Properties of a Delipidated, Detergent-Activated Ca^{2+}-ATPase†

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ABSTRACT: The Ca^{2+}-activated ATPase of sarcoplasmic reticulum has been obtained in a stable and soluble form in detergent solution, virtually free of phospholipid. Deoxycholate was used as the primary delipidating agent, with glycerol as stabilizer, yielding an inactive enzyme that can be restored to full activity by adding back phospholipid or by adding detergent in place of phospholipid. A variety of detergents can be used, but detailed experiments were carried out only with the nonionic detergent, dodecyl octaethylene glycol monoether ($C_{12}E_8$). Chromatography in this detergent in the presence of glycerol can reduce the content of residual phospholipid to as little as one molecule per ATPase polypeptide chain. Most of the enzymatic properties are unaffected by this almost complete substitution of detergent for phospholipid, including the effect of temperature on the rate of ATPase hydrolysis, which leads to a nonlinear Arrhenius plot with a break near 20 °C. The enzyme in $C_{12}E_8$/glycerol is stable and fully active as a monomer, but there is a strong tendency for self-association, which is augmented when phospholipid is bound; it is likely that the enzyme exists as an oligomer in the in vivo membrane-bound state. Binding of $C_{12}E_8$ and lipid, and accompanying changes in activity and state of aggregation, are largely reversible and thus appear to be under thermodynamic control.

The Ca^{2+}-ATPase from sarcoplasmic reticulum is an intrinsic membrane protein that until recently was considered to have an obligatory requirement for phospholipid in the maintenance of both Ca^{2+} pumping and the ability to hydrolyze ATP (Martonosi, 1972; Meissner et al., 1973; Warren et al., 1974a; Knowles et al., 1976; le Maire et al., 1976a,b). The data appeared to demonstrate that at least 25–30 mol of phospholipid per mol of ATPase polypeptide are necessary for retention of full ATPase activity. However, we have shown more recently that most of the phospholipid can be replaced with a nonionic detergent without loss of ATPase activity (Dean & Tanford, 1977). The procedure involved delipidation by deoxycholate in the presence of glycerol and poly(ethylene glycol). The delipidated enzyme had little ATPase activity, but full activity could be restored by the addition of the nonionic detergent, dodecyl octaethylene glycol monoether ($C_{12}E_8$).[1] The active, soluble $C_{12}E_8$–ATPase complex was found to be stable for several days; a slow loss of activity occurred at a rate similar to that observed for purified ATPase vesicles. The minimal lipid content in several preparations of this type was 4 mol of phospholipid per mol of ATPase polypeptide.

The present paper reports additional studies of the delipidation process, in the course of which it was found that a further reduction in lipid content to as low as 1.0 mol/mol was possible without loss of activity or stability. The enzymatic properties of the delipidated protein are compared with those of purified ATPase in phospholipid vesicles. In addition, some physical properties of the soluble, delipidated protein are described.

Experimental Section

Materials. Tween 80, Brij 58, Brij 78, Brij 98, and G-2079 were gifts of ICI United States, Inc., and Lubrol PX, Brij 35, Brij 56, Brij 96, Tween 20, and Tween 40 were purchased from Sigma Corp. Trimethyl tetradecylammonium chloride (Lachat Chemicals, Inc.) was recrystallized from methanol and diethyl ether, and sodium cholate (Sigma Corp.) was recrystallized from 80% acetone. Sodium dodecyl sulfate was purchased from BDH Chemicals, Ltd., and synthetic oleoyllysophosphatidylcholine was a product of Applied Science Laboratories, Inc. [γ-^{32}P]ATP was purchased from New England Nuclear Corp. The sources of all other materials used in the present study and their preparation are the same as those reported earlier (le Maire et al., 1976a; Dean & Tanford, 1977).

Preparation of Delipidated ATPase. Sarcoplasmic reticulum was prepared as described by Meissner et al. (1973), and purified ATPase vesicles were obtained by method 2 of Meissner et al. (1973). Delipidation was carried out as described earlier (Dean & Tanford, 1977). Briefly, ATPase vesicles (~5 mg of protein per mL) were treated with deoxycholate at a ratio of 2 g of deoxycholate per g of ATPase in the presence of 2.74 M glycerol (20% v/v), 1.0 mM MgATP, 0.1 M KCl, and 1.0 mM dithiothreitol buffered at pH 7.5 with 0.01 M Tes buffer. Vesicles were mixed with glycerol prior to the addition of deoxycholate. Solubilized ATPase was precipitated by the addition of poly(ethylene glycol) 6000, and the precipitate was resuspended in a large volume of solubilizing buffer containing poly(ethylene glycol) by vortexing and immersing in a ultrasonic bath. During this step phospholipid is stripped from the particulate ATPase to a level of 4–6 mol of inorganic phosphate (Bartlett, 1959) per mol of polypeptide, based on a molecular weight of 119 000 (Rizzolo et al., 1976). The maximum error in the determination of inorganic phosphate is no more than 20%. The wider range of lipid content reported earlier (4–10 mol/mol) was probably a result of variability in dispersion of the precipitated ATPase. The final delipidated preparation was then redissolved in 0.01 M Tes buffer, pH 7.5, containing 2.74 M glycerol, 0.1 M KCl, 0.1 mM $CaCl_2$, 1.0 mM dithiothreitol, and 1–3 mg/mL $C_{12}E_8$ to give a final protein concentration of 1–4 mg/mL. Protein was determined by the method of Lowry et al. (1951) and was corrected as described previously (le Maire et al., 1976a).

ATPase Assay. The standard ATPase assay was carried out at 37 °C as previously described (Dean & Tanford, 1977). For assays at 20 °C with up to 120 μg of ATPase per mL (data of

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[1] Abbreviations used: $C_{12}E_8$, dodecyl octaethylene glycol monoether; Tes, *N*-tris(hydroxymethyl)methyl-2-aminomethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; cmc, critical micelle concentration; $NaDodSO_4$, sodium dodecyl sulfate.

