowman spectrofluorometer equipped with a continuously stirred cuvette maintained at 25 °C. Free Ca^{2+} concentrations were calculated by taking into account all species involved in the equilibria between H^+, Mg^{2+}, Ca^{2+}, and EGTA; the association constants were taken from Martell & Smith (1974). Values for $K_{0.5}$ and n_H were obtained by least mean squares analysis of the data (Atkins, 1973). The Ca^{2+}-induced change in fluorescence of fluorescein isothiocyanate (FITC) modified ATPase was monitored at λ_{ex} = 497 nm and λ_{em} = 520 nm. FITC modification was carried out as described by Andersen et al. (1982), and excess reagent was removed by centrifugation through Sephadex G-50 (Penefsky, 1977).

Results

Binding of Triton X-100 to SR Membranes and Solubilization. The binding of Triton X-100 to SR vesicles has been measured over the detergent concentration range of 0.02–0.1% (Figure 1). There is an increase in Triton X-100 associated with the vesicles as the concentration increases. At 0.02% Triton X-100, it can be calculated, assuming 8 nmol of ATPase/mg of protein, that there is approximately 8 mol of Triton X-100/mol of ATPase in the membrane. This figure can be compared to 75–90 mol of phospholipid/mol of ATPase. Similar amounts of $C_{12}E_8$ are bound to ATPase vesicles and to SR–lipid liposomes (Andersen et al., 1983). It should be noted that in all the experiments reported below the same protein concentration of 0.4 mg/mL has been used to permit direct correlations. Increasing concentrations of Triton X-100, in the range of 0.05–0.1% (w/v), resulted in solubilization of the membranes, as monitored by 90° light scattering at 600 nm or by quantitating the amount of material pelletable by centrifugation (Figure 2). Watanabe & Inesi (1982) have reported that catalytic turnover with Ca^{2+} and ATP in the medium causes clarification of the vesicles if the vesicles are

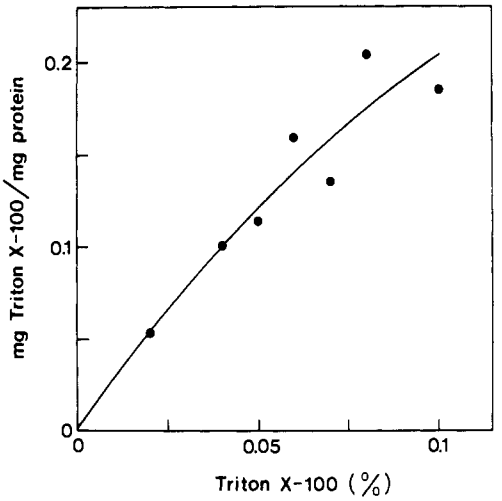

FIGURE 1: Binding of Triton X-100 to SR vesicles. SR vesicles (0.4 mg of protein/mL) were incubated at 25 °C for 5 min in 150 mM MOPS, pH 7.0, 5 mM $MgCl_2$, 50 mM KCl, and the Triton X-100 concentrations shown. The samples were centrifuged at 100000*g* for 20 min at room temperature. The data show the amount of Triton X-100 associated with the pellets.

