Behavior of Fragmented Calcium(II) Adenosine Triphosphatase from Sarcoplasmic Reticulum in Detergent Solution†

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ABSTRACT: The behavior of Ca^{2+}-ATPase from sarcoplasmic reticulum in detergent solution was compared with that of Ca^{2+}-ATPase which had been cleaved in half by limited trypsin digestion. Attempts to dissociate the fragments (I and II) with an excess of detergent micelles demonstrated that fragments I and II are structurally dependent upon each other, and that they must be denatured in order to be dissociated. Partial dissociation of the fragmented ATPase was found to occur in the bile salt detergents, deoxycholate and cholate, and optical data showed that there was an accompanying change in conformation. No dissociation of the fragmented ATPase was observed in nonionic detergents. The fragmented ATPase retained the same specific activity and stability as the intact ATPase under a variety of conditions when solubilized in Tween 80 or dodecyl octaoxyethylene glycol monoether. The data demonstrate that the noncovalent interactions that maintain the native conformation of the ATPase are not affected by either trypsin cleavage or solubilization in nonionic detergent solution.

The molecular mechanism of Ca^{2+} uptake by the sarcoplasmic reticulum has received considerable attention. The major protein component of the SR[1] is an ATPase which is involved in Ca^{2+} translocation (MacLennan et al., 1971; Meissner et al., 1973). A small polypeptide termed proteolipid by MacLennan et al. (1972) may also be involved in this process (Racker & Eytan, 1975). The ability to cleave the ATPase into equal-sized halves (fragments I and II) by limited trypsin digestion has been exploited by several laboratories to learn about the structure of the ATPase (Thorley-Lawson & Green, 1975; Rizzolo et al., 1976; Stewart et al., 1976).

The trypsin cleavage does not affect the ability of the ATPase in SR vesicles to pump calcium at the expense of ATP (Inesi & Scales, 1974; Stewart & MacLennan, 1974). Stewart et al. (1976) have labeled the active site with [γ-^{32}P]ATP and *N*-ethyl[^{3}H]maleimide and found both labels in fragment I. This fragment also displayed a Ca^{2+} specific ionophore activity, suggesting the presence of a Ca^{2+} binding site. No specific function has been ascribed to fragment II.

There are no disulfide cross-links between the fragments (Thorley-Lawson & Green, 1975) and there is evidence that both fragments are membrane embedded. Neither fragment can be selectively extracted from the membrane under a variety of conditions of pH and ionic strength or by a variety of reagents, both fragments preferring the environment provided by a detergent micelle or lipid bilayer, as opposed to aqueous media (Thorley-Lawson & Green, 1975; Rizzolo, 1977). The behavior of each separated fragment in guanidine hydrochloride is similar to that of known membrane-embedded proteins and not at all characteristic of water-soluble proteins (Rizzolo & Tanford, 1978). Thorley-Lawson & Green (1975) and Stewart et al. (1976) have been able to dissociate the fragments only with the strong denaturant sodium dodecyl sulfate. The inability of these investigators to separate the fragments in nondenaturing media on the basis of differential solubility, charge, or size could be because of the similar physical properties of the fragments rather than a strong interfragment affinity.

In the present study we wish to determine whether trypsin splits the ATPase into separate domains which are structurally and functionally independent or into domains which are bound together by noncovalent forces. We have dissolved intact and fragmented enzyme in an excess of detergent and have compared the extent of dissociation of the fragments from each other with the extent of dissociation of intact ATPase polypeptide chains from each other. Our experiments are based on the following principle: low levels of detergent will solubilize membranes forming particles of large size (effectively membrane fragments). At higher levels of detergent where there are more micelles than protein molecules, small soluble particles are formed containing only a single protein complex per particle. If the fragments remain associated under these conditions, it has to be because their associated state is the thermodynamically favored state, or a metastable state prevented from dissociation by kinetic barriers. A large excess of micelles, coupled with dilution of the protein, will favor dissociation of the fragments if the associative forces are weak. Our initial observations utilized gel chromatography. Because both fragments have approximately the same molecular weight (~60 000, Rizzolo et al., 1976), they cannot be separated by this technique, but a mixture of dissociated fragments would have a smaller particle size and hence a larger distribution coefficient than fragments that remain associated. We have used direct molecular weight measurement by sedimentation equilibrium to confirm conclusions based on elution positions in gel chromatography.

Experimental Section

Materials. Sodium deoxycholate (DOC) (Fisher Scientific Co.) and sodium cholate (Sigma Corp.) were purified by charcoal and Celite treatment and recrystallized from 80% acetone. Tween 80 was obtained from Sigma Corp. Dodecyl

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[1] Abbreviations used: SR, sarcoplasmic reticulum; ATPase, adenosine triphosphatase; ATP, adenosine triphosphate; DOC, deoxycholate; $C_{12}E_8$, dodecyl octaoxyethylene glycol monoether; TAPS, *N*-tris(hydroxymethyl)methylaminopropanesulfonic acid; Tes, *N*-tris(hydroxymethyl)-methyl-2-aminomethanesulfonic acid; CD, circular dichroism; cmc, critical micelle concentration.

