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Properties of Deoxycholate Solubilized Sarcoplasmic Reticulum Ca^{2+}-ATPase†

Marc le Maire, Karl E. Jørgensen, H. Røigaard-Petersen, and Jesper V. Møller*,‡

ABSTRACT: The Ca^{2+}-dependent ATPase of sarcoplasmic reticulum after solubilization with deoxycholate and removal of lipid by gel chromatography exists as a mixture of monomer, dimer, and smaller amounts of higher molecular weight aggregates. The binding capacity of deoxycholate by monomeric and oligomeric forms of the ATPase is 0.3 g/g of protein at pH 8 and ionic strength 0.11. Examination in the analytical ultracentrifuge results in estimates of protein molecular weight of monomer of 115 000 ± 7000 and of Stokes radius of 50–55 Å. The results indicate an asymmetric shape of both delipidated monomer and dimer. Solubilization of ATPase vesicles by deoxycholate at high protein dilutions leads to almost instantaneous loss of ATPase activity. However, ATPase may be solubilized by deoxycholate in presence of phospholipid and sucrose in a temporarily active state. Inactivation appears to be accompanied by delipidation and conformational changes of the protein as evidenced by circular dichroism measurements. Sedimentation velocity examination of enzymatically active preparations of soluble ATPase in presence of phospholipid and sucrose strongly suggests that the major part of enzymatic activity is derived from a monomer with an asymmetric shape. The extent of formation of soluble oligomers by column chromatography was dependent on the exact conditions used for initial solubilization of ATPase. No evidence for differences among monomer and dimer fractions was obtained by isoelectric focusing and amino acid analysis. The results of these studies are compatible with electron-microscopic studies by other authors which suggest that the ATPase has an elongated shape with limited hydrophobic contact with the membrane lipid. A resemblance of delipidated oligomers with the form in which ATPase occurs in the membrane is conjectural at present.

The Ca^{2+}-dependent ATPase of sarcoplasmic reticulum is characterized by tight association with membrane lipids. Studies aiming at a physicochemical characterization of the ATPase are therefore hampered by difficulties in obtaining native ATPase in a molecularly dispersed state. For this reason, detergents have to be used which solubilize the ATPase by forming micellar-like complexes with the protein. In a previous paper we have reported on the properties of detergent-solubilized ATPase, using retention of enzymatic activity as a criterion of a native-like conformation (le Maire et al., 1976). It was found that nonionic detergents under appropriate conditions could maintain the solubilized protein in an enzymatically active state. Those conditions involved initial treatment of vesicles of the ATPase with an effective solubilizer such as dodecyl octaoxyethylene glycol monoether, followed by removal of excess detergent and membrane lipid by gel chromatography in which the eluent contained a relatively low concentration of nonionic detergent. By contrast, long-term exposure of ATPase vesicles to deoxycholate, even at nonsolubilizing concentrations, was found to lead to enzymatic inactivation. From this point of view deoxycholate is a less satisfactory detergent for characterization of ATPase in the solubilized state. However, there is ample evidence in the literature showing that it is possible to reconstitute Ca^{2+}-transporting vesicles under carefully controlled conditions after solubilization with deoxycholate (Meissner and Fleischer, 1974; Warren et al., 1974a).

Thus deoxycholate appears to retain another characteristic function of the protein, viz., the capability of forming an organized structure with membrane lipids. It was therefore considered worthwhile to continue our preliminary characterization studies of ATPase solubilized by deoxycholate (le Maire et al., 1976). The work proceeded in three stages. First, we examined the properties of delipidated and enzymatically inactive enzyme in order to determine such factors as molecular weight, Stokes radius, and the aggregational state. In the second stage, we explored conditions for solubilization of dilute ATPase in an enzymatically active form. It was found that inactivation of the solubilized ATPase could be slowed down by addition of phospholipid and sucrose to the deoxycholate

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solubilization medium. Finally the physicochemical properties of enzyme solubilized in this manner were compared with those of the delipidated, but inactive, enzyme. A main finding was the demonstration that a deoxycholate solubilized monomer of ATPase temporarily could be maintained in an enzymatically active form.

Experimental Section

Materials. Sarcoplasmic reticulum vesicles were prepared by zonal centrifugation from rabbit skeletal muscle and extracted twice with a low concentration of deoxycholate to remove proteins other than the Ca^{2+}-ATPase from the membrane as described by Meissner et al. (1973). The deoxycholate-extracted membrane preparation is subsequently referred to as ATPase vesicles. The Ca^{2+}-independent ATPase activity of the preparation was around 2% of that measured in presence of 0.1 mM Ca^{2+}.

Deoxycholic acid was purchased from Sigma Chemical Co., St. Louis, Miss., and purified as previously described (le Maire et al., 1976). [*carboxyl*-^{14}C]Deoxycholic acid and iodo[1-^{14}C]acetamide were obtained from the Radiochemical Centre, Amersham, England. Egg yolk phosphatidylcholine was obtained from Serdary Research Laboratories, Ontario, Canada. [U-^{14}C]Phosphatidylcholine (algae) from New England Nuclear Corp., Dreieichenhain, West Germany, was used as a tracer of the phospholipids of sarcoplasmic reticulum in gel chromatographic experiments. Concomitant chemical determination of organic phosphate (Bartlett, 1959) and radioactivity established that the elution profile of labeled phospholipid was identical with that of phospholipid originating from the ATPase vesicles.

