The Functional Unit of Calcium-plus-Magnesium-Ion-Dependent Adenosine Triphosphatase from Sarcoplasmic Reticulum

THE AGGREGATIONAL STATE OF THE DEOXYCHOLATE-SOLUBILIZED PROTEIN IN AN ENZYMICALLY ACTIVE FORM

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Vesicles consisting of ($\text{Ca}^{2+} + \text{Mg}^{2+}$)-dependent ATPase (adenosine triphosphatase), and lipid were prepared from sarcoplasmic reticulum of rabbit skeletal muscle. As with non-ionic detergents [le Maire, Møller & Tanford (1976) *Biochemistry* 15, 2336–2342] the ($\text{Ca}^{2+} + \text{Mg}^{2+}$)-dependent ATPase after solubilization by deoxycholate showed a pronounced tendency to form oligomers in gel-chromatographic experiments, when eluted in the presence of deoxycholate and phosphatidylcholine. To evaluate the functional significance of oligomer formation the properties of enzymically active preparations of ATPase, solubilized by deoxycholate, were studied. Such preparations were obtained at a protein concentration of 2.5 mg/ml in the presence of a high salt concentration (0.4 M-KCl) and sucrose (0.3 M) in the solubilization medium. Analytical ultracentrifugation of solubilized ATPase showed one protein boundary moving at the same rate as gel-chromatographically prepared monomeric ATPase ($s_{20,w} = 6.0\text{S}$). From simultaneous measurements of the diffusion coefficient an apparent molecular weight of 133 000 was calculated, consistent with solubilization of ATPase in predominantly monomeric form. The enzymic activity of deoxycholate-solubilized ATPase when measured directly in the solubilization medium at optimal Ca^{2+} and MgATP concentrations was about 35–50% of that of vesicular ATPase. The dependence of enzymic activity on MgATP concentration indicated that the solubilized ATPase retained high-affinity binding of MgATP, but the presence of high concentrations of the nucleotide did not stimulate activity further, in contrast with that of vesicular ATPase. The dependence of enzymic activity on the free Ca^{2+} concentration was essentially the same for both solubilized and vesicular forms, indicating that interaction of ATPase with more than one molecule of Ca^{2+} is required for enzyme activity. Solubilized enzyme at 20°C was phosphorylated to about the same degree as vesicular ATPase. It is concluded that the catalytic activity of monomeric ATPase retains most of the features of vesicular ATPase and that extensive oligomer formation in gel-chromatographic experiments in the presence of deoxycholate probably reflects processes taking place during inactivation and delipidation of the protein.

Detergents are generally used for isolation and characterization of membrane proteins which interact hydrophobically with the membrane (often referred to as intrinsic membrane proteins). Such proteins together with membrane lipid constitute the building blocks of biological membranes, and some of these presumably are involved in membrane

Abbreviations used: ATPase, adenosine triphosphatase; Taps, 3-[[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]-amino]-1-propanesulphonic acid.

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transport. It is generally assumed that intrinsic membrane proteins are inserted in a membrane lipid bilayer, but there may be protein-protein interactions as well. For the ($\text{Ca}^{2+} + \text{Mg}^{2+}$)-dependent ATPase of the sarcoplasmic reticulum evidence on this point is emerging. Thus it has been shown that under appropriate conditions it is possible to solubilize the membrane-bound ATPase as a mixture of an oligomer (trimer or tetramer) and a monomer (le Maire *et al.*, 1976a). The oligomer retains Ca^{2+} -dependent ATP hydrolysis, whereas the monomer is

inactive, suggesting that protein-protein interactions are important in maintaining the transport protein in an enzymically active form. The significance of this finding is corroborated by electron-microscopic studies of the sarcoplasmic-reticulum membrane, which have shown about 3–4 times as many surface particles as intramembrane particles, as observed by freeze-fracture (Scales & Inesi, 1976a,b; Jilka *et al.*, 1975). Since the particles observed by electron microscopy of sarcoplasmic-reticulum membrane are attributable to the ATPase (Hardwicke & Green, 1974; Stewart & McLennan, 1974; Tillack *et al.*, 1974), it has been suggested that the large intramembrane particles represent association of monomers. Moreover, Murphy (1976) from cross-linking experiments has obtained evidence for the presence of tetrameric ATPase in the membrane.