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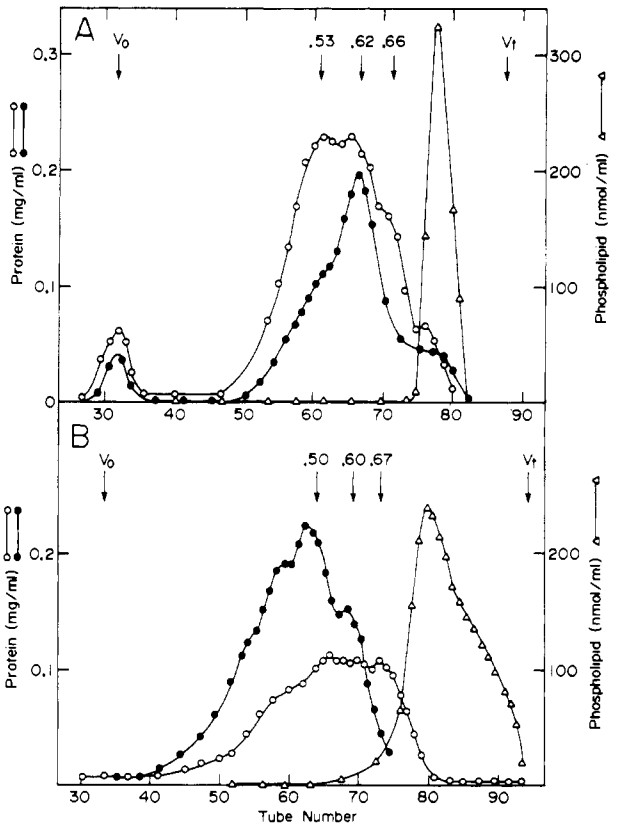

FIGURE 1: Gel filtration of ATPase in deoxycholate and cholate. ATPase vesicles (3–4 mg of protein) were solubilized in 1.0 mL of column buffer containing either (A) DOC at 1.25:1 DOC/protein weight ratio or (B) cholate at a 5:1 cholate/protein weight ratio. The sample was then loaded on a Sepharose 4B column (1.5 × 90 cm) which was equilibrated and eluted with (A) 0.01 M TAPS buffer, pH 8.0, 0.1 M KCl, 1.0 mM EGTA, and 5.0 mM DOC at room temperature, or (B) 0.01 M Tes buffer, pH 7.5, 0.1 M NaCl, 0.1 mM $CaCl_2$, 1.0 mM EDTA, and 10 mM cholate at 4 °C. (Closed circles) Intact ATPase; (open circles) fragmented ATPase.

octaoxyethylene glycol monoether ($C_{12}E_8$) was a nominally pure compound prepared by Nikko Chemicals Co., Tokyo, Japan, and it was recrystallized from hexane before use. Tos-PheCH$_2$Cl-trypsin was purchased from Worthington Biochemical Corp. *p*-Nitrophenyl *p*-guanidinobenzoate was synthesized according to the method of Chase & Shaw (1970).

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).

Preparation of Fragmented ATPase. Trypsin cleavage was carried out essentially as described by previous investigators (Ikemoto et al., 1971; Thorley-Lawson & Green, 1973; Inesi & Scales, 1974). Sarcoplasmic reticulum vesicles, at concentrations of 1–5 mg of protein/mL, were incubated for 5 min in 20 mM Tris-maleate, pH 7.0, 0.1 M NaCl, 1 M sucrose, and trypsin at a 200:1 protein/trypsin weight ratio. The reaction was stopped by adding an excess of *p*-nitrophenyl *p*-guanidinobenzoate. All of the ATPase was cleaved by this procedure, and purified fragmented ATPase vesicles were obtained as described above for intact ATPase. In some experiments the fragmented ATPase was delipidated according to the method of Dean & Tanford (1977).

ATPase Assays. Two assay systems were employed in the course of this study. In experiments with partially delipidated ATPase (containing ~30 mol of phospholipid/mol of ATPase), ATPase activity was determined at 23 °C as the rate of production of inorganic phosphate using the system described by le Maire et al. (1976a). In experiments with highly delipidated ATPase (containing <10 mol of phospholipid/mol of ATPase), ATPase activity was determined at 37 °C using the coupled assay described by Dean & Tanford (1977).

Protein Concentration. Protein concentrations were determined by the method of Lowry et al. (1951) using BSA as the standard and were corrected for the 1.2-fold higher color yield reported for the ATPase by Hardwicke & Green (1974).

Analytical Ultracentrifugation. Sedimentation equilibrium and velocity measurements were made with a Beckman Model E ultracentrifuge equipped with a photoelectric scanner. Absorbance at 280 nm was used to measure protein concentration as a function of position in the cell. As previously described (Tanford et al., 1974), the contribution of bound detergent and bound lipid (δ_D and δ_L g/g of protein, respectively) can be allowed for by the relation

$$M(1 - \phi'\rho) = M[(1 - \bar{v}_P\rho) + \delta_D(1 - \bar{v}_D\rho) + \delta_L(1 - \bar{v}_L\rho)] \quad (1)$$

where $\bar{v}_P$ is the partial specific volume of protein, and $\bar{v}_D$ and $\bar{v}_L$ are the partial specific volumes of bound detergent and lipid, respectively. A value of $\bar{v}_P = 0.738$ cm^3/g was calculated on the basis of the amino acid composition (Thorley-Lawson & Green, 1975) by the method of Cohn & Edsall (1943). The partial specific volumes of $C_{12}E_8$ (0.974 cm^3/g), DOC (0.778 cm^3/g), Tween 80 (0.896 cm^3/g), and phospholipid (0.975 cm^3/g) were taken from Tanford et al. (1974), the latter being based on the lipid composition of ATPase vesicles given by MacLennan et al. (1971).