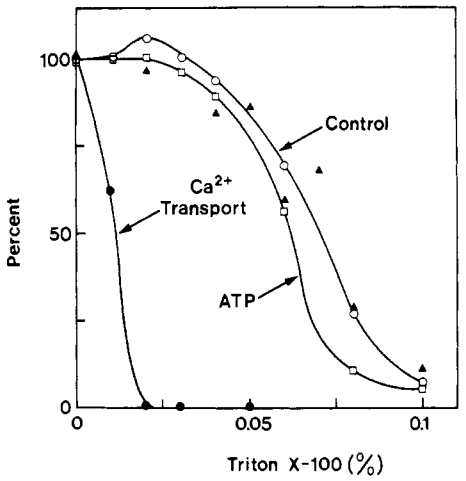

FIGURE 2: Inactivation of Ca^{2+} transport and solubilization of SR vesicles. To determine the effect of Triton X-100 on Ca^{2+} transport, SR vesicles (0.4 mg of protein/mL) were incubated at 25 °C for 5 min in 50 mM MOPS, pH 7.0, 100 mM KCl, 5 mM $MgCl_2$, 5 mM EGTA, 5 mM $^{45}CaCl_2$, 5 mM potassium oxalate, and the Triton X-100 concentrations shown. Ca^{2+} transport was intiated by addition of ATP (5 mM) and stopped after timed intervals by dilution 150-fold into a quench medium (20 mM MES, pH 6.0, 5 mM $CaCl_2$, and 125 mM KCl) and immediate Millipore filtration. Solubilization of SR vesicles (0.4 mg of protein/mL) was carried out at 25 °C for 5 min in 50 mM MOPS, pH 7.0, 100 mM KCl, 5 mM $MgCl_2$, 0.5 mM EGTA, 0.5 mM $CaCl_2$, and the Triton X-100 concentrations shown. Either the samples were then centrifuged at 100000*g* for 20 min at room temperature and the protein in the pellets was quantitated (▲) or the turbidity was measured by 90° light scattering at 600 nm (○). When ATP was present (1 mM), only the latter procedure was followed, and it was added immediately prior to measurement (□).