Gel Chromatography. Columns (1.5 × 90 cm) of Sepharose 6B were used for preparation of solubilized ATPase. Unless otherwise noted, the procedure was as follows: An aliquot of ATPase vesicles (ca. 1 ml containing 6–10 mg of protein and 10–14 μmol of dithiothreitol suspended in 1 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (pH 7.5) and 0.3 M sucrose) was clarified by addition of a stock solution of deoxycholate (0.205 M) at a detergent/protein ratio of 1.5:1. The stock solution of deoxycholate contained in addition 0.01 M *N*-tris(hydroxymethyl)methylaminopropanesulfonic acid (pH 8.0), 0.1 M KCl, and 1 mM EDTA.[1]

The eluent ordinarily contained 5 mM deoxycholate which was dissolved in either of the two following electrolyte media: (1) 0.01 M *N*-tris(hydroxymethyl)methylaminopropanesulfonic acid (pH 8.0), 0.1 M KCl, 1 mM EDTA, and 1 mM azide. This medium has an ionic strength of 0.11. (2) 0.03 M Tris-HCl (pH 8.3), 1 mM EDTA, and 1 mM azide. This medium has an ionic strength of 0.02.

Binding Experiments. Binding of [^{14}C]deoxycholate of a known specific activity by solubilized ATPase below the cmc of the detergent was estimated by equilibrium dialysis using a Lucite cell in which two 1-ml compartments were separated by Visking dialysis tubing. Binding of deoxycholate to ATPase above the cmc of the detergent was determined by gel equilibrium chromatography on Sepharose 6B according to Hummel and Dryer (1962).

Analytical Ultracentrifugation. A Beckman Model E analytical ultracentrifuge, equipped with photoelectric scanner, was used. Protein molecular weights, M_p, were determined in sedimentation equilibrium experiments from plots of ln C (light absorption of solution of 280 nm) vs. r^2 (distance from center of rotation) according to the equation given by Tanford et al. (1974)

$$M_p(1 - \phi'\rho) = M_p(1 - \bar{\nu}_p) + M_p\delta_{DOC}(1 - \bar{\nu}_{DOC}\rho) \quad (1)$$

where $(1 - \phi'\rho)$ is the factor to be employed for conversion of the buoyant weight of the protein-deoxycholate particle to protein molecular weight; $\bar{\nu}_p$ and $\bar{\nu}_{DOC}$ are the specific volume of protein and bound detergent, respectively; δ_{DOC} is the amount of bound deoxycholate, expressed on a gram per gram basis. Specific volumes of ATPase and protein bound deoxycholate were calculated as previously described (le Maire et al., 1976).

The sedimentation rate of deoxycholate-solubilized ATPase was measured at 45 000 rpm in an An-D rotor with a double sector cell. The Stokes radius of deoxycholate ATPase particles was calculated from $s_{20,w}$ and $M_p(1 - \phi'\rho)$ according to

$$R_s = \frac{M_p(1 - \phi'\rho_{20,w})}{6\pi N\eta_{20,w}s_{20,w}}$$

where N is Avogadro's number and $\eta_{20,w}$ is the viscosity of water at 20 °C.

Enzyme Activity. The effect of phospholipid and sucrose on ATP hydrolysis in presence of deoxycholate was measured by procedures previously described (le Maire et al., 1976). Egg yolk phosphatidylcholine, dissolved in chloroform, was evaporated under a stream of N_2 in the test tube to be used for the assay and dissolved by shaking with a 5 mM deoxycholate assay medium on a vortex mixer. ATPase vesicles were usually added to the media at a final concentration of 0.005–0.075 mg/ml, and phosphate liberated from ATP was measured in the supernatant by the method of Fiske and SubbaRow (1925).

Reduction and Alkylation of ATPase. A sample of ATPase vesicles (1.2 ml of 7 mg of protein/ml), solubilized by deoxycholate as described in connection with the gel column experiments, was treated with 20 μmol of dithiothreitol for 2 h under N_2. Then 0.1 ml of a 0.78 M solution of iodoacetamide (twice recrystallized) containing iodo[1-^{14}C]acetamide was added and the sample was left for 30 min at pH 8 in the dark before application to the gel column. Alkylation of ATPase without reduction was carried out in the same way except that treatment with dithiothreitol was omitted.

Other Methods. Protein concentrations of ATPase were measured by the method of Lowry et al. (1951) with correction for unequal color intensity of ATPase and the bovine serum albumin standards as previously described (le Maire et al., 1976). Radioactivity was measured by liquid scintillation counting on a Packard instrument, Model 3314, using the dioxane-based scintillator medium of Bray (1960). Dodecyl sulfate gel electrophoresis was carried out by the method of Weber and Osborn (1969), with and without reducing agent (β-mercaptoethanol) present in the medium used for formation of dodecyl sulfate-protein complex. Isoelectric focusing of detergent-solubilized ATPase was done on 5% polyacrylamide gels containing 1.5% Ampholine (pH 3.5–10). Circular dichroism measurements were carried out on a Cary 60 spectropolarimeter with CD attachment. The cmc of deoxycholate was estimated on the basis of the change in the light absorption of methyl orange accompanying the uptake of the dye by deoxycholate micelles (Benzonana, 1969).

Results

Studies on Delipidated ATPase. A typical chromatogram of deoxycholate-solubilized ATPase is presented in Figure 1A.

[1] Abbreviations used: CD, circular dichroism; EDTA, ethylenediaminetetraacetic acid; Tris, Tris(hydroxymethyl)aminomethane; cmc, critical micelle concentration; DOC, deoxycholate.