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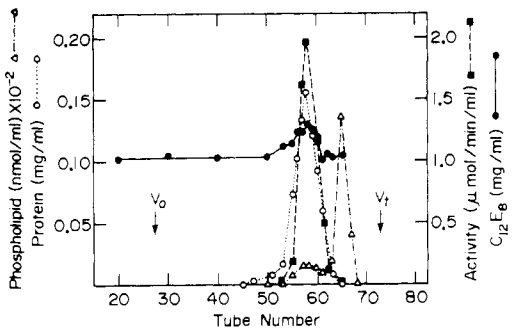

FIGURE 1: Elution profile of delipidated ATPase in 1.86 mM $C_{12}E_8$ and 2.74 M glycerol. Delipidated ATPase (2.7 mg) was dissolved in 1.0 mL of 0.01 M Tes, pH 7.5, containing 0.1 M KCl, 1.0 mM dithiothreitol, 0.1 mM $CaCl_2$, 2.74 M glycerol, and 5.57 mM $C_{12}E_8$. The sample was applied to a Sepharose 4B column at room temperature equilibrated with the same buffer except that the $C_{12}E_8$ concentration was reduced to 1.86 mM and 1.0 mM NaN_3 was added. V_0 and V_t indicate void and total volume markers. The lipid associated with the protein peak corresponds to 1.0 mol/mol polypeptide. Excess $C_{12}E_8$ corresponds to a binding of 1.3 g/g. The specific activity is 12.7 μmol min^{-1} mg^{-1}. Activity was measured at 37 °C in standard assay medium containing 1.86 mM $C_{12}E_8$.

Figure 2), the levels of NADH, phosphoenolpyruvate, pyruvate kinase, and lactate dehydrogenase were doubled, and the concentration of MgATP was increased to 10 mM to ensure that rates were maximal. The assay was initiated as before by the addition of ATPase after preincubation of the assay mixture for 3 min at 20 °C, and the absorbance at 340 nm was monitored. $C_{12}E_8$ or other detergents were included directly in the assay medium as indicated. No activity was observed in solubilized preparations when $CaCl_2$ was replaced by 0.1 mM EGTA, but the rate of ATP cleavage in the presence of EGTA was subtracted from the total ATPase activity for sarcoplasmic reticulum and purified ATPase vesicles. In the temperature dependence studies assay mixtures were preincubated at the desired temperature for 5 min prior to the addition of ATPase, and the temperature of the reaction was determined immediately after completion of the assay.

Phosphoenzyme Formation. The formation of phosphoenzyme from [γ-^{32}P]ATP was determined according to Knowles & Racker (1975) except that the washed filters (Type E, Gelman) were counted in a scintillation counter, and that the assay mixture also contained 1.86 mM $C_{12}E_8$ when delipidated ATPase was assayed. Approximately 50 μg of ATPase was used per 1.0 mL of assay mixture and the specific activity of the labeled ATP was 1.2×10^7 cpm per μmol.

Column Chromatography. Gel chromatography was carried out on 1.5 × 90 cm columns of Sepharose 4B equilibrated as indicated. Fractions (1.5–2.0 g) were collected and analyzed for protein, activity, and organic phosphorus. $C_{12}E_8$ was assayed according to Garewal (1973) except that absorbance of the ethylene dichloride extract was measured at 320 nm to increase the sensitivity of the assay. Levels of protein and lipid encountered in experiments with delipidated ATPase did not interfere with the detergent determination, although higher levels of lipid did cause interference (Goldstein & Blecher, 1975).

Circular Dichroism. Circular dichroic spectra were recorded on a Jobin-Yvon Dichrographe Mark III. A mean residue weight of 115 based on the amino acid composition (Thorley-Lawson & Green, 1973) was used for calculation of mean residue ellipiticity.

Analytical Ultracentrifugation. Sedimentation equilibrium and velocity measurements were carried out on a Beckman-Spinco Model E analytical ultracentrifuge equipped with a photoelectric scanner. Absorbance at 280 nm was used to measure protein concentration as a function of position in the cell. A partial specific volume of 0.740 cm^3/g was used for the Ca^{2+}-ATPase (le Maire et al., 1976a), 0.975 cm^3/g for bound lipid (le Maire et al., 1976a), and 0.973 cm^3/g for $C_{12}E_8$ (Tanford et al., 1977), and bound detergent and lipid were allowed for in calculating molecular weights according to Tanford et al. (1974). The viscosity and density of the solvent (1.86 mM $C_{12}E_8$ and 2.74 M glycerol) were calculated from published tables to be 2.012 cP and 1.060 g/cm^3, respectively. The density of one solution was determined on an Anton Paar Precision Density Meter DMA 02 and found to be in good agreement with the calculated value.

Results

Delipidation in $C_{12}E_8$/Glycerol. The primary delipidating agent used in our procedure is sodium deoxycholate. This reagent readily removes all of the lipid, but the enzyme is irreversibly inactivated when this is done (Hardwicke & Green, 1974). Inactivation is slowed down by the presence of glycerol, and retention of activity is further aided by precipitation of the delipidated protein by poly(ethylene glycol). The preparations of delipidated protein described in our earlier report were somewhat variable, with a residual phospholipid content ranging from 4 to 10 mol per mol of polypeptide, and there was a related variability in the state of aggregation of the protein, as judged by gel exclusion chromatography (Dean & Tanford, 1977). Minor improvements in technique, described in the Experimental Section, gave somewhat more reproducible preparations with a residual lipid content consistently between 4 and 6 mol/mol, but attempts to obtain active preparations with a lower residual lipid content by extension of the same procedure failed, undoubtedly because they involved too long a time of contact with deoxycholate.

The nonionic detergent $C_{12}E_8$ can also be used for the initial delipidation of purified ATPase vesicles. There would be an advantage in this since $C_{12}E_8$ does not inactivate the enzyme as long as glycerol is present, but the precipitate formed by the addition of poly(ethylene glycol) in $C_{12}E_8$ does not form a pellet upon centrifugation, and we have therefore continued to use deoxycholate to remove the major part of the phospholipid. We have found, however, that $C_{12}E_8$ can be employed for further delipidation by using a higher concentration of the detergent for chromatography than was used earlier. The concentration used previously (0.093 mM $C_{12}E_8$) was chosen because of the stability of the enzyme at that level of detergent. However, it was also shown that long term stability is as good as the stability of purified ATPase vesicles at a 20-fold higher concentration, in the presence of 2.74 M glycerol (Dean & Tanford, 1977; upper curve of Figure 2). Figure 1 shows the result of chromatography at the higher detergent concentration. The elution profile clearly demonstrates that additional delipidation occurs as shown by the large lipid peak eluting after the single, symmetrical protein peak. In this particular column the enzyme was delipidated to a level of 1.0 mol of phospholipid per mol of polypeptide without loss of ATPase activity in excess of that expected for the time required for chromatography. Other experiments of this kind have yielded an ATPase particle retaining 1–3 mol/mol of phospholipid; specific activities vary from 10–16 μmol min^{-1} mg^{-1} depending on the initial activity of the ATPase vesicles. Thus nearly complete delipidation in $C_{12}E_8$ results in a soluble ATPase that is no different from previous preparations with higher lipid contents with respect to activity and long-range stability.