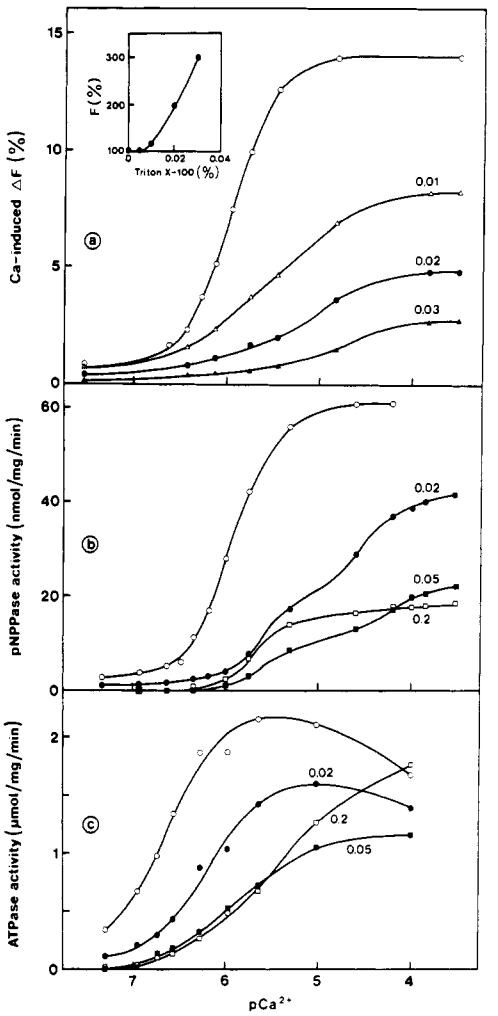

FIGURE 3: Effect of Triton X-100 on Ca^{2+} binding (a), on Ca^{2+} activation of pNPPase activity (b), and on Ca^{2+} activation of ATPase activity (c). For Ca^{2+} binding, SR vesicles (0.4 mg of protein/mL) were preincubated at 0 °C for 2 h in 150 mM MOPS, pH 7.0, 100 mM KCl, 5 mM $MgCl_2$, and 20 μM ANS. The suspension was warmed to 25 °C in the spectrofluorometer and Triton X-100 added to the concentrations shown. EGTA (0.5 mM) was added, and Ca^{2+} binding on the addition of aliquots of Ca^{2+} was followed by monitoring fluorescent changes. For measurement of pNPPase activity, SR vesicles (0.4 mg/mL) were preincubated for 5 min at 25 °C in 50 mM MOPS, pH 7.0, 100 mM KCl, 9.0 mM $MgCl_2$, 8.8 mM pNPP [to give 5.0 mM free Mg^{2+} and 4.0 mM Mg-pNPP, using a stability constant of 6 mM for Mg-pNPP (Nakamura & Tonomura, 1978)], 0.5 mM EGTA, 20 μM A23187, and the Triton X-100 concentrations shown. The rate of *p*-nitrophenol release was monitored at 405 nm and at 25 °C following the sequential addition of aliquots of Ca^{2+}. For measurement of ATPase activity, SR vesicles (0.4 mg of protein/mL) were preincubated for 5 min at 25 °C in 50 mM MOPS, pH 7.0, 100 mM KCl, 5 mM $MgCl_2$, 0.5 mM EGTA, and various Ca^{2+} concentrations to yield the free concentrations shown, 20 μM A23187 and the Triton X-100 concentration indicated in the figure. The reaction was initiated with [^{32}P]ATP (1 mM) and quenched at time intervals with an equal volume of 1 N perchloric acid.

partially solubilized with $C_{12}E_8$. A similar effect is produced with Triton X-100, as shown in Figure 2. It should be noted that at this protein concentration the effect is only detectable at concentrations of Triton X-100 greater than 0.05%. The poorly hydrolyzed substrate pNPP had no effect on the range in which solubilization occurred (data not shown). The main point to be made from these experiments for the purpose of this paper is that solubilization only begins to be initiated at approximately 0.05% Triton X-100, with or without Ca^{2+} and ATP. The interaction of Triton X-100 with the SR membrane at concentrations lower than those required for solubilization can be detected by the inhibition of Ca^{2+} transport. This activity is 50% inhibited at approximately 0.013% Triton X-100 and is nil at 0.02% (Figure 2). The reason for this inhibition of net Ca^{2+} transport is an increase in Ca^{2+} permeability. This was deduced from the accelerated rate of Ca^{2+} efflux from actively loaded vesicles following the addition of 0.01% and 0.02% Triton X-100 (results not shown). An increase in permeability also occurs with $C_{12}E_8$ binding (Andersen et al., 1983).

Ca^{2+} Binding. Ca^{2+} binding to SR membrane was monitored by using the Ca^{2+}-induced fluorescent change arising

from membrane-bound 8-anilino-1-naphthalenesulfonic acid (ANS) (Figure 3). In the absence of Triton X-100, Ca^{2+} binding to high-affinity sites ($K_{0.5}$ = 1.02 μM) produced a 13–18% reversible change in fluorescence that was cooperative (n_H = 1.6). Increasing concentrations of Triton X-100 in the 0.01–0.03% range caused an increase in the total fluorescence (inset, Figure 3a). This is probably due to an increasing concentration of detergent micelles, to which ANS binds. Nevertheless, Ca^{2+} binding could still be monitored in this low concentration range, as shown in Figure 3a. Increasing concentrations of Triton X-100 abolished the cooperative nature of Ca^{2+}-induced fluorescence, with n_H values decreasing to values below 1, and increased the $K_{0.5}$ value at 0.03% Triton X-100. This could be due either to negative cooperativity between Ca^{2+} binding sites or to a heterogeneous population of binding sites with high and with low affinity for Ca^{2+}. It should be noted that at these concentrations of detergent the proportion of solubilized ATPase is negligible.

pNPPase Activity. The effect of low concentrations of Triton X-100 on Ca^{2+} activation of pNPPase activity is shown in Figure 3b. The monophasic and cooperative activation curve of the control vesicles changed with 0.02% and 0.05% Triton X-100 to a more complex biphasic curve with lower rates at saturating Ca^{2+} concentrations. The Hill coefficients for the entire curves are approximately 1, but it is apparent that the activation in the low concentration range is similar to that of the control vesicles but with lower velocity and that there is a secondary activation in the higher concentration range. It is again worth noting that the biphasic nature of the curve cannot be ascribed to the participation of solubilized enzyme. In fact, solubilization (0.2% Triton X-100) resulted in a curve not dissimilar from the control curve but with one-third of the V_{max} and no indication of a low-affinity form of the enzyme (Figure 3b).

