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Kinetic Characterization of Detergent-Solubilized Sarcoplasmic Reticulum Adenosinetriphosphatase†

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ABSTRACT: Functional characterization of sarcoplasmic reticulum (SR) solubilized with the detergent dodecyl octaethylene glycol monoether ($C_{12}E_8$) was carried out by using both steady-state and rapid kinetic methods, and a comparison with the behavior of the adenosinetriphosphatase (ATPase) in leaky membrane vesicles was obtained. In conditions of maximal solubilization, the monomeric state (M_r 116 000 chain) of the ATPase polypeptide chains was established by comparing their ability to undergo covalent cross-linking in the membranous and solubilized states. The monomeric enzyme retains Ca^{2+}-dependent ATPase activity but is much less stable than the membranous enzyme especially in the presence of low (<1 μM) Ca^{2+} or high (pH 6.0) H^+ concentrations. Protection is afforded by occupancy of high-affinity calcium sites and pH 7.5. Depending on the duration and conditions of exposure to $C_{12}E_8$, the enzyme tends to develop a requirement for higher concentrations of activating Ca^{2+}. It is demonstrated by rapid quench methods that, in analogy to the membranous enzyme, the catalytic mechanism of the solubilized ATPase involves early formation of a phosphorylated enzyme intermediate which then undergoes hydrolytic cleavage and releases P_i. The phosphorylated intermediate can also be formed in the reverse direction of the hydrolytic reaction by exposing the enzyme to P_i in the absence of Ca^{2+} at pH 7.5 and in the presence of dimethyl sulfoxide. Addition of this organic solvent is an absolute requirement for the observation of the P_i reaction in the reverse cycle of the detergent-solubilized enzyme. Dilution with an aqueous medium containing millimolar Ca^{2+} and ADP then allows formation of ATP. In optimal conditions the enzyme retains the same number of catalytic sites as the membranous preparation. Therefore, the detergent does not uncover sites that are latent due to aggregation in the membranous state. Within the 1–100 μM range of ATP concentration, the phosphoenzyme turnover in the solubilized preparation is approximately twice as fast as in leaky vesicles. However, at millimolar ATP concentrations, the phosphoenzyme turnover is similar in both preparations, due to an activating effect of high ATP on the membranous, but not the solubilized, ATPase. In addition to a faster turnover, the solubilized enzyme undergoes a more rapid "switch off" from the calcium-activated state (e.g., loss of its ability to be phosphorylated by ATP) upon removal of calcium with ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid.

Sarcoplasmic reticulum (SR) adenosinetriphosphatase (ATPase) is most commonly prepared in the form of vesicular fragments of native membrane. However, it is possible to maintain active enzyme preparations following solubilization with detergents (McFarland & Inesi, 1971; LeMaire et al., 1976a). In some cases, the activity of the solubilized preparations has been attributed to tri- or tetrameric aggregates of the M_r ~116 000 ATPase chains (LeMaire et al., 1976b; LeMaire et al., 1978) and in others to monomeric dispersions of single chains (LeMaire et al., 1976a; Jorgenson et al., 1978; Dean & Tanford, 1978; Moller et al., 1980). Of the several detergents which are available for this purpose, dodecyl octaethylene glycol monoether ($C_{12}E_8$) is the most widely used. Therefore, we have chosen this detergent to study the kinetic behavior of SR ATPase in the solubilized state.

Previous studies on the steady-state activity of SR ATPase solubilized in $C_{12}E_8$ have shown that the enzyme retains basic functional features such as catalysis of ATP hydrolysis and its Ca^{2+} dependence (Dean & Tanford, 1978; Moller et al., 1980). On the other hand, it was reported that the solubilized ATPase does not have the complex dependence on ATP concentration which is manifested by the membranous enzyme. Furthermore, the solubilized ATPase undergoes denaturation following removal of calcium (Moller et al., 1982) and lacks the ability to react with P_i to form the phosphoenzyme in the

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absence of Ca^{2+} (Nestruck-Goyke & Hasselbach, 1981). In other cases, however, the P_i reaction was found to be preserved (Martin, 1982). A different behavior of the solubilized ATPase with respect to Mg^{2+} requirements was also reported (Yamamoto & Tonomura, 1982). Some inconsistencies are found in the literature regarding the range and apparent cooperativity of the Ca^{2+} concentration requirement for activation of the solubilized enzyme and modification of its intrinsic fluorescence (Moller et al., 1980; Murphy et al., 1982; Watanabe & Inesi, 1982; Verjovski-Almeida & Silva, 1981). The solubilized preparation was never studied with rapid quench methods, and the kinetic sequence for formation of phosphorylated enzyme intermediate and hydrolytic cleavage of P_i was not demonstrated.

We describe here a series of experiments designed to study the transient and steady-state kinetics of SR ATPase solubilized in $C_{12}E_8$. In these studies, we have used a preparation of SR vesicles which was previously characterized in detail (Inesi et al., 1982a,b). In order to avoid any functional modification or possible denaturation previous to solubilization with $C_{12}E_8$, we did not perform any preliminary extraction of ATPase with other detergents. Furthermore, all measurements were made in media containing very dilute protein to prevent reassociation of solubilized enzyme which may occur at high protein concentrations (Dean & Tanford, 1978). In this manner we obtained a direct comparison of the ATPase in its native environment and under conditions favoring maximal solubilization.

Materials and Methods

SR vesicles were isolated from rabbit leg muscle (Eletr & Inesi, 1972). Protein concentration was estimated by the Folin method.

Solubilization of SR vesicles was obtained in a medium containing 20 mM Tris–maleate[1] (pH 7.5, unless specified otherwise), 80 mM KCl, 10 mM $MgCl_2$, 0.15 mM $CaCl_2$, 1.5 mM $C_{12}E_8$, and 25–50 μg of protein/mL, at 25 °C. The protein concentration was kept low to ensure maximal solubilization as demonstrated by the measurements of Dean & Tanford (1978).

The cross-linking reagents cupric phenanthroline, glutaraldehyde, and dithiobis(succinimidyl propionate) (DSP) were used to test whether the ATPase chains in the membranous or solubilized state were susceptible to covalent cross-linking. Following incubations for increasing time or with various concentrations of cross-linking reagents, the protein was dissolved in 1% $NaDodSO_4$ and analyzed by electrophoresis on a gradient (4–8%) of polyacrylamide gel, according to Laemmli (1970).

Total calcium was measured by atomic absorption spectroscopy or by double wavelength photometry and titration with EGTA in the presence of murexide. Estimates of free Ca^{2+} were calculated with a computer program based on the Ca·EGTA binding constant given by Schwartzenbach et al. (1957), pH, and the competitive effects of Mg^{2+}, K^+, and nucleotide, as described by Fabiato & Fabiato (1979).

ATPase activity in steady-state conditions was measured in a reaction medium containing 20 mM Tris–maleate (pH 7.5, unless specified otherwise), 80 mM KCl, 10 mM $MgCl_2$, variable concentrations of Mg·ATP, variable Ca^{2+} and EGTA, 2 mM phosphoenolpyruvate, 135 μg (10.6 units) of pyruvate kinase/mL, 1.5 mM $C_{12}E_8$ where indicated, and 30–50 μg of SR protein/mL. The reaction temperature was 25 °C. Serial samples were taken for determination of P_i by the molybdovanadate reaction (Lecocq & Inesi, 1966) as described by Lin & Morales (1977). The reaction velocity was estimated from linear slopes of P_i production vs. time.