The elution profile in Figure 1 also differs from those ob-

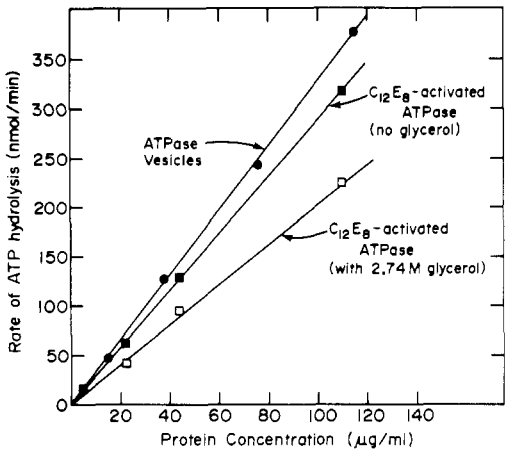

FIGURE 2: ATPase activity vs. enzyme concentration at 20 °C. Delipidated ATPase (2.2 mg/mL in 1.86 mM $C_{12}E_8$ and 2.74 M glycerol) was assayed in 1.86 mM $C_{12}E_8$ at 20 °C under conditions described in the Experimental Section. Purified ATPase vesicles were assayed by the same procedure except that $C_{12}E_8$ was not present.

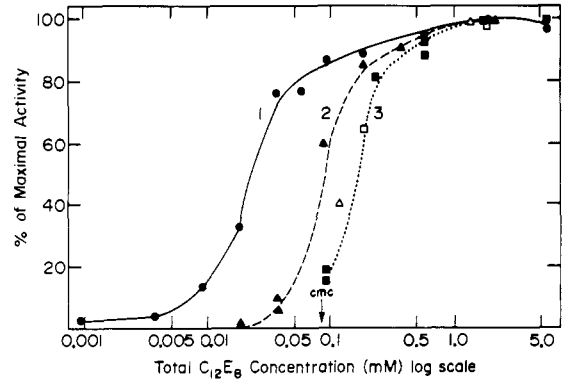

FIGURE 3: The effect of $C_{12}E_8$ concentration on the activity of delipidated ATPase. Delipidated ATPase (2.2 mg/mL in 1.86 mM $C_{12}E_8$ and 2.74 M glycerol) was diluted into assay medium containing the indicated concentrations of $C_{12}E_8$. Curve 1, circles: 1.0 μg/mL of ATPase assayed under standard conditions at 37 °C. Curve 2, triangles: 20.0 μg/mL of ATPase assayed at 20 °C under conditions specified in the Experimental Section. Curve 3, squares: 110.0 μg/mL of ATPase assayed at 20 °C. Filled symbols represent experiments in which the delipidated ATPase was diluted into assay medium already containing $C_{12}E_8$ at the specified concentrations. Open symbols represent experiments in which the ATPase was first diluted into assay medium free of $C_{12}E_8$ and the reaction was initiated by adding the specified amount of detergent. The cmc of $C_{12}E_8$ (8.7×10^{-5} M) is indicated by the arrow.

tained at lower $C_{12}E_8$ concentrations (Dean & Tanford, 1977; Figure 3) in that only a single ATPase peak is observed, whereas at the lower $C_{12}E_8$ concentration two protein peaks were seen. It appears that the residual lipid content may determine the amount of ATPase protein appearing in a rapidly eluting, aggregated fraction upon chromatography.

Factors Affecting Overall Activity in $C_{12}E_8$/Glycerol. The column fractions from Figure 1 were assayed by dilution into glycerol-free assay medium containing 1.86 mM $C_{12}E_8$ so that both protein concentration and solvent composition were different from conditions for column chromatography. To determine if activities measured under assay conditions are in fact an accurate measure of activity under chromatographic conditions, the ATPase assay was altered so that protein concentrations encountered in chromatography could be assayed directly. This was achieved by doubling the amounts of the components of the ATP regenerating system and decreasing the assay temperature to 20 °C. As shown in Figure 2, both ATPase vesicles and soluble ATPase in 1.86 mM $C_{12}E_8$ exhibited linear plots of activity vs. enzyme concentration to protein levels above 0.1 mg/mL, i.e., specific activities remain constant up to the range of protein concentrations prevailing during chromatography. The ratio of the specific activities of the soluble to vesicular ATPase was the same under these conditions as under the standard assay conditions at 37 °C.

The presence of 2.74 M glycerol, on the other hand, was found to inhibit the soluble ATPase by 30%, as shown by Figure 2. A similar amount of inhibition was observed for ATPase vesicles which indicates that the inhibition is a simple solvent effect, which is obviously reversible since the "no glycerol" line in Figure 2 is obtained using enzyme originally in 2.74 M glycerol. The & Hasselbach (1977) observed a similar inhibition of ATPase activity in sarcoplasmic reticulum by ethylene glycol.

When glycerol is omitted from the buffer of a column prepared as in Figure 1, the elution profile is identical, but the ATPase is irreversibly inactivated. Thus it appears that glycerol has two quite independent effects, one being a stabilizing effect on the protein structure required for maintaining the enzyme in an active form, the other being a trivial and reversible solvent effect on the rate of reaction in the active state.

As was shown in the previous paper, delipidated protein has little or no activity in the absence of added $C_{12}E_8$, but the loss of activity is fully reversible as long as glycerol is present. There is no simple way to quantitate the requirement for the presence of detergent because all solutions contain both free detergent and protein-bound detergent. The bound detergent represents an increasingly important fraction of the total as the protein concentration increases, and the detergent/protein ratio may then become the determining factor. At a lower protein concentration, on the other hand, the bound detergent becomes a small fraction of the whole and the total detergent concentration, independent of protein concentration, becomes the determining factor. The latter condition clearly applies to Figure 2 since the specific activity is unaffected by changes in protein concentration while the total $C_{12}E_8$ concentration remains constant.