ATPase Activity. A study similar to that described above with pNPP was done using ATP as substrate, and the results are shown in Figure 3c. Detergent binding induced a similar decrease in the velocity at saturating Ca^{2+} concentrations. However, in this case, some of this activity returned on solubilization. This phenomenon has recently been reported by others (Ludi et al., 1982), and a similar effect has been noted with $C_{12}E_8$ (Verjovski-Almeida & Silva, 1981). Binding of detergent lowered the Hill coefficient for Ca^{2+} activation to about 1 and raised the $K_{0.5(Ca)}$ value 20-fold. The activation curve of the solubilized enzyme was monophasic and showed no Ca^{2+} cooperativity (n_H = 1.0). The $K_{0.5(Ca)}$ value remained high, similar to the findings of Ludi et al. (1982) and of Kosh-Kosicka et al. (1983) for the solubilized enzyme in Triton X-100 and $C_{12}E_8$. Ludi et al. (1982) found that low concentrations of ionic detergents did not alter the Ca^{2+} activation of ATPase activity, contrary to what we found. At present, we have no explanation for this discrepancy.

The finding that solubilization reverses the inhibition of the V_{max} for ATPase activity but not of pNPPase activity shows that different steps are rate limiting in the two cycles. The very much slower hydrolysis of pNPP is likely to be due to a slower rate of enzyme phosphorylation. The results suggest that Triton X-100 inhibits this step even in the solubilized state, a phenomenon which is obscured with ATP as substrate.

The effects of Triton X-100 concentration on $K_{0.5(Ca)}$ values and the Hill coefficients for Ca^{2+} binding and Ca^{2+} activation of pNPPase and ATPase activities are shown in Figure 4a,b. The values are representative of the entire curves. From Figures 3 and 4, it is apparent that the $K_{0.5(Ca)}$ values for Ca^{2+} binding and Ca^{2+} activation of pNPPase activity of native