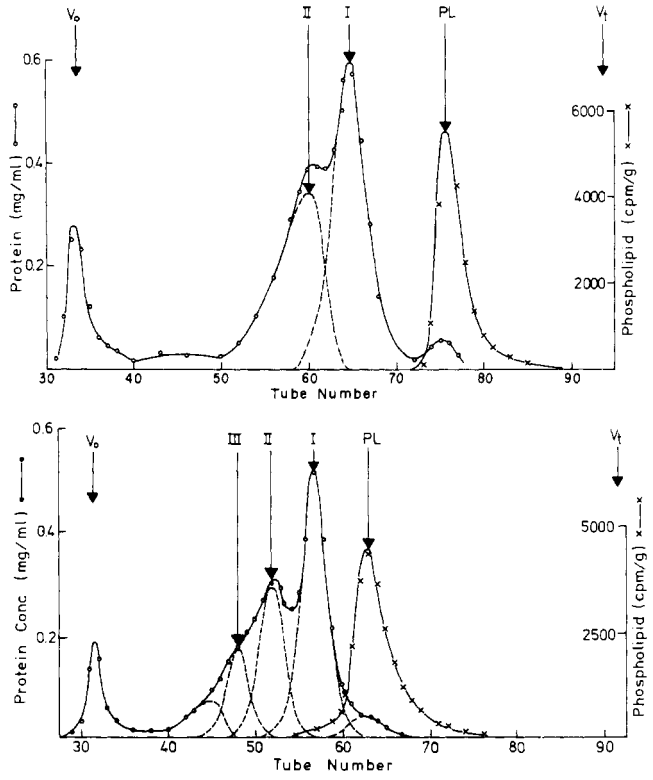

FIGURE 1: (A) Gel chromatography of deoxycholate solubilized ATPase. Vesicles of ATPase (1 ml of 8 mg of protein/ml), suspended in 1 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (pH 7.5), 0.015 M dithiothreitol, and 0.3 M sucrose, were solubilized by addition of 0.205 M deoxycholate to a detergent/protein ratio of 1.5:1. Solubilization at 20 °C took place in a test tube containing a trace of [U-^{14}C]phosphatidylcholine. The resultant mixture was applied to a Sepharose 6B column (1.5 × 90 cm) 5 min later and eluted in presence of 5 mM deoxycholate. The 5mM deoxycholate eluent medium in addition to deoxycholate contained 0.01 M *N*-tris(hydroxymethyl)methylaminopropanesulfonic acid (pH 8.0), 0.1 M KCl, 1 mM EDTA, and 1 mM azide. The arrows designate the following: V_0, void volume; I and II, peaks I and II of soluble ATPase; PL, phospholipid; V_t, total volume. (B) Gel chromatography of deoxycholate soluble ATPase. The experiment was carried out in the same way as the experiment shown in A, except that the eluent contained 5 mM deoxycholate, 0.03 M Tris-HCl (pH 8.3), 1 mM EDTA, and 1 mM azide. The broken lines represent attempted resolution of the elution profiles of soluble ATPase into constituent components.

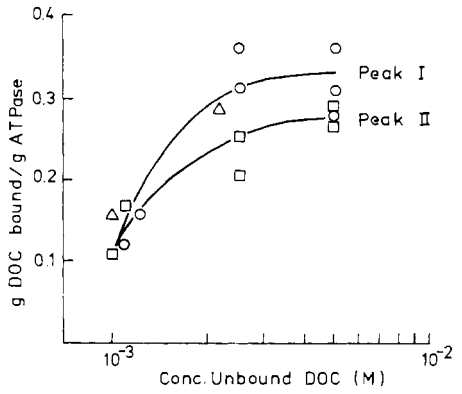

FIGURE 2: Binding of deoxycholate by delipidated monomer and dimer fractions at μ = 0.11 and 20 °C. The points below cmc of deoxycholate (ca. 3.5 mM) were obtained by equilibrium dialysis, while binding at 5 mM unbound deoxycholate was measured by gel chromatography. (○) Monomer fraction; (□) dimer fraction; (△) mixture of monomer and dimer fraction.

A suspension of ATPase vesicles was clarified by addition of a stock solution of deoxycholate, layered onto a Sepharose 6B column, and eluted with 5 mM deoxycholate dissolved in an electrolyte medium of ionic strength 0.11 (for details, see legend to Figure 1A). The main part of the protein emerges between the void and total volume as two incompletely separated peaks with partition coefficients of 0.47 and 0.42. These peaks are labeled I and II on the figure. Phospholipid is eluted as a separate peak, indicating that the ATPase is phospholipid free. A small peak of Lowry-positive material, exhibiting a maximal absorbance of light at 280 nm, is associated with the phospholipid in the elution pattern. There is also some Lowry-positive material present in the void volume, but no phospholipid, due to the presence of highly aggregated ATPase. The chromatogram resembles that previously reported with Sepharose 4B (le Maire et al., 1976), but the separation of the individual peaks is improved, indicating that the porosity of Sepharose 6B allows a better discrimination between the different components of deoxycholate-solubilized ATPase vesicles. Also, it can be seen from Figure 1A that the leading edge of the supposed dimer peak is trailing, suggesting the existence of small amounts of larger size aggregates of solubilized ATPase.

Figure 1B shows the elution pattern in an experiment with a low ionic strength (μ = 0.02), but otherwise performed in the same way as that of Figure 1A. At least four components of solubilized ATPase are discernible in this case as indicated by the broken lines. The phospholipid is not completely separated from protein, and a small amount appears to be attached to the main peak of solubilized ATPase. In comparison with Figure 1A all peaks elute with lower partition coefficients. This may be due to electrostatic repulsions originating from negatively charged groups attached to the gel which may affect the distribution to a much higher extent at μ = 0.02 than at higher ionic strengths (Andrews, 1970).

Binding of deoxycholate to solubilized ATPase at μ = 0.11 is shown in Figure 2. A plateau value of bound deoxycholate of about 0.3 g per g of protein is observed at unbound detergent concentrations above 2.5 mM. Fraction I material appears to bind slightly higher amounts of deoxycholate than fraction II material. There is a steep increase of bound deoxycholate at 1–2 mM unbound detergent, suggesting the existence of cooperative forces in the binding process. As can be seen from Table I binding of deoxycholate to solubilized ATPase is lower at μ = 0.02. This finding corresponds to observations on binding of deoxycholate by cytochrome b_5 which also shows a decrease in binding capacity by a lowering of ionic strength (Robinson and Tanford, 1975). This phenomenon is probably related to the lesser tendency of deoxycholate to self-associate at low ionic strength (Small, 1971), it being assumed that the ATPase forms a "nucleus" for formation of micelle-like deoxycholate around part of the protein as already suggested for the interaction of this detergent with cytochrome b_5 (Robinson and Tanford, 1975).