The dependence of ATPase activity on the total $C_{12}E_8$ concentration at several protein concentrations is shown in Figure 3. At the lowest ATPase concentration (1.0 μg/mL), the bound detergent is a negligible fraction of the total detergent down to at least 0.01 mM $C_{12}E_8$, and the corresponding curve of the figure can therefore be considered as describing the effect of free detergent concentration on activity.[2] The results show that $C_{12}E_8$ can restore close to full activity at a surprisingly low concentration, well below the cmc of the detergent, and under conditions where the protein must still be in an aggregated state. A relatively small, further increase in activity occurs between 0.05 and 2 mM $C_{12}E_8$, i.e., as the conditions used for chromatography in Figure 1 are approached.

The data in Figure 3 at higher ATPase concentrations reflect the binding of $C_{12}E_8$ by the enzyme, which is large enough to significantly lower the free $C_{12}E_8$ concentration. The curves of activity vs. total $C_{12}E_8$ are therefore shifted to the right. $C_{12}E_8$ binding at 1.86 mM determined from the elution profile of Figure 1 is 1.3 g/g and binding of this order of magnitude would easily account for the difference between the curves in Figure 3. The data are not precise enough to attempt a quan-

[2] The representation of similar data (at 1.0 μg/mL) in terms of the detergent/protein ratio, as in Figure 1B of our previous paper (Dean & Tanford, 1977), lends itself to misleading interpretations.

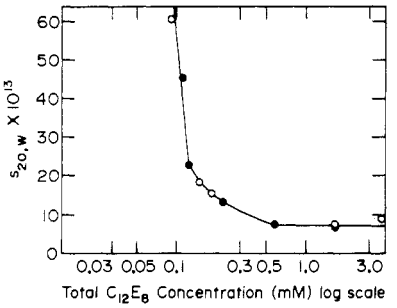

FIGURE 4: The effect of $C_{12}E_8$ concentration on the sedimentation coefficient of delipidated ATPase. Delipidated ATPase (1.75 mg/mL in 1.86 mM $C_{12}E_8$ and 2.74 M glycerol) was diluted into 0.01 M Tes buffer, pH 7.5, containing 0.1 M KCl, 0.1 mM $CaCl_2$, 0.1 mM dithiothreitol, and 2.74 M glycerol, so that the final protein concentration was 0.1 mg/mL. Samples were then submitted to sedimentation velocity centrifugation at 52 000 rpm. Filled circles represent experiments in which the delipidated ATPase was diluted into buffer already containing the specified concentration of $C_{12}E_8$. Open circles represent experiments in which the delipidated ATPase was diluted into buffer *free* of $C_{12}E_8$ (0.1 mM total $C_{12}E_8$), and after incubation for 5 min at room temperature the sample was brought to the final $C_{12}E_8$ concentration.

titative estimate of binding as a function of $C_{12}E_8$ concentration. An important aspect of the data of Figure 3 is that the observed effect of $C_{12}E_8$ is completely reversible. The same activities are obtained when enzyme originally at 1.86 mM $C_{12}E_8$ is diluted into assay medium at the final detergent concentration, and when $C_{12}E_8$ is added to enzyme that was first diluted into assay medium free of $C_{12}E_8$.

State of Aggregation in $C_{12}E_8$/Glycerol. The elution position of the active ATPase in Figure 1 is the same as that reported earlier for inactive "monomer–dimer" in Tween 80 (le Maire et al., 1976a). Disaggregation of the protein is clearly more complete here than was observed when chromatography was carried out at a much lower detergent concentration (Dean & Tanford, 1977; Figure 3), which in itself is not surprising since the large excess of $C_{12}E_8$ present corresponds to a ratio of 9 micelles per polypeptide chain in the peak fraction of Figure 1. (The average micelle aggregation number of $C_{12}E_8$ [Tanford, et al., 1977] is 120). The data indicate, however, the absence of strong cohesive forces between ATPase polypeptides, which could have preserved a higher state of aggregation despite the detergent excess. More important, the data demonstrate conclusively that the suggestion made in our earlier report, that a state of aggregation higher than a dimer is *necessary* to maintain full activity, was incorrect.

A sedimentation equilibrium study of the peak fraction from a column similar to that of Figure 1 was undertaken. Interpretation of the data is complicated by the fact that a mixed solvent system was used. To obtain the protein molecular weight, as described by Tanford et al. (1974), it is necessary to allow for preferential incorporation of water or glycerol into the sedimenting particle (Timasheff et al., 1976), as well as for the binding of detergent. Data for other proteins (Timasheff et al., 1976) suggest that preferential binding of water will occur, but it is possible that the polyoxyethylene moiety of the detergent may preferentially bind glycerol. The binding of $C_{12}E_8$ can be measured as shown in Figure 1. The results were not very reproducible, an average of four column runs yielding 1.15 ± 0.4 g of $C_{12}E_8$ bound per g of protein, but the error this introduces into the molecular weight determination is minimized by the fact that the buoyant density of the detergent is close to that of the solvent.

Sedimentation equilibrium measurements at a speed of 9000 rpm were plotted as the natural logarithm of absorbance at 280 nm in the cell vs. the squared radial distance from the center of rotation. The points were observed to fall on a straight line from the meniscus to approximately two-thirds of the length of the cell (data not shown). Allowing for $C_{12}E_8$ binding and preferential hydration of ±0.2 g/g (the negative sign signifying possible preferential binding of glycerol), the slope of this linear portion of the plot yielded a protein molecular weight of 127 000 ± 13 000. At 11 000 rpm the slope at the meniscus again yielded a weight of 127 000, but the plot was more curved at the bottom of the cell. The observed minimal molecular weight is close to the molecular weight of 119 000 reported by Rizzolo et al. (1976) for the ATPase polypeptide chain. The expected molecular weight of a "monomer" of the active ATPase may be higher than 119 000 because of the small polypeptide present in this preparation as noted earlier MacLennan et al., 1972; Racker & Eytan, 1975; Dean & Tanford, 1977).