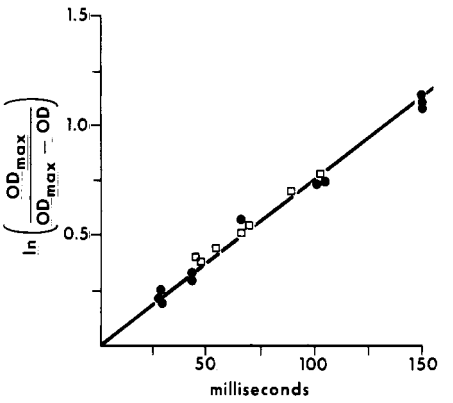

FIGURE 1: Standardization of the Dionex multimixer adapted for double quenching. Hydrolysis of 2,4-dinitrophenyl acetate (syringes A and B) was initiated by addition of an equal volume of 0.2 N NaOH (syringe C) and quenched by forced flow directly into a test tube containing 2 M HCl. OD_{max} refers to the maximal absorbance change and OD to the absorbance at the indicated times.

For the rapid kinetic experiments, mixing and quenching were carried out with the aid of a Dionex multimixing apparatus. The performance of this apparatus in the millisecond time scale was calibrated experimentally as described previously (Verjovski-Almeida et al., 1978). For experiments requiring sequential *double* mixing and *then quenching*, the Dionex apparatus was equipped with variable length delay lines at the collection syringe port following the second mixing chamber. The outflow from these delay lines was then directed into a beaker or test tube containing the quenching reagent. The accuracy of time calibration for the instrument so modified for triple mixing was tested by following the alkaline hydrolysis of 2,4-dinitrophenyl acetate (Gutfreund, 1969) as shown in Figure 1. These measurements were in close agreement with those made in the same instrument without the triple mixing modification (Verjovski-Almeida et al., 1978) and with previous determinations by stopped-flow (Barnum & Gutfreund, 1964) and rapid quench methods (Froehlich et al., 1976).

The reaction mixtures for the rapid kinetic experiments were nearly identical with those used for the steady-state experiments, except for use of [γ-^{32}P]ATP and the absence of an ATP-regenerating system. Quenching was obtained with 3.5% Cl_3CCOOH and 0.1 mM P_i (final concentrations after mixing). Due to the low protein concentration in the medium, three or four samples were collected and pooled for each reaction time in order to obtain a total of approximately 1 mg of protein per sample. The denatured protein was centrifuged and washed 4 times in 0.125 M perchloric acid and 2 mM P_i, and the final pellet was dissolved in 0.25 mL of 0.1 N NaOH, 2% Na_2CO_3, 5 mM Na_2HPO_4, and 2% $NaDodSO_4$. The dissolved protein was diluted with 1.25 mL of water and the level of phosphoenzyme determined by measurement of radioactivity (^{32}P) and protein in the sample.

The supernatant obtained after the first centrifugation of the quenched samples was used for [^{32}P]P_i determinations. Due to the low concentration of protein in the reaction mixture, 10 mL was taken from each sample, extracted with 0.5 mL of a 1% acid-washed charcoal suspension, and filtered through Millipore filters (type HAWP 0.45 μm). The filters were washed twice with 1.0 mL of Cl_3CCOOH, and the filtrate was

[1] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; $NaDodSO_4$, sodium dodecyl sulfate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; NEM, *N*-ethylmaleimide.

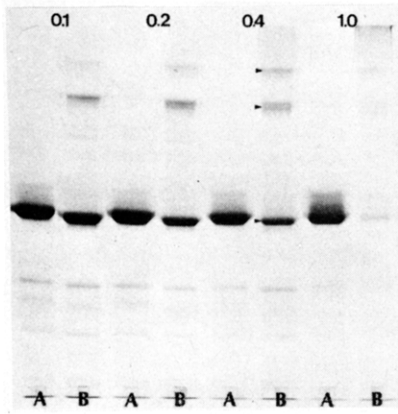

FIGURE 2: Effect of the cross-linking reagent DSP on solubilized (A) and membrane-bound (B) enzyme. Membranous or enzyme solubilized for 15 s (1.0 mg/mL in this experiment; identical results were obtained with 0.1 mg/mL) was incubated for 1 h at 25 °C in 20 mM Tris-HCl (pH 7.5), 80 mM KCl, 10 mM $MgCl_2$, and 0.1 mM $CaCl_2$ in the presence of (from left to right) 0.1, 0.2, 0.4, and 1.0 mM DSP. The reaction was then quenched with 16 mM NEM and the protein separated by $NaDodSO_4$ gel electrophoresis. It is clear that in the presence of the highest concentration of DSP, the solubilized enzyme remains as M_r 116 000 monomers, while the membranous enzyme undergoes oligomerization to dimers, trimers (arrows), and larger polymers which do not enter the gel. Similar patterns were obtained when the time of incubation with the cross-linking reagent was as short as 10 min.

collected. Fifty microliters of 100 mM nonradioactive P_i, 0.6 mL of acetone, and 3.0 mL of 10% ammonium molybdate in 5 N H_2SO_4 were then added, and the sample was vortexed. Following extraction with 4 mL of a 1/1 2-methyl-1-propanol/benzene mixture and centrifugation, an aliquot of the organic phase was counted on a scintillation counter.

Phosphoenzyme formation following incubation of the enzyme with [^{32}P]P_i was determined by a method analogous to that used for determination of phosphoenzyme formed from [γ-^{32}P]ATP. The reaction mixture is given in the legend to Figure 8.

ATP synthesis following addition of ADP and Ca^{2+} to the phosphoenzyme formed with [^{32}P]P_i was measured by a method previously described (Chaloub et al., 1979), which was adapted to be sufficiently sensitive in experiments containing low protein concentrations.

Results

Monomeric State of the Solubilized Enzyme. The monomeric state of the enzyme solubilized with $C_{12}E_8$ was previously established by analytical centrifugation and molecular sieve chromatography (Dean & Tanford, 1978). In order to have an independent evaluation, we exposed the enzyme to various cross-linking reagents (i.e., cupric phenanthroline, glutaraldehyde, and DSP) with the expectation that if two or more chains maintained a stable oligomeric relationship, they would cross-link with a similar pattern whether in the membranous or solubilized state. On the contrary, we found that all the ATPase protein was easily cross-linked while in the membranous state but remained in the monomeric state if exposed to cross-linking reagents following detergent solubilization (Figure 2). In experiments designed to check the effect of ligands on cross-linking, we found that cross-linking of ATPase chains in the membranous state was significantly reduced in the presence of ATP. This effect of ATP on the cross-linking pattern was observed when cupric phenanthroline was used, but not when DSP was used, and was due at least in part to copper complexation by the nucleotide. At any rate,