Sedimentation velocity was measured at 52 000 rpm in an AND rotor at 0.1 mg of protein/mL.

Other Methods. Circular dichroism measurements were carried out using a Jobin Yvon Dichrographe III. Sodium dodecyl sulfate gel electrophoresis was performed according to the method of Weber & Osborn (1969). Phospholipid was assayed as described by Bartlett (1959) and $C_{12}E_8$ was assayed by the method of Garewal (1973) as modified by Dean & Tanford (1978). Solution densities were measured with an Anton Paar Density Meter, DMA 02C.

Results

Studies in Bile Salt Detergents. Fragmented ATPase[2] vesicles were solubilized in DOC as described in Figure 1A. The open circles show that the elution pattern for fragmented ATPase was similar to the result obtained by le Maire et al. (1976a) for the intact ATPase (closed circles). Sedimentation equilibrium experiments established that the peak with a K_d equal to 0.62 was ATPase monomer and that the peak with a K_d equal to 0.53 was ATPase dimer. The relative amounts of monomer and dimer varied with no apparent correlation with the DOC/protein weight ratio used for initial solubilization. In each case a small protein peak, which may be the small polypeptide (le Maire et al., 1977b), elutes with the phospholipid. Both fragmented and intact ATPase have lost enzymatic activity under these conditions, as previously observed for the intact ATPase by le Maire et al. (1976a).

[2] We shall refer to ATPase which has been cleaved by trypsin as fragmented ATPase. Fragmented ATPase which has been solubilized in detergent and has the same molecular weight as intact ATPase monomer will be referred to as fragmented ATPase monomer. Detergent solubilized fragmented ATPase which has the same molecular weight as intact ATPase dimer will be referred to as fragmented ATPase dimer. Fragmented ATPase whose subdomain structure has been dissociated, i.e., fragments I and II reside in separate micelles, will be referred to as dissociated fragments.

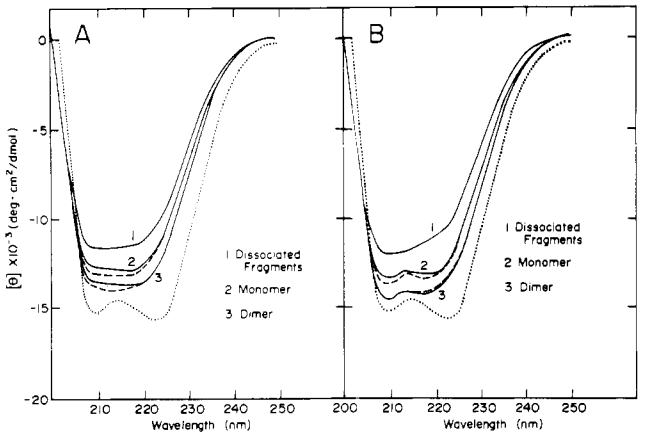

FIGURE 2: Circular dichroism spectra of fragmented ATPase in deoxycholate and cholate. Samples were taken from the columns described in Figure 1. (A) Deoxycholate; (B) cholate. (Solid curves) Fragmented ATPase; (dashed curves) intact ATPase; (dotted curve) active ATPase solubilized in Tween 80 (see Figure 4).

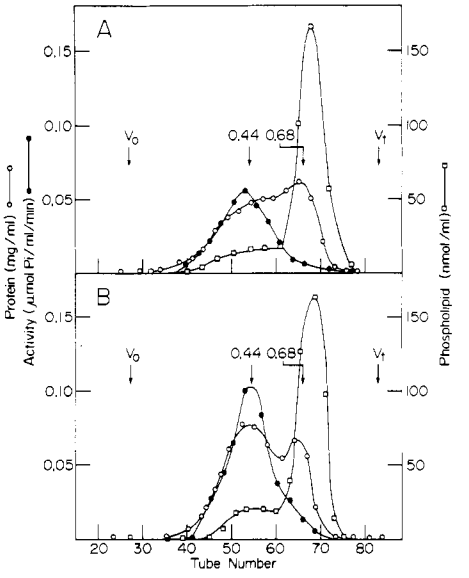

FIGURE 3: Gel filtration of ATPase in Tween 80. ATPase vesicles (3–4 mg of protein) were dissolved in 1.0 mL of 0.01 M Tes buffer, pH 7.0, 0.1 M KCl, 0.1 mM $CaCl_2$, and $C_{12}E_8$ at a 3.8:1 detergent/protein weight ratio. The sample was loaded on a Sepharose 4B column (1.5 × 90 cm) which was equilibrated and eluted at 4 °C with the same buffer containing 0.1 mg/mL Tween 80 in place of the $C_{12}E_8$. (A) Fragmented ATPase; (B) intact ATPase. V_0 and V_t mark the void and total volumes, respectively. K_d's are noted for the principal protein peaks.