Taking the molecular weight obtained from the slopes near the meniscus at both speeds as that of a pure species, we have used the method described by Schechter et al. (1976) to estimate how much of this species would have to have a higher aggregation state to account for the observed curvature of the plots. The result for the run at 9000 rpm is that no more than 15% of the total protein can be in a "dimeric" state. At 11 000 rpm the estimated fraction of "dimer" is 33%. This result suggests that concentration dependent aggregation is occurring and this possibility is now under investigation. One can conclude unambiguously from these data that the monomer is active and stable since the small proportion of dimer present could not possibly account for all of the activity. This was suggested earlier by the results of le Maire et al. (1976b) in deoxycholate, although the ATPase activity of the monomer in their preparation was lost rapidly.

Figure 4 shows sedimentation velocity experiments at various concentrations of $C_{12}E_8$ that support the existence of reversible equilibria between ATPase monomer and higher aggregation states. Samples in which $C_{12}E_8$ was first diluted to 0.1 mM before bringing the mixture to the final detergent concentration exhibited the same behavior as those in which dilution to the final detergent concentration was carried out immediately. The limiting value of the sedimentation coefficient at high $C_{12}E_8$ concentration, corrected for solvent density and viscosity, gives $s_{20,w}$ = 7.0 S. Since the protein under these conditions does not have a unique state of association, this is a weight-average value. Using the weight-average molecular weight of 127 000 derived from the sedimentation equilibrium data at the protein concentration used for sedimentation velocity experiments, and comparing it with the weight-average value of $s_{20,w}$ leads to a Stokes radius of 47 Å (Tanford et al., 1974) and a frictional ratio of f/f_{min} = 1.34. The Stokes radius obtained on the basis of the elution position (in 1.86 mM $C_{12}E_8$ and 2.74 M glycerol) on a calibrated Sepharose 4B column is 55 ± 5 Å, slightly higher than the value obtained from sedimentation velocity. The protein–detergent particle is evidently fairly asymmetric.

Thermodynamic Reversibility. The lipid exchange experiments of Warren et al. (1974a,b) in cholate and the work of le Maire (1976) in Tween 80 with radiolabeled phospholipid strongly suggest that exchange of lipid for lipid or lipid for detergent represents thermodynamic equilibrium as long as protein conformation remains the same. The reversibility of $C_{12}E_8$ activation shown in Figure 3 extends this conclusion to the binding of detergent and the related change in activity.

Further evidence of reversibility is provided by the results of Figure 5. An excess of phospholipid (2 g of phosphatidylcholine per g of protein) was added to delipidated ATPase in

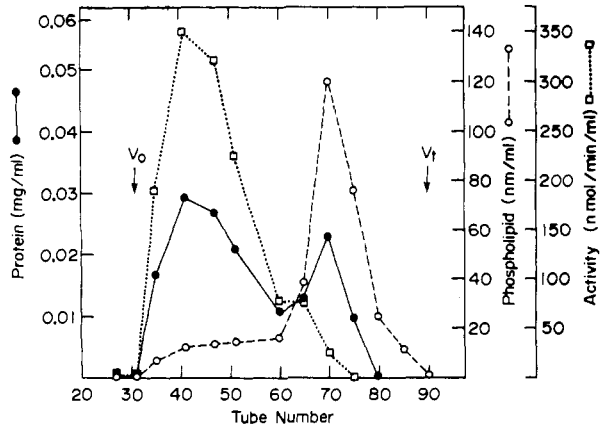

FIGURE 5: Elution profile of delipidated ATPase after readdition of phospholipid in 0.093 mM $C_{12}E_8$. Delipidated ATPase (2.0 mg) was dissolved in 0.5 mL of 0.01 M Tes buffer, pH 7.5, containing 0.1 M KCl, 1.0 mM dithiothreitol, 0.1 mM $CaCl_2$, 2.74 M glycerol, and 18.6 mM $C_{12}E_8$. Bovine phosphatidylcholine (4.0 mg in 0.4 mL of above buffer with $C_{12}E_8$ replaced with 24 mM deoxycholate) was added to the delipidated ATPase and the sample was applied to a Sepharose 4B column equilibrated with 0.01 M Tes, pH 7.5, 0.1 M KCl, 1.0 mM dithiothreitol, 0.1 mM $CaCl_2$, and 0.093 mM $C_{12}E_8$ at 4 °C. Fractions were assayed for ATPase activity at 37 °C in the absence of added $C_{12}E_8$. The lipid associated with the active protein corresponds to 50.0 mol/mol polypeptide. The specific activity is 12.1 μmol min^{-1} mg^{-1}. V_0 and V_t represent tube numbers of void and total volume markers.

$C_{12}E_8$ and the resulting solution was subjected to gel chromatography in 0.093 mM $C_{12}E_8$. The resulting elution pattern is qualitatively similar to that reported earlier for lipid-containing preparations (e.g, Dean & Tanford, 1977; Figure 3) at the same low detergent concentration. Not only has lipid become reassociated with the protein, in this case to a level of 50 mol/mol of polypeptide, but the initially largely monomeric protein again emerges as an aggregated species.

This is an important result because it indicates that the various states of the ATPase protein we have seen in this and in previous studies (excluding the irreversibly inactivated form) are under thermodynamic control. Both the retention of lipid in the presence of Tween 80 (le Maire et al., 1976a) and the rebinding of lipid in the experiment of Figure 5 indicate that the ATPase protein may have binding regions with a thermodynamic preference for phospholipid over detergent molecules. The data of Figure 5 also support the possibility mentioned earlier that there may be a direct relation between lipid binding and association of the protein to an oligomeric form.

Comparison of a Variety of Detergents. $C_{12}E_8$ is by no means unique in its ability to reactivate delipidated ATPase. A series of detergents was tested for reactivation by dilution of delipidated ATPase into assay medium containing the detergent of interest. A broad concentration range of the detergent was used so that the optimal concentration and maximal activity could be determined. The results are presented in Table I. It is immediately obvious that $C_{12}E_8$ possesses no special properties since the widely used detergent Triton X-100 shows an equal ability to reactivate ATPase activity as does Lubrol PX and Brij 56. The nonionic polyoxyethylene derivatives tested in which the polar head groups are composed of 8–10 oxyethylene groups were all able to reactivate delipidated ATPase to a high level regardless of the hydrocarbon tail which varied from 12 to 18 carbon atoms in length. Oleoyllysophosphatidylcholine also fits into this category with respect to its ability to reactivate the ATPase. In contrast, linear polyoxyethylene derivatives with 20 or more oxyethylene groups did not effectively reactivate the enzyme. The Tween series, which