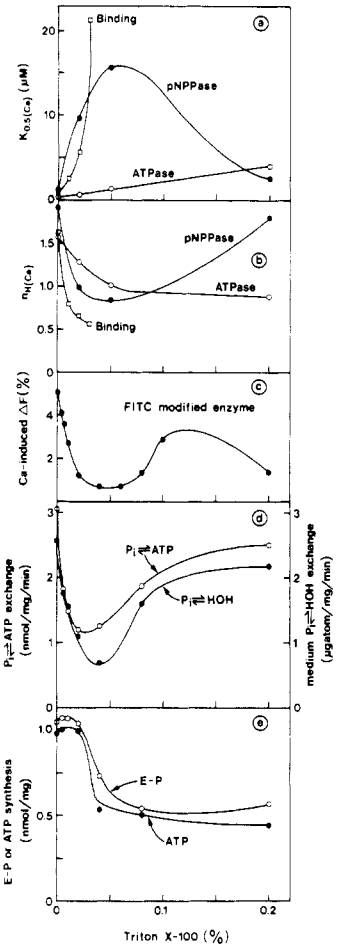

FIGURE 4: Effect of Triton X-100 on $K_{0.5(Ca)}$ (a), the apparent Ca^{2+} cooperativity (b), then Ca^{2+}-induced change in fluorescence of the FITC-modified enzyme (c), $P_i \rightleftharpoons$ HOH and $P_i \rightleftharpoons$ ATP exchanges (d), and steady-state E–P levels from P_i and the formation of ATP (e). The parameters for (a) and (b) were obtained from the curves in Figure 3 by using all the data points in each curve. The fluorescent measurements were done following preincubation at 25 °C of the FITC-modified enzyme (0.4 mg of protein/mL) in 150 mM MOPS, pH 7.0, 100 mM KCl, 5 mM $MgCl_2$, and the concentration of Triton X-100 shown. EGTA (0.5 mM) was added followed by aliquots of $CaCl_2$ to give the maximum fluorescent change. For (d) and (e), SR vesicles (0.4 mg of protein/mL) were preincubated at 25 °C in 20 mM MES, pH 6.5, 50 mM KCl, 20 mM $MgCl_2$, and 0.5 mM EGTA at the concentrations of Triton X-100 shown. $P_i \rightleftharpoons$ HOH exchange was initiated by adding 5 mM $P^{18}O_4$, $P_i \rightleftharpoons$ ATP exchange by adding 5 mM [^{32}P]P_i, 5 mM ATP, and 0.5 mM ADP, and E–P formation by adding 5 mM [^{32}P]P_i. ATP was synthesized following 10-s E–P formation by a jump in $CaCl_2$, $MgCl_2$, and KCl concentrations and pH as described under Materials and Methods. All assays were at 25 °C. Oxygen exchange was calculated from the relationship $v_{ex} = [(4 - 3P_c)/(4 - 4P_c)]4k_{av}([P_i]/[protein])$ where P_c represents the partition coefficient and is equal to $(4 - k_4/k_{av})/3$, k_4 is the pseudo-first-order rate constant for the disappearance of $P^{18}O_4$, and k_{av} is the corresponding rate constant for the average loss of all ^{18}O in each of the ^{18}O-enriched species. The use of these equations in such analyses has been explained in Hackney et al. (1980).

vesicles are the same. The $K_{0.5(Ca)}$ value for Ca^{2+} activation of ATPase activity is lower. This suggests that ATP binding but not pNPP binding alters the affinity of the enzyme for Ca^{2+}. Following solubilization, the $K_{0.5(Ca)}$ value for pNPPase activity changes little (1.2–2.5); that for ATPase activity changes about 20-fold (0.2–3.7). The values for the solubilized enzymes are similar for both substrate types. It can be concluded that solubilization results in a loss of the ATP enhancement of Ca^{2+} binding affinity and that this is largely the reason for the change in the $K_{0.5(Ca)}$ value with ATP as substrate. It is also significant that Ca^{2+} activation of pNPPase

activity of the solubilized enzyme is cooperative, with the n_H value similar to that of the native enzyme, whereas with ATP the cooperativity is decreased compared with the native enzyme (Figure 4b). Hence, the change in the apparent cooperativity in the latter case is also due to an effect of ATP.

The dependence of the magnitude of the Ca^{2+}-induced change in fluorescence of the ATPase, modified by fluorescein isothiocyanate (FITC), on detergent concentration is shown in Figure 4c. FITC reacts covalently and stoichiometrically with an ATP binding site and can be used to report on the Ca^{2+}-induced conformational change (Andersen et al., 1982). In the 0.02–0.06% Triton X-100 concentration, the Ca^{2+}-induced fluorescent change (ΔF) is 10% of that of detergent-free vesicles. Higher concentrations of detergent partially restore the ΔF which decreases again at yet higher concentrations. These changes in fluorescence with detergent concentration do not appear to be due to quenching or some other direct effect of detergent. This can be deduced from the restoration of some of the ΔF at higher concentrations of detergent and because a similar effect on ΔF, arising from the intrinsic fluorescence, can be produced by $C_{12}E_8$ binding in the same concentration range (Verjovski-Almeida & Silva, 1981; Ludi et al., 1982; Andersen et al., 1983). With $C_{12}E_8$, the ΔF on Ca^{2+} binding is fully restored on solubilization. It can also be calculated from the data in Figure 3 that the decrease in ΔF on Ca^{2+} binding, using ANS to report binding, is larger than would be expected from the increase in total fluorescence.