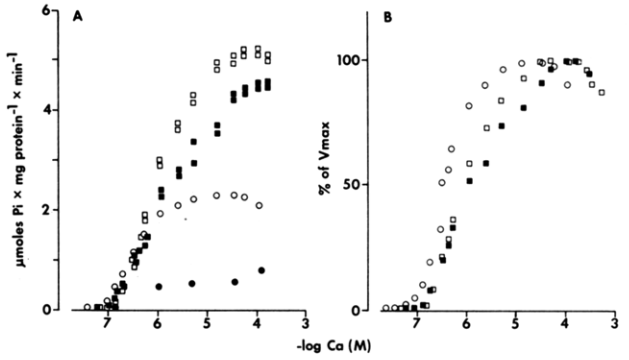

FIGURE 3: Hydrolytic activity of SR ATPase in its membranous form or following solubilization in various conditions. Control activity (○) was obtained with SR vesicles doped with a divalent cation ionophore (10 μM A23187). Solubilization of SR vesicles (30–50 μg of protein/mL) was obtained by addition of $C_{12}E_8$ (1.5 mM) in the presence of 20 mM Tris–maleate (pH 7.5), 80 mM KCl, 10 mM $MgCl_2$, and Ca^{2+} and/or EGTA to yield 150 (□, ■) or 0.13 μM (●) free Ca^{2+}. The ATPase reaction was started 20 s (□) or 10 min (■, ●) following solubilization, by the addition of 0.1 mM ATP, 2 mM phosphoenolpyruvate, 135 μg/mL (10.6 units/mL) pyruvate kinase, and Ca^{2+} and/or EGTA to yield the indicated pCa. Temperature 25 °C. Serial samples were quenched with an equal volume of the phosphate determination reagent (Lin & Morales, 1977). Steady-state velocities were obtained from linear plots of P_i production.

in the presence of detergent, no significant cross-linking was observed under any conditions. It is then apparent that essentially all the enzyme solubilized in our conditions was in the monomeric state.

Steady-State ATPase Activity. It is known that the ATPase activity of native SR vesicles is subject to "back-inhibition" by the high concentration of Ca^{2+} which is transported inside the vesicles as a consequence of ATP utilization (Makinose & Hasselbach, 1965). Therefore, manifestation of intrinsic ATPase activity in steady-state conditions requires the use of SR vesicles treated with ionophores to induce leakage of accumulated Ca^{2+} and prevent back-inhibition (Scarpa et al., 1972). It is then possible to obtain constant rates of P_i production and demonstrate a Ca^{2+} requirement for activation of the enzyme. When SR vesicles are solubilized in $C_{12}E_8$ in the presence of 150 μM Ca^{2+} at pH 7.5 and the ATPase reaction is started by the addition of ATP, a hydrolytic activity significantly higher than that of leaky vesicles is obtained (Figure 3A). If aliquots of EGTA are added along with the ATP in order to lower free Ca^{2+} during the ATPase runs, the Ca^{2+} concentration dependence of solubilized ATPase is similar to, but not identical with, that of leaky vesicles (Figure 3).

In the presence of 150 μM Ca^{2+}, little difference is observed whether the reaction is started 10 min or 20 s following solubilization. However, if the enzyme is incubated in the presence of very low Ca^{2+} (0.13 μM) for 10 min after solubilization, and the reaction is started by adding ATP along with aliquots of $CaCl_2$ to raise the free Ca^{2+} to activating concentrations, a much lower activity is obtained (Figure 3A). It is then apparent that ATPase solubilization with $C_{12}E_8$ produces rapid loss of hydrolytic activity unless protection is afforded by occupancy of calcium sites on the enzyme. Even in the presence of 150 μM Ca^{2+}, a slow inactivation is observed, and within 20–30 min, the activity of the solubilized enzyme decreases 50% (Figure 4). If the solubilization is carried out at pH 6.0, a rapid loss of activity occurs independent of the presence or absence of Ca^{2+} (Figure 4, inset). It is noteworthy that, in contrast to solubilized ATPase, membranous ATPase is quite stable at pH 6.0. Therefore lability at pH 6.0 is a specific feature of the solubilized ATPase. In our experiments, no significant protection was obtained with 20% (v/v) glycerol.

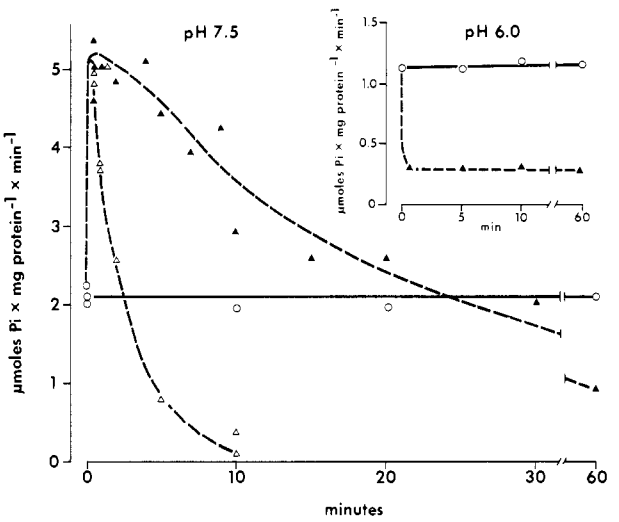

FIGURE 4: Loss of hydrolytic activity following ATPase solubilization in various conditions. Control activity (○) following exposure to solubilization media (with no $C_{12}E_8$) was obtained with SR vesicles doped with a divalent cation ionophore (10 μM A23187). Solubilization was carried out as described in Figure 3 at pH 7.5 or 6.0 and in the presence of 150 (▲) or 0.13 μM (△) Ca^{2+}. The ATPase reaction was started at different times (15 s to 60 min, as indicated) following solubilization.

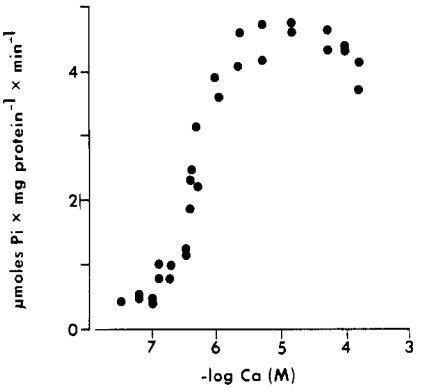

FIGURE 5: Concentration dependence of the protecting effect of Ca^{2+} over the loss of hydrolytic activity by SR ATPase following solubilization. SR vesicles were solubilized at pH 7.5 as described in Figure 3, with the exception of the Ca^{2+} concentration which was adjusted to the levels indicated above. The ATPase reaction was started 10 min following solubilization by adding ATP, the ATP regenerating system, and $CaCl_2$ to yield 150 μM free Ca^{2+} in all samples.

The affinity of the enzyme protecting sites for Ca^{2+} at pH 7.5 can be estimated by solubilizing SR vesicles in the presence of variable concentrations of Ca^{2+} and, after 10 min, starting the ATPase reaction with ATP and saturating Ca^{2+}, which, in the absence of denaturation, should give maximal activation. The residual ATPase indicates that the protecting effect is obtained within the same Ca^{2+} concentration range required for occupancy of the high-affinity sites (Inesi et al., 1980) which are involved in enzyme activation of the nonsolubilized ATPase (compare Figure 5 with Figure 3).