TABLE I: Ability of Detergents to Reactivate Delipidated ATPase.[a]

Detergent	Maximal act. (%)	Concn of detergent at max act. (mg/mL)
$C_{12}E_8$[c]	100	1.0
Lubrol PX ($C_{12}E_{9.5}$)	100	1.0
Triton X-100 [(*tert*-octylphenyl)$E_{9.5}$]	100	0.1
Brij 56 ($C_{16}E_{10}$)	100	0.4
Brij 96 ($C_{18:1}E_{10}$)	89	0.1
$C_{18:1}$lysophosphatidylcholine[b]	89	0.004
Tween 20 ($C_{12}E_{20}$ sorbitan ester)	81	0.5
Tween 40 ($C_{16}E_{20}$ sorbitan ester)	71	6.0
Tween 80 ($C_{18:1}E_{20}$ sorbitan ester)	24	5.0
Brij 98 ($C_{18:1}E_{20}$)	17	0.04
Brij 58 ($C_{16}E_{20}$)	15	0.04
G-2079 ($C_{16}E_{20}$ ester)	13	4.0
Brij 35 ($C_{12}E_{23}$)	13	0.5
$NaDodSO_4$[b]	30	0.03
$C_{14}N(CH_3)_3Cl$[b]	0	
Deoxycholate[b]	0	
Cholate[b]	0	

[a] Delipidated ATPase (20 μg) was assayed in the presence of a series of detergent concentrations for each detergent at 20 °C as described in the Experimental Section. Maximal activities are compared with the activity of ATPase in 1.0 mg/mL $C_{12}E_8$ (1.86 mM) which is normalized to 100%. [b] Assays were carried out with approximately 2.0 μg/mL ATPase at 37 °C. [c] C_n refers to the number of carbons in the long chain alcohol or fatty acid and E_x refers to the average number of oxyethylene units in the polar head groups. Compounds of the type C_nE_x are polyoxyethylene *ethers* except where an *ester* link is specified.

have 20 oxyethylene groups distributed among 4 positions on a sorbitol moiety, creating a relatively short but bulky polar head group, were also effective activators, except for Tween 80. Thus it appears that the length of the hydrocarbon chain is not a critical factor, but that the size of the polar head group is an important determinant for the ability of nonionic detergents to activate the ATPase. The concentration of maximal stimulation does not appear to be related to the cmc of the detergent. For example, $C_{12}E_8$ gives maximal stimulation at a concentration 20-fold higher than its cmc, whereas maximal stimulation occurs very near the cmc for Triton X-100.

Sodium dodecyl sulfate reactivated the enzyme to 30% of the maximal level at a concentration below the cmc. This detergent might well be a better activator were it not that denaturation takes over before the optimal concentration is reached. This suggests that a negative charge does not interfere with activation, whereas the result with the cationic detergent trimethyl tetradecylammonium chloride suggests that a positive charge may not be tolerated. Deoxycholate and cholate are definitely denaturing detergents for this particular protein and this process is irreversible as already demonstrated (Hardwicke & Green, 1974).

It should be noted that Martonosi et al. (1968) observed reactivation of ATPase activity of phospholipase treated sarcoplasmic reticulum by lysolecithin, anionic and nonionic detergents. Green (1975) and Knowles et al. (1976) also have shown that partially delipidated enzyme can be reactivated with various lipid preparations, but stable and fully active enzyme in detergent alone was not achieved.

Kinetics of ATP Hydrolysis. Several laboratories have re-

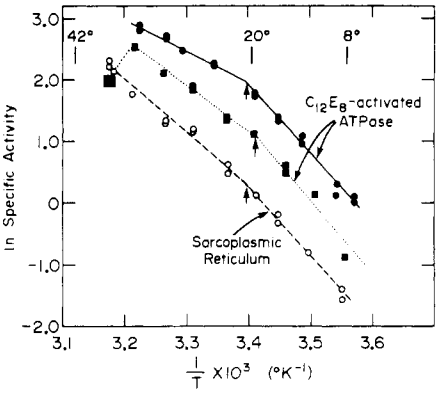

FIGURE 6: Temperature dependence of ATPase hydrolysis for $C_{12}E_8$-activated ATPase and sarcoplasmic reticulum. Delipidated ATPase preparations were assayed in the presence of 1.86 mM $C_{12}E_8$ at the temperatures specified under standard assay conditions (2.0 μg/mL ATPase). Sarcoplasmic reticulum was assayed under the same conditions except that $C_{12}E_8$ was omitted and a higher protein concentration (6.0 μg/mL) was used. The delipidated preparation retaining 5.6 mol of phospholipid per mol of ATPase (squares) was prepared by the usual procedure, whereas the preparation retaining only 2.5 mol/mol (filled circles) was obtained after further delipidation in $C_{12}E_8$/glycerol as in Figure 1. Break points in the plots, determined by least-squares analysis of the points above and below 20 °C, are indicated by the arrows.

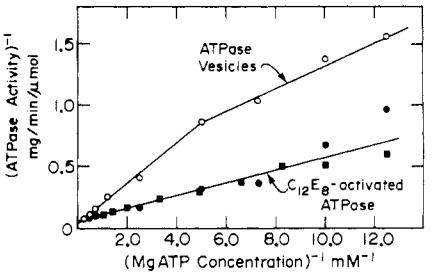

FIGURE 7: Double-reciprocal plot of ATPase activity vs. MgATP concentration for purified ATPase vesicles and delipidated ATPase. Delipidated ATPase (2.7 mg/mL in 1.86 mM $C_{12}E_8$ and 2.74 M glycerol) was diluted into standard assay medium at 37 °C at the indicated MgATP concentrations. ATPase vesicles were assayed in identical fashion except that $C_{12}E_8$ was omitted. Data are shown for purified ATPase vesicles (open circles); delipidated ATPase assayed in 1.86 mM $C_{12}E_8$ (filled circles); and delipidated ATPase assayed in 0.093 mM $C_{12}E_8$ (filled squares). Each data point is the average of two or three determinations. Two different delipidated ATPase preparations were used for assay at 1.86 and 0.093 mM $C_{12}E_8$.