Stability of the ATPase in the Presence of Detergent and EGTA. High concentrations of detergent, which are considered to result in monomer formation, also render the enzyme unstable in the absence of Ca^{2+} (Dean & Tanford, 1978; le Maire et al., 1978; Moller et al., 1980). We have determined whether low concentrations of detergent, below those necessary for solubilization, could produce the same instability in the absence of Ca^{2+} and thereby affect the Ca^{2+} activation curves. Preincubation of the enzyme in EGTA and 0.05% Triton X-100 resulted in less than 10% change in ATPase activity over a period of 4 min (not shown). In contrast, solubilization (0.2% Triton X-100) resulted in the loss of 40% of the activity over the course of 2 min.

Preincubation in detergent, prior to pNPPase assay, was performed in the presence of pNPP, and little inactivation was apparent over 20 min in EGTA, even at 0.2% Triton X-100. Protection of the detergent-solubilized enzyme by substrate has been described by Moller et al. (1980).

$P_i \rightleftharpoons$ ATP and $P_i \rightleftharpoons$ HOH Exchange, E–P Formation, and ATP Synthesis. Low concentrations of Triton X-100 decreased both $P_i \rightleftharpoons$ ATP and $P_i \rightleftharpoons$ HOH exchanges by about 65% (Figure 4c). This effect was reversed at higher concentrations of detergent. Since $P_i \rightleftharpoons$ HOH exchange activity = k[E–P], where k is the rate constant for phosphoenzyme hydrolysis (Hackney et al., 1980) and [E–P] is the steady-state level of phosphoenzyme, it is possible to determine which of these two parameters is responsible for the decreased oxygen exchange. As can be seen from Figure 4d,e, the correlation between the dependence of oxygen exchange and the steady-state level of E–P is poor. The latter increases up to 0.025% Triton X-100 and declines at higher concentrations, just the opposite to the oxygen exchange curve. Hence, the reason for the decrease in $P_i \rightleftharpoons$ HOH exchange in the 0.02–0.05% Triton X-100 range and the increase in the 0.1–0.2% range is an alteration in the rate constant for phosphoenzyme hydrolysis. It can be calculated that k decreases from 38 to 11 s^{-1} at 0% and 0.02% Triton X-100, respectively, and reaches a value of 64 s^{-1} at 0.2% detergent. It should be noted that inhibition of steady-state E–P levels occurs at concentrations of Triton X-100 just lower than that required for solubilization and hence it is not a consequence of solubilization. A similar dependence on Triton X-100 concentration has been obtained by Ludi et al. (1982) and on $C_{12}E_8$ concentration by Ikemoto et al. (1981).

In order to determine whether the decreased $P_i \rightleftharpoons$ ATP exchange is due to a decreased capacity of the enzyme to synthesize ATP, the synthesis of ATP from E–P derived from P_i, in single enzyme turnover experiments, was investigated and is shown in Figure 4e. Nearly stoichiometric amounts of ATP could be formed from E–P both in the absence and in the presence of Triton X-100, including solubilizing concentrations.

Discussion

The results show that several properties of the Ca^{2+}-ATPase of SR vesicles are altered by the presence of Triton X-100 at concentrations below that required for solubilization. Above these concentrations, under solubilizing conditions, many of these effects are reversed. The alterations induced by detergent binding to the vesicles, such as Ca^{2+} binding, Ca^{2+} activation of enzyme turnover in the presence of a Ca^{2+} ionophore, and the inhibition of $P_i \rightleftharpoons$ ATP exchange in the presence of ionophore and of $P_i \rightleftharpoons$ HOH exchange, measured in the absence of Ca^{2+}, cannot be influenced by a change in permeability of the vesicles. This suggests that intercalation of Triton X-100 into the bilayer is directly modulating enzyme activity.