In experiments in which solubilization is carried out in the presence of variable Ca^{2+} concentrations and the ATPase reaction is started 10 min after solubilization by adding only ATP, a clear displacement to higher Ca^{2+} concentrations and a partial loss of the cooperative appearance of the Ca^{2+} activation curve are obtained [Figure 6; see also Verjovski-Almeida & Silva (1981); Watanabe et al., 1981]. This is a compound effect including variable protection from $C_{12}E_8$ denaturation and variable activation of the nondenatured enzyme which is still present when ATP is added. In fact, if

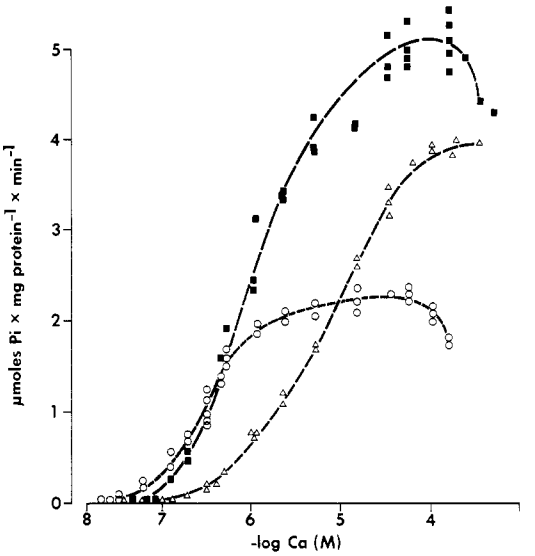

FIGURE 6: Ca^{2+} concentration dependence of SR ATPase in the membranous form or following solubilization in the presence of variable pCa. Control activity (○) was obtained with SR vesicles doped with a divalent cation ionophore (10 μM A23187). Solubilization was carried out in a mixture containing 30–50 μg of protein/mL, 20 mM Tris–maleate (pH 7.5), 150 μM total calcium (pCa adjusted with EGTA), 1.5 mM $C_{12}E_8$, 80 mM KCl, and 10 mM $MgCl_2$. The ATPase reaction was started 30 s (■) or 10 min (△) following solubilization by adding ATP (0.1 mM), phosphoenolpyruvate (2 mM), and pyruvate kinase (10.6 units/mL). Temperature 25 °C.

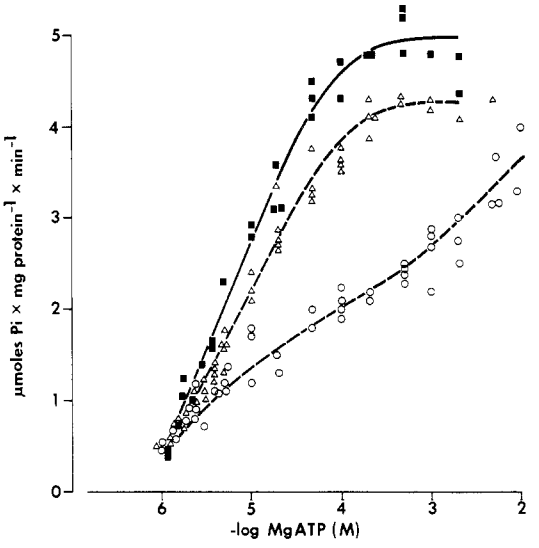

FIGURE 7: Substrate (Mg·ATP) concentration dependence of SR ATPase in the membranous form and following solubilization. Control activity (○) was obtained with SR vesicles doped with a divalent cation ionophore (10 μM A23187). Solubilization was carried out at pH 7.5 and in the presence of 150 μM Ca^{2+}, as described in Figure 3. The ATPase reaction was started 30 s (■) or 10 min (△) following solubilization by adding ATP and $MgCl_2$ to yield the indicated concentrations of Mg·ATP complex. Temperature 25 °C. The ATPase reaction was run in the presence of saturating Ca^{2+} (150 μM) and an ATP regenerating system (see legend to Figure 3).

ATP is added only 30 s following solubilization, the dependence of ATPase activation on low Ca^{2+} concentration is retained (Figure 6).

It has been shown repeatedly (Inesi et al., 1967; Yamamoto & Tonomura, 1967; deMeis & deMello, 1973; The & Hasselbach, 1972; Neet & Green, 1977; Dupont, 1977) that the ATP dependence of the ATPase of leaky vesicles extends over a very wide concentration range and has a pattern that cannot be fitted with a simple Michaelis–Menten scheme (Figure 7). This pattern has been explained by postulating an activating

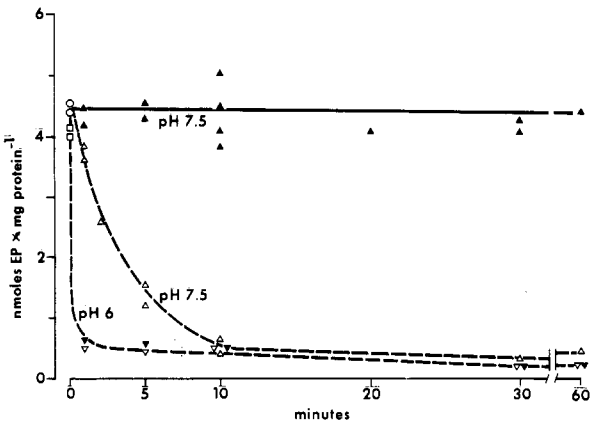

FIGURE 8: Phosphorylation of SR ATPase with ATP following solubilization in various conditions. Control values for phosphoenzyme levels [(○) for pH 7.5; (□) for pH 6.0] were obtained with SR vesicles doped with a divalent cation ionophore (10 μM A23187). Solubilization was carried out at pH 7.5 (▲, △) or pH 6.0 (▼, ▽) in the presence of 150 μM (▲, ▼) or 0.15 μM (△, ▽) Ca^{2+}. Other conditions are as described for Figure 3. The phosphorylation reaction was started 15 s to 60 min following solubilization by adding ATP and $CaCl_2$ (when necessary) to reach 150 μM $CaCl_2$. The reaction mixture for the phosphorylation reaction contained 20 mM Tris–maleate (pH 7.5 or pH 6.0), 80 mM KCl, 6 mM $MgCl_2$, 150 μM Ca^{2+}, 30–50 μg of protein, 1.5 mM $C_{12}E_8$ (except for controls), and 50–100 μM [γ-^{32}P]ATP. Following a 5-s incubation, the reaction was quenched with 3.5% Cl_3CCOOH and 0.1 mM P_i (final concentrations). Temperature 25 °C.