The striking difference between the elution profiles of Figure 1A is a protein shoulder with a K_d of 0.66 in the profile for fragmented ATPase suggesting dissociated fragments were formed. Under the conditions of ionic strength employed in these experiments, the cmc of DOC was approximately 1 mM and the micelle aggregation number was approximately 17 (Carey & Small, 1972). The micellar concentration of the column buffer described in Figure 1A was 2.4×10^{-4} M, while the protein concentration of the fragmented monomer peak was 1.7×10^{-6} M. This 100-fold excess of micelles was sufficient to dissociate a fraction of the ATPase into fragments I and II.

Sedimentation equilibrium experiments confirmed this conclusion. If the DOC binding of 0.3 g/g of protein determined by le Maire et al. (1976a) is assumed for the fragments, the molecular weight of the protein in the shoulder ($K_d = 0.66$) can be determined. A sample from the trailing edge of this shoulder was heterogeneous with a molecular weight approaching 60 000 at the top of the centrifuge cell and 120 000 at the bottom of the cell. These are the weights expected for dissociated fragments and ATPase monomer. Fitting the data to a model which assumed only two sedimenting species were present (Schechter et al., 1976) indicated that approximately 27% of the sample was dissociated fragments and 65% was fragmented monomer. Obtaining similar values at two rotor speeds indicated the sedimenting particles did not interact during the course of the experiment. $NaDodSO_4$ gel electrophoresis of the same sample demonstrated the absence of small degradation products and indicated that fragments I and II were present in equal amounts.

Sedimentation velocity experiments performed at 52 000 rpm on the same sample used for the sedimentation equilibrium experiment resulted in a single broad boundary which yielded an average $s_{20,w}$ value of 4.9 S. This is lower than the value of 5.5 S obtained by le Maire et al. (1976b) for ATPase monomer in DOC but must be greater than the true value for the dissociated fragments, since a distinct boundary could not be seen for each of the two different size species known to be present.

Gel filtration experiments analogous to those in DOC were performed in cholate. The elution profiles of intact (closed circles) and fragmented (open circles) ATPase are shown in Figure 1B. In contrast to the experiments in DOC, the phospholipid–cholate mixed micelles eluted as a bimodal peak which was not clearly resolved from the protein peak, and the small polypeptide was not resolved from either the intact or fragmented ATPase. The separate peak for dissociated fragments, however, stands out clearly in the elution profile of fragmented ATPase. Under the conditions of ionic strength employed in these experiments, there was a 1000-fold excess of micelles over fragmented monomer which was sufficient to dissociate a fraction of the fragmented ATPase into fragments I and II. Sedimentation equilibrium experiments confirm the protein eluting with a K_d of 0.67 contained dissociated fragments.

The circular dichroism spectra presented in Figure 2 show that dissociation of the fragments by the bile salt detergents is accompanied by a conformational change. First, it should be noted that the conformation of these enzymatically inactive preparations differ from that of the active enzyme and that the conformation is somewhat sensitive to the aggregation state of the protein. Fragmented monomer, however, has the same CD spectra as intact monomer, and fragmented dimer has the same CD spectra as intact dimer. The dissociation of the fragments resulted in further conformational change in both cholate and deoxycholate. Despite the fact that the samples of dissociated fragments were contaminated with fragmented monomer the difference between spectra 1 and 2 in Figures 2A and 2B exceeds experimental error. Since the spectrum of a mixture is an average of the optical activities of the individual components, the CD spectrum of the dissociated fragments should have been identical with that of fragmented monomer if the fragments had retained the same conformation that they had in the parent molecule.

Partially Delipidated ATPase. Fragmented ATPase vesicles were solubilized in 10 mM Tes buffer, pH 7.0, 0.1 M KCl, 0.1 mM $CaCl_2$, and $C_{12}E_8$ at a detergent/protein weight ratio of 3.8 and immediately loaded on a Sepharose 4B column; the column was equilibrated and eluted at 4 °C with the same buffer substituting 0.1 mg/mL Tween 80 for the $C_{12}E_8$. The column took approximately 24 h to run. The elution pattern is shown in Figure 3 along with the elution pattern for intact ATPase run under similar conditions. The results were in good

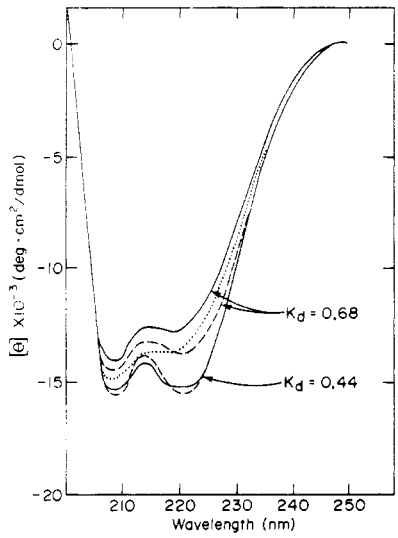

FIGURE 4: Circular dichroism spectra of partially delipidated ATPase in Tween 80. Samples were taken from the columns described in Figure 3, 36 h after initial solubilization of the protein. (Solid curves) Fragmented ATPase; (dashed curves) intact ATPase. The dotted curve is ATPase eluting with a K_d of 0.44, 4 days after the initial solubilization of the protein.