ported that a break occurs in Arrhenius plots of ATPase activity for sarcoplasmic reticulum or purified ATPase vesicles (Inesi et al., 1973; Lee et al., 1974; Deamer, 1973). These discontinuities have been attributed to liquid-crystalline transitions in the lipid bilayer (Inesi et al., 1973), lipid cluster formation (Lee et al., 1974) or protein-lipid interactions of the ATPase with a tightly associated lipid annulus (Hesketh et al., 1976). Since the $C_{12}E_8$-activated ATPase is more than 95% delipidated, if the break in the Arrhenius plot is due to protein-lipid interactions, then no break should be observed with the delipidated preparation. This is shown not to be the case in Figure 6. The break occurs at approximately 20 °C for sarcoplasmic reticulum which is in agreement with Inesi et al. (1973) and Deamer (1973), but is somewhat lower than the value reported for purified ATPase by Lee et al. (1974). The behavior of two preparations of $C_{12}E_8$-activated ATPase, one retaining 5.6 mol of phospholipid/mol of ATPase and the other 2.5 mol/mol, is nearly identical with that of sarcoplasmic reticulum. The apparent activation energies obtained from the linear portions of Figure 6 for sarcoplasmic reticulum above and below 20 °C are 16.6 and 22.1 kcal/mol, respectively, which are in good agreement with the values reported by Inesi et al. (1973). The corresponding values for the $C_{12}E_8$-activated ATPase are 15.9 and 26.5 kcal/mol, thus demonstrating the striking similarity between the soluble and vesicular preparations. These results suggest that protein-lipid interaction is not responsible for the observed breaks in the Arrhenius plots for the Ca^{2+}-ATPase and argue instead for temperature dependent changes intrinsic to the polypeptide as have been observed for some soluble enzymes (Massey, 1953; Trujillo & Deal, 1977). The two functional states of the ATPase proposed by Inesi et al. (1976) cannot account for the observed behavior since no Ca^{2+}-independent activity is present in this preparation.

The formation of a phosphorylated ATPase from ATP in the presence of Ca^{2+} was determined for both purified ATPase vesicles and the $C_{12}E_8$-activated enzyme. The delipidated ATPase in 1.86 mM $C_{12}E_8$ was phosphorylated to a level of 5.0 nmol of [^{32}P]phosphate per mg of enzyme (0.60 on a mol/mol basis), while freshly prepared ATPase vesicles were phosphorylated to the extent of 4.7 nmol/mg. The value for vesicles is in good agreement with the results of Knowles et al. (1976) of 4.76 nmol/mg under the same assay conditions, but is somewhat lower than that reported by Meissner et al. (1973) and MacLennan et al. (1971) who included Mg^{2+} in the assay mixture. This result demonstrates that $C_{12}E_8$ efficiently replaces lipid in supporting ATP-dependent phosphorylation.

A detailed study of the effect of Ca^{2+} on ATP hydrolysis was not undertaken because of the difficulty in the determination of free Ca^{2+}, especially in light of the possibility of Ca^{2+} binding to the polar head group of $C_{12}E_8$. However, it was determined that the low affinity Ca^{2+} site on the ATPase that shuts down the enzyme when occupied (Ikemoto, 1974) appears to be unperturbed by delipidation and solubilization, since at 10 mM Ca^{2+} the activity of both the soluble and vesicular preparation was completely inhibited. Removal of nearly all free Ca^{2+} by EGTA results in a complete loss of activity at all temperatures for the $C_{12}E_8$-activated ATPase whereas a low level of Ca^{2+}-independent activity remains for purified ATPase vesicles or sarcoplasmic reticulum. Inesi et al. (1976) also observed this effect when sarcoplasmic reticulum was solubilized with Triton X-100.

In Figure 7, double-reciprocal plots of ATPase activity vs. MgATP concentration are presented. A nonlinear plot for ATPase vesicles is observed which is in agreement with earlier observations (Inesi et al., 1967; Neet & Green, 1977). The substrate concentration at half maximal velocity is 1.75 mM and V_{max} is 16.0 μmol min^{-1} mg^{-1}. In contrast, the reciprocal plot for $C_{12}E_8$-activated ATPase is linear within the precision of the determinations at $C_{12}E_8$ concentrations of 0.093 and 1.86 mM and shows a K_m of 0.84 mM and a V_{max} of 12.5. Obviously solubilization in $C_{12}E_8$ has eliminated the "negative cooperativity" observed in vesicular preparations as was also observed with Triton X-100 by Inesi et al. (1976). This difference in steady-state kinetics does not appear to be a result of changes in the state of aggregation, since the assay was carried out under both conditions favoring monomer (1.86 mM $C_{12}E_8$) and those favoring aggregates (0.093 mM $C_{12}E_8$).

These collective studies on the hydrolysis of ATP by the delipidated, $C_{12}E_8$-activated ATP suggest an inherent similarity between vesicular and solubilized enzymes, but the data are not complete enough to justify a stronger conclusion.

Circular Dichroic Spectra. The circular dichroic spectra of $C_{12}E_8$-activated, lipid-activated, and vesicular ATPase were compared in an attempt to determine if any gross conforma-

tional differences could be detected after delipidation and solubilization. Particle size increases for the preparations used in Figure 8 from monomeric ATPase in $C_{12}E_8$ (curve A) to aggregated ATPase in $C_{12}E_8$ (curves B and C) and finally to ATPase in phospholipid vesicles (curve D). The progressive increase in the molar ellipticity at 209 nm from curve A to curve D is correlated with the increase in particle size which suggests that the conformation of the ATPase may change as a result of aggregation. However, it is equally possible that the spectral differences are caused by the type of optical artifacts described by Holzwarth (1972) or by conformational changes induced by binding of $C_{12}E_8$. Solubilization does not cause the type of change in circular dichroic spectra observed by le Maire et al. (1976b) with deoxycholate, i.e., an increase in molar ellipticity at 222 nm with little change at 209 nm as activity is lost with time. The spectrum exhibited by delipidated ATPase in $C_{12}E_8$ and glycerol is stable for several days and is similar to that observed for purified ATPase vesicles immediately after solubilization in deoxycholate (le Maire et al., 1976b).