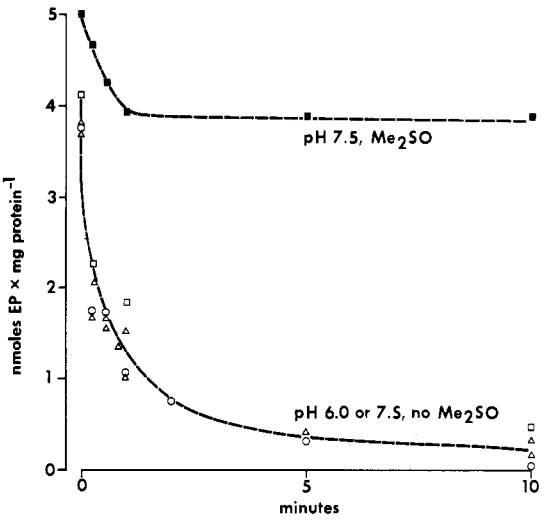

FIGURE 9: Phosphorylation of SR ATPase with P_i following solubilization in various conditions. Controls (zero time) were obtained with SR vesicles doped with a divalent cation ionophore (10 μM A23187) and in the absence of detergent. Solubilization was carried out at pH 6.0 (○, △) or 7.5 (□, ■) and in the presence of 150 μM Ca^{2+} and no EGTA (□, ■) or 1 mM EGTA and no added Ca^{2+} (○, △). Other components of the solubilization mixture were 1.5 mM $C_{12}E_8$, 30–50 μg of protein/mL, 20 mM $MgCl_2$, and 10–30 mM Tris–maleate to yield the pH indicated. The phosphorylation reaction was started either 15 s previous, or 15 s to 10 min following solubilization by adding 10.0 (△, ○, □) or 0.5 mM (■) [^{32}P]P_i, 1 mM EGTA (when not already present in the medium), sufficient buffer to lower the pH to 6.0 when indicated (□), and 30% (v/v) dimethyl sulfoxide when indicated (■). The phosphorylation reaction was quenched 15 s (○, □) or 5 min (■) after the addition of [^{32}P]P_i or 15 s to 10 min following solubilization (△). Temperature 25 °C. Quenching reagent 0.5 N PCA and 4 mM P_i.

effect of high ATP concentrations on the turnover of the phosphorylated ATPase intermediate (Verjovski-Almeida & Inesi, 1979; Ariki & Boyer, 1980). On the other hand, in agreement with Dean & Tanford (1978), we find that the solubilized ATPase is fully activated within a lower range of ATP concentrations and exhibits a simple pattern that can be fitted assuming only a substrate role for the Mg·ATP complex (Figure 7). Therefore, the solubilized ATPase can reach its maximal ATPase velocity with no need for the activating effect of high ATP.

Enzyme Phosphorylation with ATP or P_i, and ATP Synthesis. ATP utilization by SR ATPase includes formation of a phosphorylated enzyme intermediate (Yamamoto & Tonomura, 1967; Makinose, 1969) which is obtained by transfer of the ATP terminal phosphate onto an aspartyl residue at the catalytic site (Degani & Boyer, 1973; Bastide et al., 1973). With our preparation, the maximal level of phosphoenzyme observed with ATP in the presence of Ca^{2+} is 4–5 nmol/mg of protein. We found that the phosphoenzyme level remains unchanged when the ATPase is solubilized in the presence of 150 μM Ca^{2+} at pH 7.5. High phosphoenzyme levels are obtained even when ATP is added 60 min after solubilization (Figure 8), while a 75% reduction of hydrolytic activity is observed at this time (Figure 4). On the other hand, if SR vesicles are solubilized in the presence of very low Ca^{2+} (0.15 μM), the enzyme loses its ability to undergo phosphorylation when ATP and saturating Ca^{2+} are added (Figure 8). If the solubilization is carried out at pH 6.0, in the presence or the absence of Ca^{2+}, the enzyme loses its ability to be phosphorylated even more rapidly (Figure 8).

Phosphorylation of SR ATPase can also be produced by exposing the enzyme to P_i in the absence of Ca^{2+} (Masuda & deMeis, 1973; Kanazawa & Boyer, 1973) as a reversal of the hydrolytic step of the ATPase cycle. Phosphorylation of the membranous ATPase with P_i is greatly favored by a moderately acid pH. However, when the solubilized ATPase is exposed to pH 6.0 or to pH 7.5 in the absence of Ca^{2+}, a rapid denaturation occurs [Figures 4 (inset), 8, and 9]. In the case of the P_i reaction, the denaturation is quite rapid and is expressed by a loss of the enzyme's ability to be phosphorylated (when the solubilization is carried out previous to the phosphorylation reaction) or (when the vesicles are solubilized following the phosphorylation reaction) by a rapid decrease in the phosphoenzyme level (Figure 9). Therefore, phosphorylation with P_i can barely be observed under these conditions. The question is then whether the lack of phosphorylation with P_i is due to the denaturing conditions used for this reaction (pH 6.0 and no Ca^{2+}) or to an intrinsic property of the solubilized preparation.

The phosphorylation reaction with P_i also occurs in the presence of dimethyl sulfoxide. This solvent markedly increases the apparent affinity of the enzyme for P_i and displaces the reaction equilibrium in favor of phosphoenzyme formation (deMeis et al., 1980), thereby rendering the detection of high levels of phosphoenzyme possible even at alkaline pH. In these conditions, we obtained phosphorylation with solubilized ATPase as well as with membranous ATPase (Figure 9). It is then apparent that when denaturation is avoided, the solubilized ATPase can be phosphorylated either with ATP in the presence of Ca^{2+} or with P_i in the absence of Ca^{2+}. In optimal conditions, nearly identical levels of phosphoenzyme (4–5 nmol/mg of protein) are obtained with solubilized or membranous ATPase. It should be pointed out, however, that addition of dimethyl sulfoxide is an absolute requirement for the reaction of P_i with the detergent-solubilized enzyme.

Following formation of phosphoenzyme with P_i in the presence of dimethyl sulfoxide, it is possible to obtain ATP synthesis by adding an aqueous medium containing ADP and Ca^{2+} (deMeis & Inesi, 1982). It is shown in Table I that this can be obtained even with the solubilized enzyme. The yield of ATP from phosphoenzyme is the result of equilibration of

Table I: Phosphorylation of Membranous and Solubilized ATPase with P_i and Subsequent Formation of ATP[a]

enzyme	E-P (nmol/ mg of protein)	ATP (nmol/ mg of protein)
membranous	4.8 ± 0.4	1.18 ± 0.2
solubilized	5.0 ± 0.3	0.74 ± 0.2

[a] SR vesicles (100 μg/mL) were solubilized for 15 s in 20 mM Tris-HCl (pH 7.5), 20 mM $MgCl_2$, and 1.5 mM $C_{12}E_8$. The medium was then diluted with a solution that resulted in a final concentration of 20 mM Tris-HCl (pH 7.5), 20 mM $MgCl_2$, 1.5 mM $C_{12}E_8$, 1 mM EGTA, 30% (v/v) dimethyl sulfoxide, 0.1 mM [^{32}P]P_i, and 50 μg/mL solubilized protein. Following 5-min incubation, part of the sample was quenched in acid for determination of phosphoenzyme. Alternatively, 4.0 mL of the reaction mixture was added to 8.0 mL of a medium containing 20 mM Tris-HCl (pH 7.5), 20 mM $MgCl_2$, 9 mM $CaCl_2$, 3 mM ADP, 7.5 mM glucose, and 48 units (total) of hexokinase. At this point the concentration of solubilized enzyme was 16.6 μg/mL. The reaction was stopped after 15 s with 1 mL of 5 N PCA and 12 mM ATP. The [^{32}P]ATP formed was measured as described under Materials and Methods.