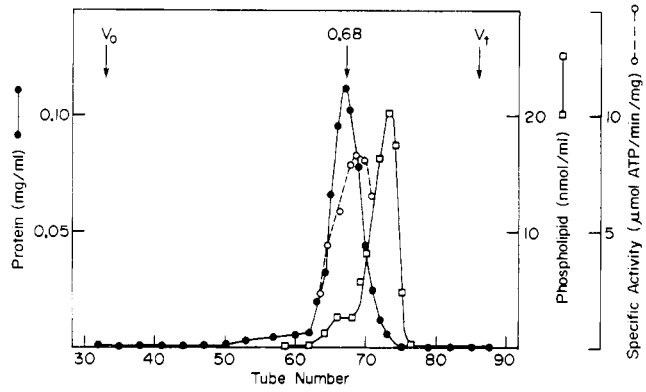

FIGURE 5: Gel filtration of highly delipidated, fragmented ATPase in 20% glycerol and $C_{12}E_8$. Fragmented ATPase was delipidated with DOC by the method of Dean & Tanford (1977) and ~3 mg of protein was solubilized in 1.0 mL of eluent buffer containing a 3:1 $C_{12}E_8$ micelle/fragmented monomer molar ratio. The sample was loaded on a Sepharose 4B column (1.5 × 90 cm) which was equilibrated and eluted with 0.01 M Tes buffer, pH 7.5, 0.1 M KCl, 0.1 mM $CaCl_2$, 0.1 mM DTT, 20% (v/v) glycerol, and 1.0 mg/mL $C_{12}E_8$ at room temperature.

agreement with le Maire et al. (1976a) and there were no significant differences between the behavior of intact and fragmented ATPase. The protein eluted as a broad peak which can be divided into two approximately Gaussian components. Sedimentation equilibrium experiments identified the leading peak, K_d equal to 0.44, as a trimer or tetramer and the trailing peak, K_d equal to 0.68, as a monomer or dimer. Enzyme activity was associated with both protein peaks, but the specific activity of the leading peak was about tenfold higher than the specific activity of the trailing peak. The experiments in Figure 3 were repeated several times, with similar results. The fraction of protein in each peak was somewhat variable, the persistence of larger particles being favored by the use of relatively low solubilizing concentrations of $C_{12}E_8$. Both fragmented and intact ATPase retained approximately 30 mol of phospholipid/mol of ATPase monomer and the fragmented ATPase had the same stability when stored at 4 °C as observed by le Maire et al. (1976a) for partially delipidated ATPase and ATPase vesicles.

Most of the phospholipid appeared as a sharp peak, eluting later than the protein, but incompletely separated from it. The poor separation from the protein, in comparison to the results in the bile salts, can be ascribed to the fact that $C_{12}E_8$ micelles are considerably larger than bile salt micelles, so that the mixed micelles are also larger. This feature made it difficult to determine by sedimentation equilibrium if any dissociated fragments were formed. (This difficulty was resolved by first delipidating the fragmented ATPase as described below.)

The circular dichroism spectra shown in Figure 4 indicate the conformations of ATPase eluting at different positions in the column were not the same, but the spectra of fragmented ATPase were very similar to the corresponding spectra of intact ATPase. After 4 days when the protein eluting with a K_d of 0.44 had lost 50% of its activity, the CD spectrum of both fragmented and intact ATPase resembled that of the trailing protein peak as is shown by the dotted curve in Figure 4.

Essentially the same results were obtained when the column described in Figure 3 was equilibrated and eluted with buffer containing 0.04 mg/mL $C_{12}E_8$. As far as could be determined, there were no differences in the behavior of intact and fragmented ATPase when solubilized in Tween 80 or in $C_{12}E_8$.

Highly Delipidated ATPase. An active preparation of ATPase containing less than 6 mol of phospholipid/mol of ATPase monomer, dissolved in $C_{12}E_8$, has been described by Dean & Tanford (1978). This preparation is better suited for detecting small amounts of dissociated fragments because there is no longer a large population of detergent-phospholipid mixed micelles to interfere with the analysis.

Fragmented ATPase vesicles with a specific activity of 12–15 μmol of ATP per min per mg were delipidated with DOC by the method of Dean & Tanford (1977) yielding a preparation with a specific activity of approximately 10 μmol of ATP per min per mg (comparable to that of intact ATPase) when assayed at 37 °C. Since the bulk of the phospholipid had been removed, most of the $C_{12}E_8$ micelles used to solubilize the delipidated ATPase contained little or no phospholipid. Using the $C_{12}E_8$ aggregation number of 120 determined by Tanford et al. (1977), and the cmc, 9×10^{-5} M, determined by Becher (1967), the initial solubilizing concentration of the detergent could be expressed as the number of micelles per fragmented ATPase monomer.