Studies of the various fractions of solubilized ATPase in the analytical ultracentrifuge showed that sedimentation equilibrium plots were linear at μ = 0.02, while they were often slightly curved at μ = 0.11, in which case initial slopes were used for calculation of the buoyant weight, $M_p(1 - \phi'\rho)$, of the protein-deoxycholate particles. The buoyant weight was independent of the initial protein concentration in the cell over a range of 0.1–0.3 mg/ml and of rotor speed (range 7200–15 000 rpm), arguing against the existence of reversible protein aggregation in the samples. Sedimentation equilibrium studies were usually performed at a detergent concentration of 5 mM deoxycholate, but a few measurements at 10 mM detergent

TABLE I: Molecular Weights and Stokes Radii of Different Fractions of DOC Solubilized and Delipidated ATPase.

Sample[a]	Ionic Strength	$M_p(1-\phi'\rho)$[b]	DOC Binding (g/g of protein)	Mol Wt[c]	$s_{20,w}$ (10^{-13} s)	R_s[d] (Å)	f/f_{min}[e]
I	0.11	37 600 ± 2100(22)	0.32	115 000	6.5	50	1.41
I	0.02	34 900 ± 2600(10)	0.20	116 000	5.5	55	1.60
II	0.11	70 700 ± 9800(12)	0.28	222 000	9.3	66	1.51
II	0.02	69 700 ± 7800(6)	0.18	235 000	8.5	71	1.64
III	0.02	127 500 ± 21 000(4)	0.18	430 000			

[a] Peak samples of fractions as defined in Figure 1. [b] Average values ± SD with number of determinations in parentheses. Measurements were performed over a range of initial protein concentrations of 0.1–0.3 mg/ml and rotor speeds 7200–15 000 rpm. [c] Calculated according to eq 1. [d] Mean of three determinations. Initial protein concentration of 0.15–0.25 mg/ml. R_s calculated according to eq 2. [e] Frictional ratios calculated as described by Tanford et al. (1974).

concentration gave identical buoyant particle weights. Therefore the calculated buoyant particle weights are probably not affected by changes in the deoxycholate concentration throughout the sample column as a result of sedimentation of deoxycholate micelles. A summary of protein molecular weights of the different fractions of solubilized ATPase as calculated from the sedimentation equilibrium results are presented in Table I. The protein molecular weight of fraction I is 115 000, corresponding to protein in monomer form. The molecular weight of fraction II corresponds to a dimer of ATPase. The aggregation state of fraction III material is attended by some uncertainty; it may correspond to a tetramer or a trimer of ATPase, or possibly to a mixture of both forms. The sedimentation of the small protein fraction in association with the lipid peak was examined on one occasion. The buoyant particle weight was 3400 indicating a maximal molecular weight around 13 000 (assuming no binding of deoxycholate and a buoyancy factor of 0.74). This material may be of a similar nature as the proteolipid of sarcoplasmic vesicles described by MacLennan et al. (1972).

Table I also provides a summary of $s_{20,w}$ and Stokes radii of monomer and dimer ATPase. Depending on the composition of the electrolyte medium, Stokes radius of the monomer varies from 50 to 55 Å. This corresponds to a frictional ratio (f/f_{min}) of 1.4–1.6 which is a higher value than that of a typical globular protein. Accordingly the dimensions of the delipidated monomer appear to be large in relation to the molecular weight. The table also shows that dimer ATPase is characterized by a similar degree of asymmetry.

Studies on Deoxycholate Solubilized and Enzymatically Active ATPase. The delipidated ATPase prepared by deoxycholate column chromatography is enzymatically inactive, and activity could not be restored by addition of phospholipid in accordance with the observations of Hardwicke and Green (1974). However, other investigators have reported retention of enzymatic activity by deoxycholate-solubilized ATPase at high protein concentrations (e.g., Martonosi, 1968; MacLennan, 1970) and in the presence of sucrose (Selinger et al., 1969). Also it has been found that Ca^{2+}-transporting vesicles can be reconstituted under these conditions by gradual removal of deoxycholate (Meissner and Fleischer, 1974). It is not apparent from these reports to what extent the lipid associated with the sarcoplasmic reticulum has been effective in protecting the ATPase against the inactivating effect of deoxycholate, although it may be surmised from the lipid-exchange experiments of Warren et al. (1974a,b) that lipid remains bound to reactivatable and reconstitutable ATPase. Furthermore, it should be noted that in the previous reports the deoxycholate solubilized samples were added to an enzymatic assay medium which did not contain detergent so that ATPase–phospholipid bilayers presumably were reformed. It is therefore not known whether activity was reversibly lost in the solubilized state. Since the analytical ultracentrifuge characterization of ATPase of the present paper requires dilute solutions of the protein, we examined conditions for maintaining activity of ATPase solubilized by excess detergent.

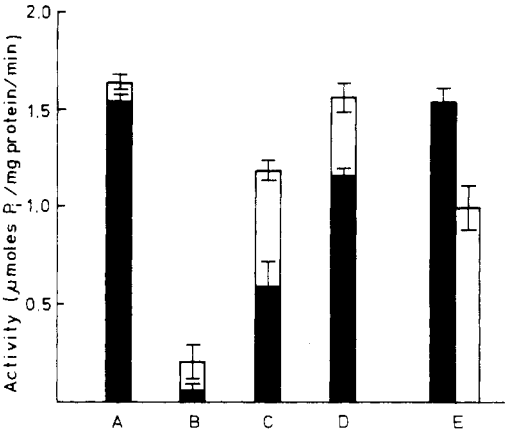

FIGURE 3: Initial ATPase activity in presence of deoxycholate, phospholipid, and sucrose. The media in all experiments contained 7.5 mM Mg-ATP, 0.1 mM Ca^{2+}, 0.01 M *N*-tris(hydroxymethyl)methylaminopropanesulfonic acid (pH 8.0), and 0.1 M KCl, with additions of deoxycholate, phosphatidylcholine, and sucrose as mentioned below. ATPase vesicles were added at zero time to a final concentration of 0.1 mg/ml, and the reaction which proceeded at 20 °C was stopped after 10 min by addition of perchloric acid. The unfilled columns refer to media containing 0.3 M sucrose, while dark columns are results obtained in absence of sucrose. The letters designate the following: (A) no further addition to medium than mentioned above; (B) addition of 5 mM deoxycholate; (C) 5 mM deoxycholate and 0.5 mM phosphatidylcholine; (D) 5 mM deoxycholate and 1 mM phosphatidylcholine; (E) 5 mM deoxycholate and 2 mM phosphatidylcholine. The results are presented as means ± SD of four experiments.