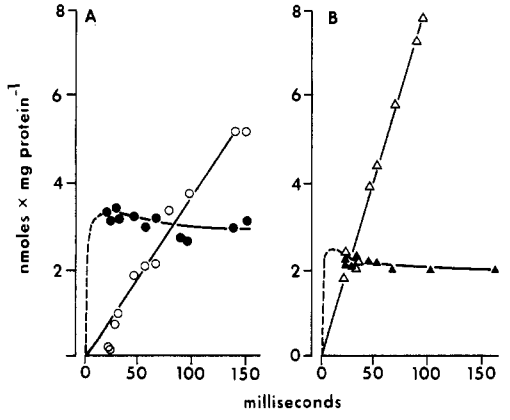

FIGURE 10: Early phase of phosphoenzyme (●, ▲) and P_i (○, △) production following addition of ATP to membranous (A) or solubilized (B) ATPase. The control experiment (A) was done with SR vesicles doped with a divalent cation ionophore (10 μM A23187). Solubilization for the (B) experiment was obtained by exposing SR vesicles (30–50 μg/mL) to 1.5 mM $C_{12}E_8$ in the presence of 20 mM Tris–maleate (pH 7.5), 10 mM $MgCl_2$, 150 μM $CaCl_2$, and 80 mM KCl. The reaction was started within 1–2 min after solubilization by adding an equal volume of the same medium containing 20 μM [γ-^{32}P]ATP (10 μM final concentration) and quenched with an equal volume of trichloroacetic acid (final concentration 3.5% trichloroacetic acid and 0.1 mM P_i). Mixing was obtained with the aid of a Dionex multimixer. Several pushes were pooled in order to collect approximately 1 mg of protein per sample.

ADP·E–P·Ca_2 with ATP·E·Ca_2 and is limited by significant E–P hydrolysis. Nevertheless, it is clear that the reaction can be obtained with the monomeric enzyme as well as with the membranous system.

Rapid Kinetics. The use of [γ-^{32}P]ATP and rapid mixing devices permits time resolution of enzyme phosphorylation and P_i production in the initial phase of the reaction even at 25 °C (Froehlich & Taylor, 1975; Kurzmack & Inesi, 1977). It is shown in Figure 10A that upon addition of ATP to leaky vesicles a burst of enzyme phosphorylation is obtained, followed by a linear production of P_i. A similar pattern, which is consistent with the role of phosphoenzyme as an intermediate in the ATPase reaction, is obtained with solubilized ATPase (Figure 10B). In this case, however, P_i production is faster even though the increased turnover results in phosphoenzyme levels which are slightly lower than that in leaky vesicles.

In the large set of experiments summarized in Figure 11, steady-state phosphoenzyme levels of 3.5 and 3.0 nmol/mg of protein were obtained with leaky vesicles and solubilized protein, respectively. The ATP concentration dependence for

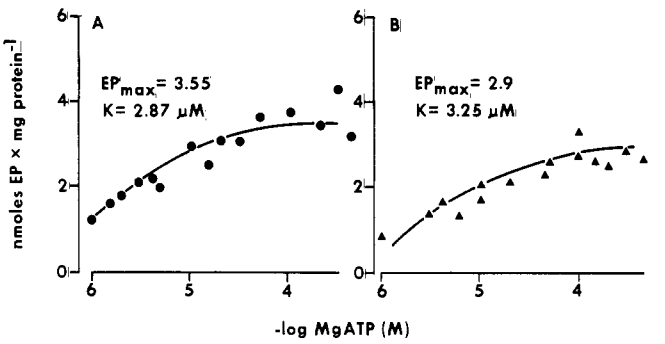

FIGURE 11: Dependence of phosphoenzyme levels on the concentration of substrate (Mg·ATP), as determined in rapid mixing experiments. Control (A) and solubilized (B) ATPases were tested as described for Figure 10, except for the concentration of Mg·ATP which was varied. Fitting of the experimental points was obtained by using an iterative nonlinear regression method (Koeppe & Hamann, 1980) based on eq 1 [n is the Hill coefficient (which had a value very close to 1.0)].

$$[EP]_{obsd} = \frac{[EP]_{max}[Mg\cdot ATP]^n}{K + [Mg\cdot ATP]^n} \quad (1)$$

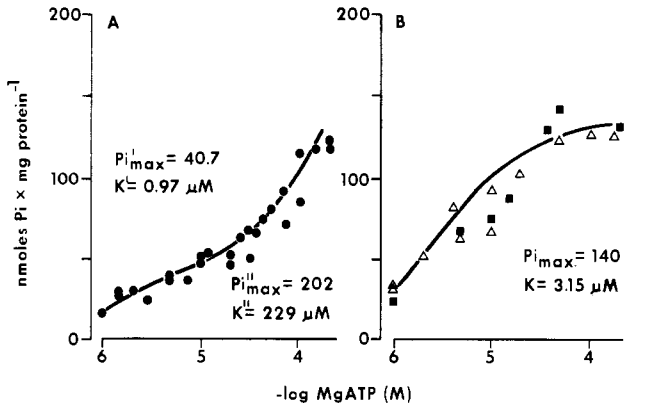

FIGURE 12: Dependence of the initial velocity of P_i production (nmol/mg^{-1} s^{-1}) on the concentration of substrate (Mg·ATP), as determined in rapid mixing experiments. Experimental conditions for control (A) and solubilized (B) ATPase were as described for Figure 10, except for the concentration of Mg·ATP which was varied. The reaction was started 10 min (△) or 30 s (■) following solubilization (B). It is shown that for the membranous ATPase (A), the dependence of initial velocity on substrate concentration could not be fitted with an equation based on a single Mg·ATP site but could be fitted with an equation with two affinity constants for the Mg·ATP site(s) (eq 2). For the solubilized ATPase, a very good fitting was obtained

$$[P_i]_{obsd} = \frac{[P_i]'_{max}[Mg\cdot ATP]^n}{K' + [Mg\cdot ATP]^n} + \frac{[P_i]''_{max}[Mg\cdot ATP]^n}{K'' + [Mg\cdot ATP]^n} \quad (2)$$

with a single-site equation. The n value was very close to 1.0 in all cases.

establishment of phosphoenzyme levels is similar for both preparations, with saturation at 0.1 mM ATP. In the experiments done with solubilized ATPase, establishment of phosphoenzyme levels and the velocity of P_i production exhibit a similar ATP dependence (Figures 11B and 12B), so that the phosphoenzyme turnover is approximately 45 s^{-1} at all ATP concentrations. In the experiments done with leaky vesicles, however, the ATP dependence of P_i production differs from that of phosphoenzyme formation (Figures 11A and 12A). In fact, the phosphoenzyme turnover varies from 15 s^{-1} in the presence of 10 μM ATP to 35–40 s^{-1} at 200 μM ATP. These features of solubilized as opposed to membranous ATPase are apparent upon inspection of Figures 10–12. It should be pointed out that the turnover numbers observed at pH 7.5 are higher than those observed at pH 6.8 which is most commonly