The solubilized ATPase was fractionated according to size by gel filtration chromatography. As observed by Dean & Tanford (1977) for the intact ATPase, low micelle/monomer mol ratios favored large oligomeric forms of the enzyme which were active, while high micelle/monomer mol ratios favored dissociation to monomer and dimer forms of the enzyme which were inactive. No differences could be observed by circular dichroism between fragmented and intact ATPase solubilized under similar conditions and sedimentation equilibrium experiments performed on fragmented ATPase solubilized at a micelle/monomer mol ratio of 12 did not detect the presence of dissociated fragments.

Ideally, the association of fragments I and II should be studied under conditions where the intact enzyme remains active, but a molar excess of $C_{12}E_8$ micelles irreversibly inactivates the enzyme. When 20% glycerol is included in the buffer solution, however, the intact enzyme remains fully active for several days at room temperature (Dean & Tanford, 1977). In the present study, fragmented or intact ATPase was delipidated and solubilized with a $C_{12}E_8$ concentration of 3 micelles per ATPase monomer and eluted from the column at room temperature. Identical elution profiles were obtained for fragmented and intact ATPase, the former presented in Figure 5. The protein eluted as a symmetrical peak with a K_d of 0.68. The concentration of fragmented monomer units was 9.4 ×

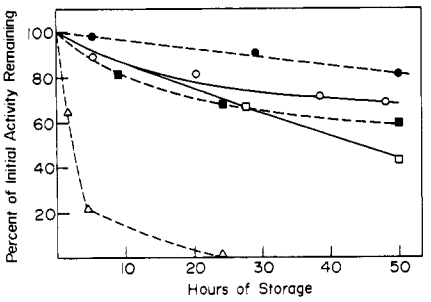

FIGURE 6: Stability of highly delipidated ATPase in 20% glycerol and $C_{12}E_8$. Samples were stored in 0.01 M Tes buffer, pH 7.5, 0.1 M KCl, 20% (v/v) glycerol and 1.0 mg/mL $C_{12}E_8$ at room temperature, and assayed at 37 °C. Fragmented (○) and intact (●) ATPase stored at 1.0 mg of protein/mL. Fragmented (□) and intact (■) ATPase stored at 0.15 mg of protein/mL. Intact ATPase (△) stored at 0.16 mg of protein/mL in the absence of glycerol. The assay medium included 1.0 mg/mL of $C_{12}E_8$, 1 μg/mL of protein, and no glycerol. The data for intact ATPase were taken from Dean & Tanford (1977).

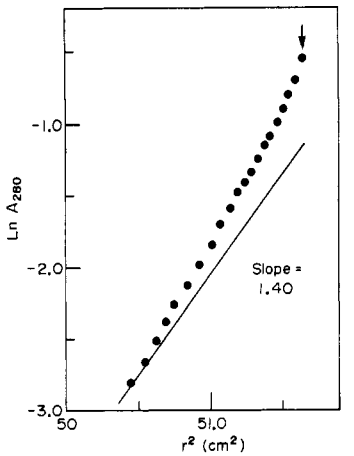

FIGURE 7: Sedimentation equilibrium of highly delipidated, fragmented ATPase in 20% glycerol and $C_{12}E_8$. Sample from the protein peak of the column described in Figure 5 was spun at 14 000 rpm at room temperature. The initial protein concentration was 0.18 mg/mL and when equilibrium was reached, the meniscus was depleted. A line with the slope (1.40) expected for fragmented monomer is included for reference; dissociated fragments would have a slope of 0.70.

10^{-7} M, compared with 1.5×10^{-5} M, the micellar concentration of the column buffer. Although a number of columns were run with fragmented or intact ATPase, the elution profile never gave any evidence of the formation of dissociated fragments or the separation of the small polypeptide from the ATPase. The phospholipid binding was 1–3 mol/mol of fragmented monomer, while the $C_{12}E_8$ binding was approximately 1.0 g/g of protein in agreement with Dean & Tanford (1978).

The ATPase was active in contrast to the protein eluting with a similar K_d when glycerol was absent from the eluent. The assays were performed 26 hours after the initial solubilization in $C_{12}E_8$ when the specific activity was 11 μmol of ATP per min per mg. The stability of the enzyme in this buffer at room temperature is shown in Figure 6. The solid lines represent the intact ATPase, while the dashed lines represent the fragmented ATPase. The enzyme was stored at two protein concentrations represented by circles and squares. The fragmented ATPase appeared to be somewhat less stable than the intact ATPase. As would be expected from their similar specific activities and stabilities, the circular dichroism spectra of the fragmented and intact ATPase agreed within experimental error and were similar to that of the active, partially delipidated ATPase in Tween 80.

The sedimentation behavior of the fragmented ATPase from the peak fraction of the column described in Figure 5 was studied. There is the possibility of preferential incorporation of water or glycerol into the sedimenting particle in these experiments. Dean & Tanford (1978) have shown that ignoring this binding would lead to a possible error of ±7% in the molecular weight determination. The solvent density, ρ, was determined by densimetry to be 1.060 g/cm^3.