Figure 3 shows the effect of phosphatidylcholine and 0.3 M sucrose on ATPase activity immediately after solubilization of a small amount of ATPase vesicles in 5 mM deoxycholate. It can be seen that there is virtually no enzymatic activity when ATPase vesicles are solubilized by 5 mM deoxycholate alone, while a low level of activity is retained by inclusion of 0.3 M sucrose in the medium in accordance with the observations of Selinger et al. (1969). Appreciable breakdown of ATP occurs in presence of phospholipid, and the activity attains the same level as in the absence of deoxycholate at a 1–2 mM concentration of phospholipid. Sucrose enhances ATPase activity, except at 2 mM phospholipid where more activity is observed in the absence of sucrose. It should be noted that all activity

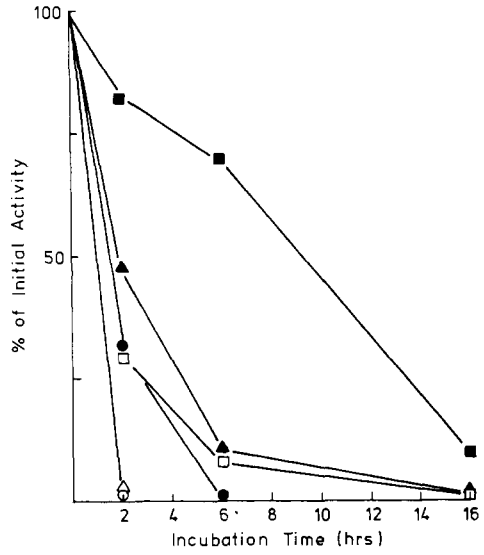

FIGURE 4: Decline of ATPase activity as function of time in presence of deoxycholate, phosphatidylcholine, and sucrose. ATPase vesicles were added to the different media at a final concentration of 0.1 mg/ml and assayed for activity after 0, 2, 6, and 16 h of incubation by addition of Mg-ATP (temperature 20 °C, reaction period 10 min). The media contained 5 mM deoxycholate, 0.1 mM Ca^{2+}, 0.01 M *N*-tris(hydroxymethyl)methylaminopropanesulfonic acid (pH 8.0), and 0.1 M KCl, with the following additions: (○, ●) 0.5 mM phosphatidylcholine; (△, ▲) 1 mM phosphatidylcholine; (□, ■) 2 mM phosphatidylcholine. Filled symbols refer to presence of 0.3 M sucrose in the media. Mean values of four experiments are shown.

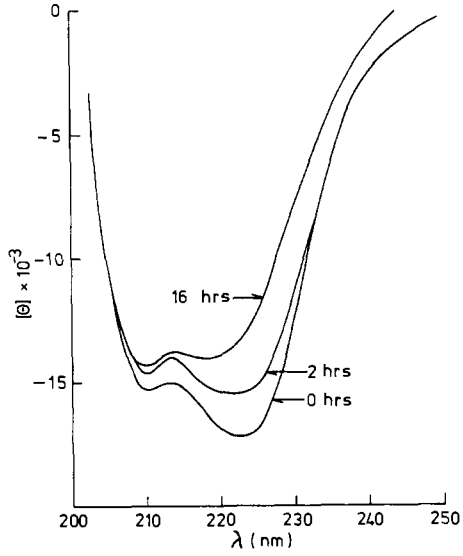

FIGURE 5: Circular dichroism spectra of ATPase vesicles at different times after solubilization in 5 mM deoxycholate, 1 mM phosphatidylcholine, and 0.3 M sucrose. (Ordinate) Molar ellipticity (deg cm^2 $dmol^{-1}$). (Abscissa) Wavelength.

measurements were performed in the detergent medium used for solubilization, i.e., the possibility of reversible inactivation of ATPase in the solubilized state is ruled out.

The effect of time on ATPase activity in various mixtures of deoxycholate, phospholipid, and sucrose is shown in Figure 4. It should be noted that 100% activity refers to the initial activity as shown in Figure 3, regardless of the different activity levels observed under various conditions. It is seen that activity decays in the presence of deoxycholate in all cases and is reduced to negligible levels after 6 h of incubation at 20 °C, except at the highest phospholipid concentration (2 mM) and 0.3 M sucrose. The presence of sucrose retards the inactivating effect of deoxycholate on ATPase activity. Thus approximately 50% activity remains after 2 h of incubation in 1 mM phospholipid and 0.3 M sucrose, while almost complete inactivation occurs at the same level of phospholipid in absence of sucrose.

The sedimentation velocity behavior of samples of enzymatically active ATPase treated with 5 mM deoxycholate, different concentrations of phospholipid, and 0.3 M sucrose was examined in the analytical ultracentrifuge. At a phospholipid concentration of 2 mM, which was optimal for retaining activity, the material was immediately pelleted indicating the existence of highly aggregated ATPase. At 1 mM phospholipid and 0.3 M sucrose, part of the material (representing approximately 20% of the protein) sedimented quickly, but the height of the plateau region in the ultraviolet scan remained constant during the subsequent sedimentation for 60 min at 45 000 rpm. The sedimentation rate of the slowly moving boundary immediately suggested that the solubilized ATPase predominantly was in a monomeric form: A value of $s_{20,w} = 4.7$ was obtained which is somewhat lower than that of delipidated monomer (Table I). Repetition of the scan after storing of the rotor with contents at room temperature for 23–24 h resulted in a small increase of $s_{20,w}$ of solubilized ATPase to 5.1 S. No evidence of a more quickly sedimenting component that might represent a dimer was discernible in the scans. Simulation experiments in which delipidated monomer and dimer were mixed in various proportions indicated that we should have been able to detect a dimer content of about 20% in the preparation. This result was confirmed by a sedimentation equilibrium study which indicated that the major part of solubilized ATPase was present as a monomer under these conditions.