Several mechanisms, which are not necessarily exclusive of each other, can be proposed. First, detergent molecules could be interacting directly with the ATPase, at the protein–lipid interface, substituting for the surrounding phospholipid and thereby altering the properties of the enzyme. The enhanced permeability of the membrane to Ca^{2+} could also be through alterations at the protein–lipid interface. In view of the low proportion of detergent relative to the phospholipid which elicits changes in catalytic function, Triton X-100 may preferentially associate with the ATPase rather than in the bulk phospholipids. A stronger interaction between the ATPase and Triton X-100 compared with the ATPase and phospholipid could arise from hydrogen bonding between the poly(ethoxyethylene) moiety of the detergent and the amino acid side chains. Another mechanism for the changes could be through detergent affecting the fluidity and packing of phospholipids. Andersen et al. (1983) have shown that insertion of $C_{12}E_8$ into the SR membranes increases the fluidity of the lipid phase and the rotational diffusion of the ATPase. As pointed out by the authors, a rise in temperature induces similar changes and is associated with an increased enzyme turnover, contrary to what is observed with detergent. A third mechanism could be through alteration of lipid distribution and possibly surface charge distribution. That the lipid distribution surface charge is not critical for activity is shown by the finding that solubilization results in enhanced ATPase activity. However, these factors may still be modulating enzyme activity, and much higher activity may be possible if, for example, the surface charge of the detergent micelle was identical with that of the SR membrane. A fourth mechanism, possibly stemming from the increased fluidity or else via direct interaction with the ATPase, is disruption of protein–protein interactions. This could be through either the prevention of oligomeric associations or the inhibition of proteins which modulate the ATPase. Dissociation of modulator proteins from the membrane surface could also occur. SDS–polyacrylamide gel electrophoresis of pelleted material reveals that some extrinsic membrane proteins, notably calsequestrin, do partly dissociate at 0.04% and

0.06% Triton X-100 (not shown). However, since the purified ATPase does not exhibit properties similar to the detergent-modified ATPase, the former having a high turnover rate (MacLennan, 1971) and a full Ca^{2+}-induced change in tryptophan fluorescence (Verjovski-Almeida & Silva, 1981; Andersen et al., 1982), a mechanism which invokes modulator proteins is not likely. Although the quaternary state of the Ca^{2+} pump has not been resolved, there is much evidence to suggest that the enzyme is a functional oligomer [see Ikemoto (1982) for a review]. Thus, intercalation of detergent between ATPases or else the enhanced fluidity could inhibit expression of oligomeric interactions. The enhanced rotation of the ATPase reported by Andersen et al. (1983) on $C_{12}E_8$ binding could be due to both the increased fluidity and the smaller size of the rotating unit. There is evidence that the kinetic heterogeneity of Ca^{2+} binding sites, attributed to oligomeric associations, is eliminated by nonsolubilizing concentrations of $C_{12}E_8$ (Ikemoto et al., 1981). Also, energy transfer between two populations of ATPases, containing donor and acceptor fluorophores reconstituted together, is diminished under these conditions (Champeil et al., 1982). Excimer formation between a pyrene derivative covalently attached to the enzyme is also decreased (Ludi & Hasselbach, 1982, 1983).

Under conditions used for Ca^{2+} binding and Ca^{2+} activation of *p*-nitrophenyl phosphate hydrolysis, biphasic curves were obtained contributing to overall lower values for n_H. Although this could be due to negative cooperativity, the characteristics of the curves in the lower Ca^{2+} concentration range suggest that this part of the curve reflects native ATPase and that in the higher concentration range detergent-modified ATPase. Thus, Triton X-100, under some conditions, apparently induces heterogeneity of the ATPases. Biphasic curves for Ca^{2+} activation of ATPase activity have been obtained for the solubilized enzyme in $C_{12}E_8$ (Silva & Verjovski-Almeida, 1983). If the detergent does inhibit ATPase interaction, our results would suggest that one reason for ATPases to associate is to increase Ca^{2+} affinity and accelerate turnover of the pump. The latter, we demonstrate, is achieved at least partly by increasing the rate constant for phosphoenzyme hydrolysis.