Depleting the meniscus at 14 000 rpm resulted in a curved semilogarithmic plot of the data with a molecular weight of approximately 130 000 ± 11 500 at the top of the cell (Figure 7). The standard deviation reflects the combined error in δ_D and in the preferential binding of water. The observed minimal weight was close to the weight of the ATPase polypeptide. The expected molecular weight of fragmented monomer might be higher than 119 000, the intact ATPase polypeptide molecular weight (Rizzolo et al., 1976), because the small polypeptide (which was resolved from the ATPase by the bile salt detergents) could be present in the complex. Molecular weights of 350 000 were measured at the bottom of the cell. At 9000 rpm the semilogarithmic plot of the data was linear for most of the cell and yielded a molecular weight of 130 000 similar to the result obtained by Dean & Tanford (1978) for the intact ATPase. These results indicate that the fragments are not dissociated at low protein concentrations in the presence of an excess of micelles, and that the ATPase aggregates at high protein concentration.

Clearly there is a strong affinity of fragment I for fragment II. The association site may or may not be buried in the hydrophobic interior of the membrane or detergent micelle. If the association site is located in the exterior aqueous medium the fragments might be bound to each other by general electrostatic interaction or linked together via divalent cation bridges. Fragmented ATPase was eluted from columns similar to those described in Figure 5 except that the buffer contained 1.0 M KCl to minimize ionic interactions between the fragments. The protein eluted with a K_d of 0.61, indicating the enzyme was aggregated. This was confirmed by sedimentation equilibrium. When 1.0 M KCl, 20 mM EDTA, and 20 mM EGTA were included in the buffer, the protein was again aggregated, eluting with a K_d of 0.58. In a second series of experiments fragmented ATPase was prepared at low ionic strength in the absence of chelators on the column described in Figure 5. The KCl concentration was then adjusted to various levels between 0.1 M and 1.0 M, with and without chelating agents. The samples were incubated for 1 or 24 h and then analyzed by sedimentation equilibrium at high rotor speeds so as to deplete the meniscus. The results obtained under these conditions were similar to the result obtained at low ionic strength in the presence of calcium, showing electrostatic interactions alone cannot account for the high affinity of fragment I for fragment II.

Discussion

The Ca^{2+}-ATPase polypeptide can be split in half by trypsin without affecting its ability to pump Ca^{2+} across a membrane against a concentration gradient at the expense of ATP (Inesi & Scales, 1974; Stewart & MacLennan, 1974). Several other proteins contain peptide bonds that are especially labile to proteolytic enzymes. In many of the examples known so far, these bonds are located in an amino acid sequence which links structurally and functionally independent domains. Each domain has a distinct function which is unaffected by cleavage of the linkage sequence. Cytochrome b_5 reductase (Spatz & Strittmatter, 1973) and cytochrome b_5 (Spatz & Strittmatter,

1971; Visser et al., 1975) contain a water-soluble, globular domain which possesses the enzyme activity and a hydrophobic domain which anchors the enzyme to the membrane. The trypsin labile bond is located in the strand of amino acids joining the two domains. Myosin contains an insoluble, filamentous domain which self-associates to form long fibers. A globular domain, possessing the enzyme activity is linked to the fiber via a tightly coiled, rod-like arm. These three domains are joined by flexible hinge regions which are labile to limited proteolysis (Lowey et al., 1969). The immunoglobulins contain three globular domains linked by papain susceptible hinges to form a Y-shaped structure (Porter, 1959; Noelken et al., 1965).

Unlike these examples, the ATPase fragments are not structurally independent of each other. While it has been possible to dissociate ATPase oligomers and virtually completely delipidate the enzyme without affecting activity, it has not been possible to dissociate fragments I and II, without inactivating the enzyme. When the fragments are dissociated in cholate or DOC, they do not retain the same conformation that they had in the parent molecule.

There is evidence that the ATPase is present in an aggregated form in the sarcoplasmic reticulum (Packer et al., 1974; Malan et al., 1975; le Maire et al., 1976a; Vanderkooi et al., 1977), and Dean & Tanford (1978) provide evidence that the ATPase can reversibly associate in $C_{12}E_8$. The forces of association, however, are weak and the aggregates are easily disrupted. By contrast, the association between fragments I and II is very strong and cannot be disrupted by a tenfold excess of $C_{12}E_8$ micelles. Warren et al. (1974) and Dean & Tanford (1978) demonstrated the ATPase has a strong affinity for phospholipid; yet most of this lipid may be dissociated from the enzyme by procedures which do not dissociate the fragments.

The nature of the noncovalent forces holding the fragments together is not well understood. Thorley-Lawson & Green (1975) established there are no interfragment disulfide bonds linking fragments I and II, and it is evident from the results of this study that electrostatic interactions do not play a major role. There is no way of telling whether the attractive forces between the fragments lie within the membrane or in the externally exposed parts of the protein, but it is clear that both halves of the polypeptide chain mutually interact to form the folded structure of the ATPase. This is in contrast to the protease labile proteins cited earlier in which different parts of the molecule folded independently of the other.