The existence of some fast-moving material in presence of 1 mM phospholipid and 0.3 M sucrose in deoxycholate-solubilized ATPase vesicles raises the question if the enzymatic activity of such solutions is solely associated with highly aggregated material. In order to study this possibility, we examined enzymatic activity and sedimentation behavior after removal of insoluble material by centrifugation for 20 min at 220 000*g*. The supernatant of the preparative ultracentrifugation step exhibited a specific activity of 80% of that of the noncentrifuged control. Examination in the analytical ultracentrifuge confirmed that insoluble material had been completely removed by the preparative ultracentrifugation step, and that the boundary of solubilized ATPase moved with a sedimentation rate similar to that observed previously (4.7 S). The experiments establish that solubilized protein in presence of phospholipid and sucrose retains enzymatic activity, although presumably to a somewhat lesser extent than aggregated material.

CD spectra of ATPase vesicles dissolved in 5 mM deoxycholate with or without phospholipid and sucrose were recorded initially and at different times after solubilization. Figure 5 shows that progressive changes in the spectra occur with time in presence of 1 mM phospholipid and 0.3 M sucrose. The curve recorded initially is characterized by a high negative ellipticity in the 200–250-nm region with a pronounced double-trough feature. After 16 h ellipticity is somewhat reduced, especially of the band exhibiting a minimum near 222 nm. Intermediate changes were observed after 2 h which correspond to about 50% decrease of enzymatic activity. In all cases the CD spectra at different phospholipid concentrations (0, 0.5, and 1 mM phospholipid) did not differ after inactivation, but the minima in 0.3 M sucrose were somewhat more pronounced,

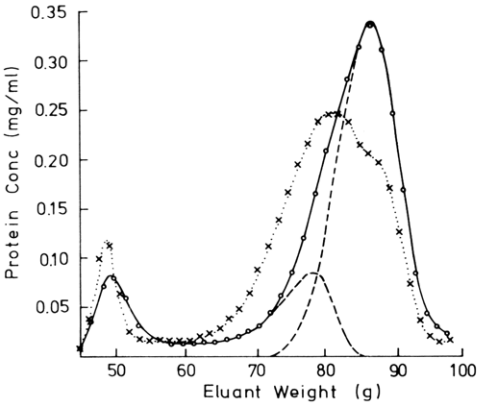

FIGURE 6: Elution profiles of ATPase solubilized by deoxycholate under different conditions and chromatographed on Sepharose 6B in presence of 5 mM deoxycholate. (X . . . X) Chromatogram of ATPase (7.5 mg of protein) obtained by solubilization of ATPase vesicles with 0.205 M deoxycholate in presence of dithiothreitol and chromatographed in 5 mM deoxycholate at $\mu = 0.11$ as described in the legend of Figure 1A. (●—●) ATPase vesicles (7.5 mg) solubilized by addition of 100 ml of 2.5 mM deoxycholate and 0.015 M dithiothreitol and concentrated to 2 ml by ultrafiltration on Amicon Dia-flo XM 100A filter, before application to the column. (- - -) Resolution of the latter curve into monomer and oligomer fractions.

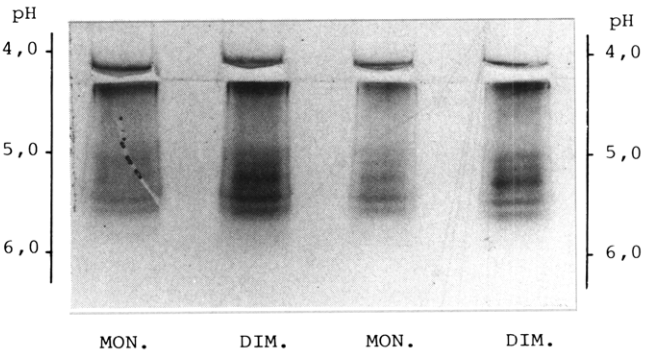

FIGURE 7: Isoelectric focusing of chromatographically prepared ATPase. Monomer and dimer fractions prepared by column chromatography as described in the legend of Figure 1A were concentrated by XM 100A Dia-flo ultrafiltration. Deoxycholate was removed by repeated dialysis after addition of Triton X-100 (0.1%) to the concentrated sample and to the dialysate. Isoelectric focusing was performed in a 5% polyacrylamide gel containing 0.1% Triton X-100 and 0.5% Ampholine (pH 3.5–10) at 440 V for 2.5 h.

both in enzymatically active solutions and after inactivation. Thus the mean value of the 222-nm band was −12 900 in absence of sucrose and −14 500 deg cm dmol^{-1} in 0.3 M sucrose after inactivation. The changes occurring as a function of time appeared to be correlated with inactivation of the enzyme in all cases. The CD spectra after inactivation were indistinguishable from that of delipidated monomer ATPase, prepared by gel chromatography.