The lower Ca^{2+}-induced change in fluorescence of the FITC-modified enzyme with Triton X-100 binding suggests that the enzyme, in the absence of Ca^{2+}, is in a conformation similar to that of the native ATPase with Ca^{2+} bound. Similar results are obtained by using intrinsic tryptophan fluorescence as a measure of conformation (Verjovski-Almeida & Silva, 1981; Andersen et al., 1983). On the other hand, the capacity of the enzyme to be phosphorylated from P_i does not fall off until about 0.04% Triton X-100. Since the conformation of the enzyme with Ca^{2+} bound cannot be phosphorylated from P_i, it must be concluded that the conformation induced by detergent binding and that by Ca^{2+} differ in this important respect. The decrease in the E–P levels from P_i at 0.04% Triton could be taken as evidence that at this concentration the conformation becomes closer to the Ca^{2+}-bound form. However, upon solubilization at 0.06–0.1% Triton X-100 and at higher concentrations, the lower E–P level is maintained, and yet the Ca^{2+}-induced change in fluorescence of the FITC-modified enzyme and of the tryptophan residues (Verjovski-Almeida & Silva, 1981; Andersen et al., 1982) is restored. Hence, the decrease in the capacity of the enzyme to be phosphorylated from P_i in the absence of Ca^{2+} is unrelated to the equilibrium of the Ca^{2+}-bound and Ca^{2+}-free conformations of the enzyme and is likely a result of a change in the rate constants governing the reaction of P_i with the enzyme. In particular, we show that, for the solubilized enzyme, the rate constant for E–P hydrolysis is increased.

Our results show that ATP enhances the affinity of the native enzyme for Ca^{2+}. pNPP does not exert this effect. Since the apparent cooperativity of Ca^{2+} activation is unaltered by ATP, it is likely that ATP enters the catalytic cycle prior to binding of the first Ca^{2+}. Early entry of ATP into the catalytic cycle has been suggested recently from ATP modulation of catalytic intermediates subsequent to phosphorylation of the ATPase (McIntosh & Boyer, 1983). The ATP modulation of Ca^{2+} affinity is lost on solubilization; $K_{0.5(Ca)}$ values for pNPPase and ATPase activities of the solubilized enzyme are similar. Thus, in large measure, the 20-fold change in the $K_{0.5(Ca)}$ value for ATPase activity following solubilization reported here and by others (Moller et al., 1980; Watanabe et al., 1981; Ludi et al., 1983; Kosk-Kosicka et al., 1983) is due to the loss of the ATP effect. This is in keeping with minor differences in the $K_{0.5(Ca)}$ value for pNPPase activity (our results) and for Ca^{2+} binding (Verjovski-Almeida & Silva, 1981; Andersen et al., 1982; Ludi et al., 1982) between the native and the solubilized enzyme. Hence, with the solubilized ATPase, either ATP and Ca^{2+} bind in a random manner or the ATP modulation is obscured or defunct.

The mechanism for the changes noted at low Triton X-100 concentrations cannot at this stage be ascertained with any certainty. However, we consider that it is likely either due to interaction of the detergent with the ATPase, at the protein–lipid interface, exerting a direct modulatory effect, or due to inhibition of protein–protein interactions, producing an altered ATPase which, in the absence of Ca^{2+}, exhibits a conformation similar to that of the native ATPase with Ca^{2+} bound and yet can react with P_i, has lower Ca^{2+} affinity and cooperativity, and has a slower turnover. Solubilization and introduction of the ATPase into a detergent micelle cause other changes which partly reverse these effects.

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Registry No. ATPase, 9000-83-3; Ca, 7440-70-2; Triton X-100, 9002-93-1.

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