Two other membrane proteins, rhodopsin (Pober & Stryer, 1975) and band 3 from erythrocyte ghosts (Jenkins & Tanner, 1977), are similar to the ATPase in this regard. Rhodopsin, the visual receptor protein of retinal rods of vertebrates, can be split by thermolysin into two membrane embedded fragments without affecting the bleaching-regeneration cycle, and there is some evidence that the rhodopsin fragments remain associated. Band 3, involved in anion transport, can be cleaved from one side of the membrane by trypsin and from the other side by thermolysin. Two of the fragments formed are membrane embedded, but no study has been made of the affect of the cleavage on anion transport or of the association of the fragments.

Finally, these results are interesting in a general way because they provide a striking illustration of the generally benign influence of nonionic detergents on the noncovalent interactions that maintain the native conformation of membrane proteins. Fragmented and intact ATPase prepared under similar conditions in the presence of $C_{12}E_8$ or Tween 80 displayed similar specific activities, stabilities, and CD spectra. Nor was the ability of the enzyme to reversibly associate impaired when the fragmented ATPase was solubilized in nonionic detergent.

On the other hand, bile salts clearly are less benign for the ATPase. Fragments are separated in bile salt solution and enzyme activity is lost. A similar result has been obtained for the glycoprotein spike complex of the Semliki Forest Virus membrane (Helenius et al., 1978). DOC dissociates this complex into its constituent glycoproteins (without denaturing the individual proteins), whereas the nonionic detergent Triton X-100 solubilizes the complex in an oligomeric form that is almost certainly the form in which the spikes exist in the membrane.

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Low pH Dimerization of Chymotrypsin in Solution†

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ABSTRACT: The mechanism of the acid dimerization of α-chymotrypsin in solution was reexamined using a number of chemical derivatives. Blocking of the carboxyl of Tyr-146, while that of Asp-64 remained free, eliminated completely the ability of α-chymotrypsin to dimerize, as did methylation of His-57. O-Acetylation of Tyr-146 reduced the dimerization constant to that of γ-chymotrypsin, and deacetylation did not reverse it to the value of α-chymotrypsin. Acetylation and deacetylation of the other accessible tyrosines did not affect the dimerization. It is concluded that the mechanism proposed by Aune and Timasheff [Aune, K. C., and Timasheff, S. N. (1971) *Biochemistry 10,* 1609–1617] for the solution dimerization which involves the electrostatic interaction between the His-57 imidazolium ring and the terminal carboxyl of Tyr-146 is still most consistent with all the experimental observations. The interactions in dilute solution may differ somewhat from those observed in crystals. In particular, the two intermolecular bridges formed by sulfate ions in crystals cannot be present in solution.

The self-association in solution of chymotrypsin has been broadly investigated over the past 20 years. Below pH 5, α-chymotrypsin has been shown to exist as a monomer–dimer system in equilibrium, with a pH optimum of 4.1–4.3 (Egan et al., 1957; Timasheff, 1969; Aune and Timasheff, 1971; Aune et al., 1971; Horbett and Teller, 1973, 1974; Neet and Brydon, 1970). A thermodynamic analysis of the pH profile of this dimerization, together with an examination of the intersubunit contacts in the crystallographic dimer (Sigler et al., 1968; Birktoft et al., 1969), led Aune and Timasheff (1971) to conclude that the pH dependence was determined most probably by the formation of a pair of salt bridges between ionizable groups on the protein surface, the most likely groups being the imidazolium of histidine-57 and the carboxylate of tyrosine-146.

More recent, refined analyses of the contacts between ionizable groups in the dimer in the crystal state (Birktoft and Blow, 1972) have revealed the presence of other contacts and have questioned the validity of the interactions identified by Aune and Timasheff (1971) in the solution dimer. In a very detailed and elegant analysis of the crystal structure, Vandlen and Tulinski (1973) have found that, in the crystal, the contact in the dimer interface region across dyad A between Tyr-146 of one molecule and His-57 of the other molecule is either by the formation of a salt bridge between the carboxylate of Tyr-146 and the imidazolium ring of His-57 or by hydrogen bonding between the protonated carboxyl of Tyr-146 and the carbonyl of His-57, the second mode of interaction being more likely below pH 6. Another major interaction in the crystal state is the formation of an ion pair between the carboxylate of Asp-64 of one molecule and the α-amino of Ala-149 of the other molecule, a second pair being possibly formed in symmetrical fashion. In the crystal, an important source of dimer stabilization is afforded by two identical bridges formed by sulfate ions, each bridge being between the phenolic hydroxyl of Tyr-146 of one molecule and the hydroxyl of Ser-195 and the imino of Gly-193 of the other molecule (Vandlen and Tulinski, 1973). In solution, addition of sulfate ions weakens the dimer formation (Aune et al., 1971), which is contrary to what should be expected if a sulfate ion bridge were involved in this interaction. In view of these apparent contradictions between the crystal and solution results, further solution studies were undertaken using derivatives of chymotrypsin which affect the residues in question, and the results are reported in this paper.

Materials and Methods

Materials. α-Chymotrypsin, three times crystallized (lots OLC, CDI, 6084-5, 6102-3, 8JA, 8LK, 36J835, and 345888),

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