Dimer Content in Different Preparations of Solubilized ATPase. The elution pattern of deoxycholate solubilized ATPase shown in Figure 1 is typical of that obtained with most preparations of ATPase vesicles. However, during the course of this investigation some preparations were observed to give rise to a dominance of dimer ATPase when the preparation was carried out according to the procedure described in Figure 1A (see the elution pattern represented by the dotted line in Figure 6). It should be noted that this procedure entails that the initial solubilization of ATPase vesicles with deoxycholate is performed at a high protein concentration (6–10 mg/ml). When the same preparation of ATPase vesicles was solubilized at a low protein concentration (0.2 mg/ml) in a large volume of deoxycholate solution and thereafter concentrated to a small volume suitable for application to the column, the major part eluted as a monomer as indicated by the unbroken line of Figure 6. This result is of interest in connection with the sedimentation experiments on enzymatically active, solubilized ATPase described above. Those studies which were performed at low protein concentrations did not give evidence for the presence of substantial amounts of dimer either. A possible explanation for the different proportion of dimer produced by the two procedures is considered under Discussion. At present we note that in the previous study (le Maire et al., 1976) rechromatography of delipidated monomer and dimer fractions of solubilized ATPase gave rise to essentially symmetrical peaks with no evidence of interconversion of the components during passage of the Sepharose column. The delipidated forms of ATPase, once being formed, thus are in a rather stable state.

Occurrence of Intermolecular Disulfide in ATPase. The stability of delipidated dimer ATPase raises the question if it is held together by covalent bonds such as might be formed by oxidation of cysteinyl residues. It was previously shown that the elution of dimer ATPase was unchanged after treatment with dithiothreitol and iodoacetamide (le Maire et al., 1976). Additional dodecyl sulfate gel electrophoresis experiments showed that the monomer fraction of deoxycholate chromatographed ATPase moved as a single band whether or not a reducing agent (β-mercaptoethanol) was added to the protein–dodecyl sulfate complex applied to the gel. The dimer fraction behaved essentially as the monomeric one, except that in the absence of β-mercaptoethanol a faint, more slowly moving band was observed. This observation is consistent with only a minor part of the dimer existing as an intermolecular disulfide.

The number of reactive -SH groups of deoxycholate chromatographed ATPase was estimated by incorporation of radioactivity in the protein after treatment with ^{14}C-labeled iodoacetamide (see Experimental Section). According to these measurements, the delipidated monomer contains 17.3 ± 0.5 and the delipidated dimer 17.8 ± 0.8 reactive -SH groups (averages of six peak fractions of two column experiments ± SD). After pretreatment of the deoxycholate soluble ATPase with dithiothreitol the corresponding values were 18.9 ± 0.5 and 18.5 ± 0.3 for the delipidated monomer and dimer, respectively. The results confirm that at most a fraction of dimer ATPase could exist as intermolecular disulfide. According to previously published amino acid analyses of the ATPase (MacLennan et al., 1971; Meissner et al., 1973) and our own measurements, there are a total about 25 cysteine residues in the protein. Thus a large number of the cysteine residues are in a reduced state in the protein.

Heterogeneity of ATPase. The observation that the dimer content of delipidated ATPase, prepared by the usual column chromatographic procedure, differed markedly among various preparations led us to consider that the basis of the variation could be the existence of ATPase with differences in the peptide structure. This possibility was examined by isoelectric focusing on an Ampholine–polyacrylamide gel in presence of Triton X-100. Difficulties were encountered as a result of precipitation of the protein–Triton X-100 complex in presence of Ampholine, but with the setup described in the legend of Figure 7 the major part of the protein entered the gel. Monomer and dimer fractions consistently gave rise to at least five different bands, suggesting heterogeneity of ATPase, at around pH 5.0–5.7. However, there was no discernible difference

among the monomer and dimer fractions, except that a somewhat larger proportion of dimer did not enter the gel. Amino acid analysis of deoxycholate-solubilized ATPase gave results which agreed with those previously published by MacLennan et al. (1971) and Meissner et al. (1973). No difference could be detected between monomer and dimer fractions in this way either.

Discussion

The results obtained in the present study indicate that solubilized ATPase prepared by gel chromatography in presence of deoxycholate elutes as a mixture of monomer, dimer, and higher molecular weight oligomers. This finding corresponds to our observations on the elution of ATPase in presence of nonionic detergents which also gives rise to solubilized ATPase in different aggregation states (le Maire et al., 1976). However, solubilization of ATPase vesicles with deoxycholate invariably leads to delipidation and inactivation by long-term exposure in contrast to the nonionic detergents where activity may be retained by ATPase lipoprotein in the solubilized state for a considerable time (le Maire et al., 1976).

Molecular weights of the ATPase peptide ranging from 90 000 to 115 000 have commonly been estimated by dodecyl sulfate gel electrophoresis (McFarland and Inesi, 1971; MacLennan et al., 1972; Louis and Shooter, 1972; Meissner et al., 1973; Thorley-Lawson and Green, 1973). In the present study a protein molecular weight of monomer ATPase of 115 000 was obtained by sedimentation equilibrium determinations which agrees with the value of 119 000 ± 6500 obtained by similar measurements on ATPase solubilized by dodecyl sulfate (Rizzolo et al., 1976). The Stokes radius of the delipidated monomer as determined by sedimentation velocity and sedimentation equilibrium measurements according to eq 2 is 50–55 Å which corresponds to a frictional ratio, f/f_{min}, of 1.4–1.6. A value of f/f_{min} exceeding 1.24 is generally considered as an upper limit for a typical globular protein, including the effect of bound water (Tanford et al., 1974). Our results would be compatible with a description of the protein–deoxycholate monomer particle as a prolate ellipsoid with a ratio of the long and short axes of 6–10:1, but does not of course exclude other asymmetric shapes of the molecule. It is of interest in this connection that Hardwicke and Green (1974) have observed an elongated shape by electron microscopic observations on negatively stained preparations of deoxycholate solubilized and delipidated ATPase. It may be argued that the comparison of frictional ratios of deoxycholate ATPase with those of water soluble globular proteins may not be strictly correct in case that the deoxycholate moiety is much more hydrated than that of the protein. However, at least 1.6 g of H_2O would have to be bound per g of deoxycholate if the large Stokes radius is to be compatible with a close to spherical shape of the protein.