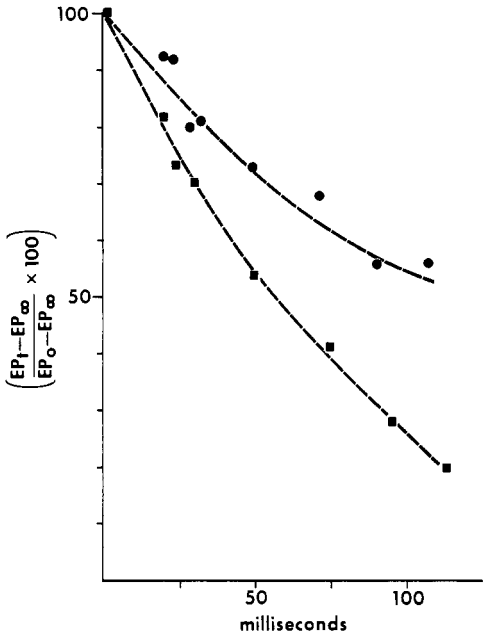

FIGURE 13: Decay of [^{32}P]phosphoenzyme following a 12-fold dilution of [γ-^{32}P]ATP with nonradioactive ATP. Membranous (●) or solubilized (■) ATPase was first exposed to 10 μM radioactive ATP for 40 ms, and the 120 mM nonradioactive ATP was added. Serial quenching with 3.5% Cl_3CCOOH and 0.1 mM P_i was then carried out at the time intervals indicated in the figures (zero time is the dilution with nonradioactive ATP). The triple mixing was obtained with the Dionex multimixer adapted as described under Materials and Methods. The reaction mixture contained 20 mM Tris–maleate (pH 7.5), 80 mM KCl, 10 mM $MgCl_2$, and 150 μM $CaCl_2$. When indicated, SR vesicles were solubilized several minutes before the addition of radioactive ATP as described for Figure 3. The protein concentration was below 50 μg/mL in all cases. Temperature 25 °C.

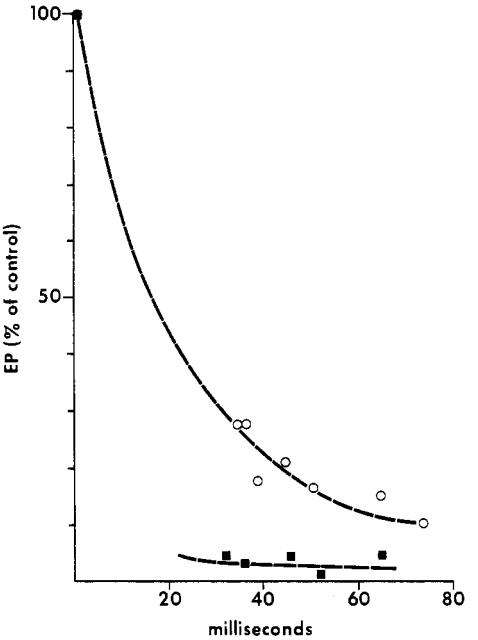

FIGURE 14: Loss of Ca^{2+}-dependent activation following addition of EGTA. Membranous (○) or solubilized (■) ATPase was first exposed to 6 mM EGTA for a variable time as indicated, and then 10 μM [γ-^{32}P]ATP was added. Finally the reaction was quenched in 3.5% Cl_3CCOOH and 0.1 mM P_i 0–73 ms after the addition of ATP. The Dionex multimixer was adapted for triple mixing as described under Materials and Methods. The reaction mixture contained 20 mM Tris–maleate (pH 7.5), 80 mM KCl, 10 mM $MgCl_2$, and 150 μM $CaCl_2$. When indicated, SR vesicles were solubilized previous to the addition of EGTA as described for Figure 3. The protein concentration was below 50 μg/mL in all cases. Temperature 25 °C.

used for studies of SR ATPase.

The experiments described above indicate that, in the presence of low ATP concentrations, the phosphorylated intermediate of solubilized ATPase has a more rapid turnover than that of leaky vesicles. This can be further demonstrated in experiments in which [^{32}P]phosphoenzyme is first formed by incubation of ATPase with [γ-^{32}P]ATP (10 μM). Then an excess of nonradioactive ATP (120 μM) is added, followed by acid quenching at serial times in order to monitor the dilution of [^{32}P]phosphoenzyme with nonradioactive phosphate (Figure 13). We found that dilution of [^{32}P]phosphoenzyme formed with solubilized ATPase is completed in a shorter time, which again is indicative of a faster turnover.

Another feature of SR ATPase which can be shown by rapid quench methods is a relatively slow switch off of its calcium activated state, e.g., the loss of its ability to be phosphorylated by ATP following addition of EGTA to remove activating calcium from the enzyme (Sumida et al., 1978). In the experiments shown in Figure 14, solubilized ATPase or leaky vesicles were exposed to EGTA for times varying from 0 to 73 ms; then ATP was added and allowed to react for 50 ms before quenching. When leaky vesicles were used, we still found some residual phosphorylation following limited exposure to EGTA, and we were able to resolve the tail end of an exponential with a half-time of approximately 20 ms (pH 7.5), representing the switch off of the activated state. On the contrary, no time resolution was obtained for solubilized ATPase as its ability to be phosphorylated was totally lost after the shortest exposure to EGTA permitted by our mixing device (when adapted for this particular experiment). It is then apparent that following addition of EGTA to remove calcium from the high-affinity (activating) sites of the enzyme, re-activity to ATP is lost much faster by the solubilized ATPase than by the leaky vesicles. This may be related to the tendency of the enzyme to acquire a dependence on higher Ca^{2+} concentrations following solubilization.

Discussion

In agreement with previous observations (Dean & Tanford, 1978; Moller et al., 1980), our experiments demonstrate that high catalytic activity is retained by SR ATPase following solubilization with $C_{12}E_8$ (Figure 3). At saturating ATP and Ca^{2+} (pH 7.5, 25 °C), we obtain a maximal velocity of 5–6 μmol (mg of solubilized protein)$^{-1}$ min^{-1}. Faster rates are obtained if a higher temperature (37 °C) is used for the ATPase reaction. Phosphorylation of the monomeric enzyme can be obtained with ATP or P_i, and reversal of the cycle to ATP synthesis can also be obtained in the appropriate conditions.

Protecting and Activating Effects of Ca^{2+}. ATPase activity can be maintained for days in the presence of $C_{12}E_8$ if the protein is stored at high concentrations (Dean & Tanford, 1978). On the other hand, if the protein is diluted (25–100 μg/mL in 1.5 mM $C_{12}E_8$) to ensure maximal solubilization, we find that the enzyme activity is unstable and decays with a half-time of 20–30 min in the presence of 150 μM Ca^{2+} and with a half-time of 3–4 min in the presence of 0.15 μM Ca^{2+} (Figure 4). The Ca^{2+} concentration requirement for protection of the catalytic sites can be determined by keeping the solubilized enzyme for 10 min in the presence of variable Ca^{2+} and then adding ATP and excess free Ca^{2+} (150 μM) to activate the residual (nondenatured) ATPase. These experiments show that the Ca^{2+} concentrations required for protection of catalytic activity are similar to those necessary for occupancy of the activating sites in membranous ATPase (Figures 3 and 5).