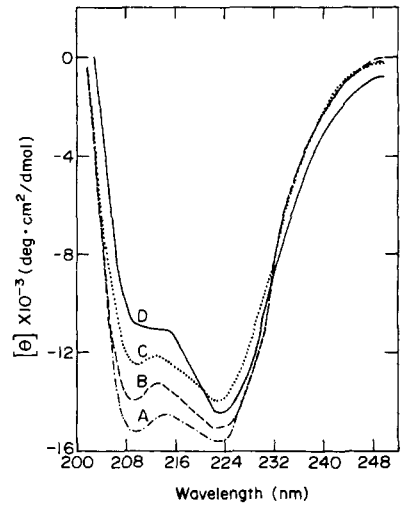

FIGURE 8: Circular dichroic spectra of $C_{12}E_8$- and lipid-reactivated ATPase and purified ATPase vesicles. The buffer for the ATPase preparations was 0.01 M Tes, pH 7.5, 0.1 M KCl, 1.0 mM dithiothreitol, 0.1 mM $CaCl_2$, and 2.74 M glycerol. $C_{12}E_8$ was included as indicated. (A) Delipidated ATPase (0.28 mg/mL) in 1.86 mM $C_{12}E_8$; (B) lipid-activated ATPase (0.028 mg/mL) in 0.093 mM $C_{12}E_8$ from the active fractions of the column described in Figure 6; (C) delipidated ATPase (0.28 mg/mL) in 0.093 mM $C_{12}E_8$; (D) ATPase vesicles (0.14 mg/mL protein).

Discussion

The results of this paper have shown that the Ca^{2+}-ATPase of sarcoplasmic reticulum does not require phospholipid for the expression of ATPase activity. Replacement of phospholipid by $C_{12}E_8$ leads to the formation of a soluble, delipidated enzyme with overall activity at the same level as in purified ATPase vesicles obtained directly from sarcoplasmic reticulum, with a similar temperature dependence, and a similar ability to form a phosphorylated intermediate. The lowest phospholipid content for any of the preparations we have described is 1.0 mol/mol of ATPase polypeptide, but there is no reason to believe that this last residual phospholipid molecule is "essential". Its retention with the protein is probably ascribable to the fact that the ATPase has a higher affinity for phospholipid than for detergent (see below). It could probably be removed by rechromatography of the protein peak in Figure 1 in an excess of $C_{12}E_8$. The results of Table I show that $C_{12}E_8$ is not unique in its ability to act as replacement for phospholipid; many other amphiphiles have the same property. It is interesting that bile salts are not able to substitute for phospholipid in the local environment of the protein. Active soluble enzyme in deoxycholate can be prepared (le Maire et al., 1976b), but only if bound phospholipid is retained.

Two differences in the enzymatic activity between delipidated and vesicular ATPase were noted. One is that the dependence of ATPase hydrolysis on the MgATP concentration is altered (Figure 7) and the other is that Ca^{2+}-independnet activity is lost. Several explanations for the nonlinear double-reciprocal plot observed for vesicular preparations of the ATPase, but lacking with soluble preparations, have been proposed. Froelich & Taylor (1975) suggested that a second, low affinity ATP binding site on the polypeptide could account for the kinetics of vesicular preparations, although the available data indicate only one ATP site per ATPase polypeptide (Meissner, 1973). Other possibilities are a slow transition between two active forms of the ATPase or ATP-dependent protein–protein interactions (Neet & Green, 1977). The data presented here appear to rule out the third possibility since aggregated ATPase in $C_{12}E_8$ gave the same result as the monomer, i.e., a linear double-reciprocal plot, although it is possible that lipid is necessary for proper protein–protein interactions.

In addition, it must be noted that the property most critical for the biological action of the ATPase, the coupling of ATPase activity to the active transport of Ca^{2+}, cannot be tested when the enzyme is in a homogeneous solution.

An important aspect of our results is that the changes in the ATPase protein as a function of environmental conditions that we have studied have proved to be reversible, so that the data obtained for binding of lipid or detergents and for the related alterations in state of aggregation and enzymatic activity can be interpreted in terms of thermodynamic equilibria. The following conclusions may be drawn:

(1) The ATPase protein has one or more sites or extended regions with a very high affinity for amphiphiles in general, and it is enzymatically inactive if these sites are not occupied. The association between the protein and $C_{12}E_8$ is not simply an insertion of the protein into a detergent micelle, such as was postulated for the reaction between cytochrome b_5 and amphiphiles (Robinson & Tanford, 1976). The binding of $C_{12}E_8$ occurs well below the cmc of the detergent (Figure 3), whereas the binding of detergents to cytochrome b_5 occurs near or even above the cmc. Moreover, the amount bound at 1.86 mM $C_{12}E_8$ (which by Figure 3 can be inferred to be the saturation level) is about 1.15 g of $C_{12}E_8$ per g of protein, which corresponds to 2.2 micelles per polypeptide chain or even more per particle if the partial association to a dimeric state at this detergent concentration is taken into account.

(2) The high affinity binding sites or regions have a significantly higher affinity for phospholipid than for detergent. This is demonstrated by the difficulty in removing the last few molecules of phospholipid even in the presence of a large excess of detergent, and by the reincorporation of phospholipid under the conditions of Figure 5. The retention of phospholipid in Tween 80-solubilized particles (le Maire et al., 1976a) has also been shown to be a result of a higher thermodynamic affinity (le Maire, 1976). It would seem that interactions of the phospholipid head group with the protein may be more important in this selectivity than the hydrocarbon moiety as suggested by the reactivation studies in Table I, and especially so by the very low concentration required for activation when lysophospholipid was used.

(3) Our data have demonstrated that the ATPase is stable and fully active as a monomer, but that the protein has a propensity for reversible self-association (e.g., Figure 4). The tendency for formation of oligomers is clearly greatly increased

by the presence of phospholipid, as is demonstrated most forcefully by Figure 5, and of course also by an increase in protein concentration. It is possible that the high effective protein concentration present in sarcoplasmic reticulum will lead the protein to be present there in an aggregated form, as has been suggested by others (Packer et al., 1974; Malan et al., 1975; Murphy, 1976; le Maire et al., 1976a; Vanderkooi et al., 1977).

It should be pointed out in conclusion that the ATPase protein can readily undergo irreversible changes in state in addition to the reversible processes we have described. It has been shown, for example, that exposure to high concentration of deoxycholate leads to delipidation and inactivation that are irreversible (e.g., Hardwicke & Green, 1974), and that monomeric and dimeric states of the protein under these conditions are not readily interconvertible (le Maire et al., 1976a). Furthermore purified ATPase vesicles, prepared directly from sarcoplasmic reticulum, as well as solubilized enzyme, slowly lose ATPase activity on standing for a period of days.

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