Binding of deoxycholate to soluble ATPase appears to attain a plateau value of 0.3 g per g of protein above a concentration of unbound detergent of 5 mM at an ionic strength of 0.11. This level is considerably smaller than that observed for binding of deoxycholate to cytochrome b_5 (Robinson and Tanford, 1975). The results of the latter study indicate that the hydrophobic portion of cytochrome b_5, which is anchored to the membrane lipid, combines with about 2.5 g of deoxycholate per g of protein. To the extent that cytochrome b_5 can be taken as a representative of the interaction of the hydrophobic part of a membrane protein with deoxycholate, it can be concluded that only a limited part of the ATPase probably is involved in hydrophobic interactions with membrane lipid.

The delipidated preparations of deoxycholate-solubilized ATPase, prepared by gel chromatography, were enzymatically inactive, but we were able to preserve enzyme activity for a limited time period if phospholipid and sucrose were added to the deoxycholate solution used for solubilization of the protein. These findings are consistent with previous observations by other authors in which soluble ATPase, prepared by the action of deoxycholate on concentrated suspensions of sarcoplasmic reticulum, were found to retain enzymatic activity for some time in presence of 0.3 M sucrose (Selinger et al., 1969) and to be capable of forming Ca^{2+}-transporting vesicles by gradual removal of deoxycholate (Meissner and Fleischer, 1974). Presumably the sarcoplasmic reticulum lipid has afforded protection against the inactivating effect of deoxycholate in these latter experiments since we found that dilute solutions of ATPase in deoxycholate have very low enzymatic activities.

By sedimentation velocity and equilibrium studies on dilute solutions of ATPase, prepared by direct solubilization in 5 mM deoxycholate and removal of insoluble residues by preparative ultracentrifugation, it was only possible to demonstrate small amounts of dimer or other oligomers of the protein. We estimate that the maximum level of oligomers that might be present would amount to around 20%. Therefore the enzymatic activity exhibited by soluble ATPase in presence of phospholipid and sucrose is most probably referable to monomer ATPase in the solution. An initial specific activity of about 1 was obtained for soluble ATPase. If the enzymatic activity of the soluble ATPase had been associated exclusively with, e.g., 20% oligomer, then the specific activity of the oligomer would have to be around 5. However, this is a much higher value than that attained by ATPase vesicles at the same pH, temperature, and Ca^{2+} concentration (Figure 3), and therefore monomers probably participate in enzymatic hydrolysis of ATP.

The sedimentation coefficient of the monomer in enzymatically active preparations of ATPase was somewhat lower than that of delipidated ATPase monomer. Thus the monomers derived from enzymatically active solutions also are characterized by an asymmetric shape. The progressive inactivation of ATPase with time was accompanied by a small increase in the sedimentation rate of the protein suggesting a less asymmetric shape in the enzymatically inactive state. It was possible to obtain definite evidence for conformational changes of the protein accompanying inactivation by CD experiments. These changes were characterized particularly by a reduction in the ellipticity of the band which exhibits a minimum at around 222 nm. The CD changes are probably indicative of a decrease of α-helix content accompanying inactivation. Because of the similarity of the CD spectrum of the inactivated preparations with that of chromatographically prepared ATPase, it appears reasonable to suggest that inactivation is also accompanied by delipidation. In agreement with this view, Warren et al. (1974b) have observed that ATPase activity is reduced to zero when the phospholipid content of deoxycholate-treated ATPase is reduced from 30 to 15 mol of phospholipid per mol of protein.

A major question arising in connection with the results of the present paper is if the deoxycholate-solubilized monomer ATPase has a structural resemblance with the form in which it occurs in the membrane. It is therefore of interest to compare our results with those obtained by other investigators by microscopic techniques. The existence of protuberances on the sarcoplasmic reticulum facing the cytoplasmic side has been known for some time (Deamer and Baskin, 1969; Ikemoto et al., 1971; Greaser et al., 1969). Freeze-fracture data indicate a preponderance of structures on the side of the membrane

facing the cytoplasm (Packer et al., 1974; Inesi and Scales, 1974). These results suggest that the ATPase has an elongated shape and that, in accordance with the deoxycholate binding data, only part of the molecule interacts hydrophobically with lipids. However, recent freeze-fracture studies suggest that ATPase may be present in the membrane as oligomeric aggregates (Malan et al., 1975). The tendency for chromatographically prepared ATPase in presence of nonionic detergents (le Maire et al., 1976) and deoxycholate to exist as oligomers may be taken as support for this view. However, it should be noted that, in the case of deoxycholate, soluble oligomers of ATPase present some puzzling features which make it necessary to exercise caution in the interpretation of this result. Firstly we have been unable to demonstrate interconversion of monomeric and dimeric species by rechromatography (le Maire et al., 1976) or by analytical ultracentrifugation. Dodecyl sulfate gel electrophoresis of the dimeric fraction in absence of reducing agent was consistent with the presence of only small amounts of disulfide-linked ATPase in the dimer fraction. The possibility that the stability of aggregates of deoxycholate-soluble ATPase might be caused by heterogeneity in the ATPase was not substantiated by isoelectric focusing experiments. Although several bands of ATPase were observed with an apparent isoelectric point around 5, the appearance of the monomer and dimer fraction was essentially alike. Finally, it was evident that the formation of soluble oligomers was very dependent on the procedures used for solubilization of ATPase vesicles. By solubilization of a dilute suspension of ATPase vesicles in deoxycholate, a much smaller extent of oligomer formation was noted than by solubilization of a concentrated suspension with the detergent (Figure 6). The mechanism of oligomer formation is under further study, but at the present stage the following may be proposed as a working hypothesis: In the initial period after solubilization ATPase exists as a mixture of monomer, dimer, and other oligomers of ATPase. The system is in a state of dynamic equilibrium (apart from any covalently linked ATPase) so that the proportion of each species depends on total protein concentration. Subsequently inactivation occurs, accompanied by gradual removal of lipid. The inactivated forms of ATPase are present as "frozen" conformations of the protein that are not readily interconvertible. This sequence of events would account for the formation of oligomers by solubilization with deoxycholate at high protein concentrations, and, if correct, would be compatible with the presence of aggregated ATPase in the sarcoplasmic reticulum membrane.

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