The dependence of ATPase activity on Ca^{2+} is clearly displaced to higher Ca^{2+} concentrations when denaturation is permitted following solubilization (Figure 6). In the presence of minimal denaturation the Ca^{2+} concentration dependence of the solubilized preparation is similar to, but not identical with, that of the membranous enzyme (Figure 3B). Generally, a partial loss of the cooperative appearance of the activation curve is noted. It should be pointed out, however, that the cooperative character of enzyme activation curves is not related in a simple manner to the actual behavior of calcium binding in equilibrium conditions. Unfortunately, calcium binding to the monomeric ATPase cannot be measured reliably, due to the very low concentrations of protein which must be used to maintain the monomeric state.

Effects of H^+. Another important factor for maintenance of catalytic activity in solubilized preparations is the H^+ concentration. We find that if the solubilized preparation is exposed to pH 6.0, the enzyme rapidly loses its catalytic activity (Figure 4) and its ability to be phosphorylated by ATP or P_i (Figures 8 and 9). On the contrary, pH 7.5 affords a better protection of the enzyme, especially in the presence of Ca^{2+}. These observations must be viewed in the light of previous work, demonstrating that the specific cation sites of the SR ATPase may be occupied by Ca^{2+} or H^+ and the enzyme resides in a different conformational state depending on the identity of the ligand (Hill & Inesi, 1982). It is then likely that the calcium and the proton states confer stability or instability to the enzyme under conditions of solubilization.

It is noteworthy that the ability of the enzyme to be phosphorylated, the intrinsic catalytic activity, and the kinetic regulation of the enzyme are affected with selective patterns by the solubilization procedure (Figures 4, 8, 9, 12, and 14). It is likely that these three functional features involve different mechanistic adaptations of the enzyme structure, which are not denatured simultaneously. Denaturation is not necessarily due to solubilization, but it may be due to direct effects of $C_{12}E_8$. Other detergents such as lysolecithins have been reported to produce solubilization without denaturation of the enzyme (Nestruck-Goyke & Hasselbach, 1981).

Stoichiometry of the Phosphorylation Sites. Our experiments show that in optimal conditions the stoichiometry of the catalytic sites remains unchanged following maximal solubilization with $C_{12}E_8$. The *maximal* (confirmed by enzyme equilibration with P_i in the presence of dimethyl sulfoxide) levels of phosphoenzyme range between 4 and 5 nmol/mg of protein, independent of whether the vesciles are used in the original membranous form or after solubilization. Therefore, solubilization with $C_{12}E_8$ does not uncover any enzyme that may be though to be latent due to aggregation of vesicles or oligomerization of polypeptide chains.

In the assumption that each phosphorylation site corresponds to one monomer of the M_r 110000–120000 ATPase polypeptide chains, the observed phosphorylation levels account for only 45–60% of the total protein in our SR vesicles. It is noteworthy that in $NaDodSO_4$ gels stained with Coomassie Blue, the ATPase band seems to account for a higher percentage of the total protein. The discrepancy may be due to inadequacy of gel densitometry for quantitative assessment of nonspecific protein components.

Considering that our preparation of SR vesicles contains 8–10 nmol of high-affinity calcium binding sites/mg of protein (Inesi et al., 1980), and that a *maximal* level of 4–5 nmol of phosphoenzyme/mg of protein is formed either in the membranous or monomeric state, it is apparent that one ATPase chain contains one catalytic (phosphorylation) site and two specific calcium sites. Similar conclusions were reached by Murphy et al. (1982) by comparing enzyme velocity and calcium binding of membranous solubilized SR ATPase.

On the basis of our present findings, one would expect that preparations of purified ATPase should contain approximately 7–8 nmol of catalytic sites/mg of protein. In fact, this figure has been obtained by titration of the sites with fluorescein isothiocyanate (Andersen et al., 1982; Mitchinson et al., 1982). However, the actual phosphorylation level was found to be somewhat lower (Andersen et al., 1982), probably due to some denaturation of the enzyme during the purification procedure.

Regulation of Enzyme Turnover. It was previously suggested (Andersen et al., 1982; Inesi et al., 1982a,b) that detergent solubilization increases the ATPase turnover. We find that in the presence of saturating Ca^{2+}, the steady-state velocities of membranous (in leaky vesicles) and solubilized ATPases must be compared in the presence of various ATP concentrations since the two preparations differ in their dependence on ATP concentration. The membranous ATPase displays a complex dependence including a first activation between 1 and 100 μM ATP and a further activating effect at higher ATP concentrations. The solubilized ATPase, on the contrary, displays a simple ATP dependence which is totally expressed within the 1–100 μM range, with no activating effect at higher ATP concentrations. Within the low ATP range the hydrolytic activity is approximately twice that of the membranous enzyme. However, at high ATP concentrations ($\sim$1 mM), the velocities of the two preparations are approximately the same due to the activating effect of high ATP on the membranous enzyme. It should be pointed out that we are comparing the solubilized enzyme to SR vesicles whose activity is already stimulated by the addition of A23187.

From the kinetic point of view, these observations are explained by the rapid mixing experiments (Figures 10–12), showing that the phosphorylated intermediate is formed with nearly identical stoichiometry in the two preparations and the higher velocity of the solubilized preparation is due to a higher turnover of the intermediate. The turnover of the membranous enzyme rises to levels comparable to those of the solubilized enzyme only in the presence of millimolar ATP. Therefore, the kinetic constraints imposed by the membrane structure on the turnover of the intermediate is released either by solubilization of the enzyme or by the effect of millimolar ATP.

Another observation which is suggestive of a lesser kinetic constraint in the solubilized enzyme is the rapid switch off of its calcium-activated state upon removal of calcium by EGTA (Figure 14). A relatively slow deactivation of the membranous enzyme upon removal of calcium has been attributed to reversal of a calcium-dependent conformational state (Sumida et al., 1978). Such a reversal is evidently facilitated by the treatment with $C_{12}E_8$. This effect may be related to a tendency to require higher Ca^{2+} concentrations for enzyme activation.

Conclusions. The Ca^{2+}-dependent ATPase of SR vesicles retains a stoichiometric equivalent number of catalytic sites and full enzymatic activity under conditions of maximal solubilization with $C_{12}E_8$. However, stability of the solubilized enzyme is much more sensitive to low Ca^{2+} and high H^+ concentrations than membranous ATPase. Furthermore, the solubilized enzyme behaves differently with respect to its turnover regulation and is less constrained in its acquisition of diverse functional states. Therefore, assembly in the native membrane provides an environment which protects the enzyme against denaturation and affects its kinetic regulation.

These findings should be considered in light of the ultrastructural observations (Scales & Inesi, 1976), indicating that

the native assembly of SR ATPase consists of amphiphilic enzyme units retaining their individuality on the outer surface of the vesicles, while coming into close proximity to other units within the membrane bilayer. It is clear that the catalytic activity is a function of the individual polar heads protruding from the membrane. On the other hand, it seems that enzyme stability and kinetic regulation are significantly influenced by the protein assembly in the membrane and near-neighbor interactions. It is likely that detergent solubilization affects kinetic regulation by changing the hydrophobic character of the enzyme environment.

Registry No. ATPase, 9000-83-3; calcium, 7440-70-2; Mg·ATP, 1476-84-2; $C_{12}E_8$, 81208-94-8.

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