It should be noted that, if deoxycholate rather than non-ionic detergents is used as a solubilizing agent, formation of a transiently active monomer of ATPase can be demonstrated if solubilization is carried out in the presence of phospholipid and sucrose (le Maire *et al.*, 1976b). It has also been found that Ca^{2+} -transporting vesicles may be reconstituted from similar solubilization media by removal of deoxycholate (Meissner & Fleischer, 1974; Repke *et al.*, 1976), raising the question whether a monomer of ATPase is indeed a functional unit in Ca^{2+} transport and associated ATP hydrolysis. Although our previous characterization of active ATPase (le Maire *et al.*, 1976b) was performed at a low protein concentration (0.2–0.3 mg/ml), reconstitution experiments after solubilization with deoxycholate have been carried out at a relatively high protein concentration (2.5–7 mg/ml). The question remains of whether enzymically active ATPase, solubilized by deoxycholate, undergoes association at the higher protein concentrations used for reconstitution studies before insertion in the membrane. Evidence for the existence of concentration-dependent association reactions was obtained in our previous paper (le Maire *et al.*, 1976b). Delipidated and inactive ATPase, prepared by solubilization in deoxycholate and subsequently gel chromatographed in the presence of this detergent, was eluted as a mixture of monomer, dimer and larger aggregates of ATPase. The extent of aggregate formation was increased by a high protein concentration during the initial solubilization of membrane-bound ATPase with deoxycholate. It was speculated that the inactive oligomers, which are eluted from the gel column, might reflect the presence of aggregated forms of enzymically active ATPase in the medium used for initial solubilization. The present paper describes experiments in which we have further characterized deoxycholate-solubilized ATPase, paying attention to properties at high protein concentrations.

Methods

Preparation of $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -dependent ATPase from sarcoplasmic reticulum

Sarcoplasmic reticulum was prepared from a homogenate of rabbit skeletal muscle, and proteins other than the $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -dependent ATPase were removed by extraction with a low concentration of deoxycholate as described by Meissner *et al.* (1973). The phospholipid content of the preparation was estimated to be 0.45 mg/mg of protein, and $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -dependent ATP hydrolysis accounted for more than 98% of total ATPase activity. In most experiments the membrane-bound enzyme was solubilized by addition of deoxycholate (final concn. 2.8 mg/ml) to the ATPase vesicles (final concn. 3.5 mg of protein/ml), and non-solubilized ATPase was removed by centrifugation at 200 000g for 30 min. The electrolyte and sucrose composition of the media used for solubilization is described in the legends of the Figures

Analytical ultracentrifuge experiments

A Beckman Model E analytical ultracentrifuge, equipped with a photoelectric scanner, was used for characterization of deoxycholate-solubilized ATPase. Sedimentation rates were determined at 44 000 rev./min and 20°C in a double-sector cell in an An-D rotor. Owing to the high protein concentrations, the scans were performed at 294 nm rather than at 280 nm. The diffusion coefficient of solubilized protein was estimated in a synthetic-boundary double-capillary cell after layering of solvent on top of the deoxycholate-solubilized sample after the start of the centrifugation. The spreading of the protein boundary was followed as a function of time, usually at a rotor speed of 6000 rev./min. The diffusion coefficient, D , was calculated on the basis of the expression for diffusion across an initially sharp boundary for an infinitely long fluid column:

$$C = \frac{C_0}{2} \left[1 - \frac{2}{\pi^{\frac{1}{2}}} \int_0^{\frac{x}{2\sqrt{Dt}}} e^{-y^2} dy \right] \quad (1)$$

where C_0 is the initial protein concentration in the sample, and C is the concentration of protein as a function of time t and distance x from the initially sharp boundary; y is an independent variable,

integration being carried out from $y = 0$ to $y = \frac{x}{2\sqrt{Dt}}$.

The apparent molecular weight of deoxycholate-solubilized ATPase (including bound deoxycholate and lipid), M , was calculated from the Svedberg equation:

$$M = \frac{RTs}{D(1 - \bar{v}\rho)} \quad (2)$$

where $\bar{v}$ is the partial specific volume of the solubilized particle, ρ is solution density and s is the sedimentation coefficient. Eqn. (3) was used for estimation of $\bar{v}$ and protein molecular weight, M_p :

$$M(1 - \bar{v}\rho) = M_p[(1 - \bar{v}_p\rho) + \delta_{\text{DOC}}(1 - \bar{v}_{\text{DOC}}\rho) + \delta_{\text{lip}}(1 - \bar{v}_{\text{lip}}\rho)] \quad (3)$$

where $\bar{v}_p$, $\bar{v}_{\text{DOC}}$ and $\bar{v}_{\text{lip}}$ are the partial specific volumes of the protein, deoxycholate and lipid moieties of the particle, and δ_{DOC} and δ_{lip} the amounts of bound deoxycholate and lipid, expressed on a weight basis. The following values were used for partial specific volumes: $\bar{v}_p = 0.74 \text{ cm}^3/\text{g}$ (le Maire *et al.*, 1976a), $\bar{v}_{\text{DOC}} = 0.778 \text{ cm}^3/\text{g}$ (Tanford *et al.*, 1974) and $\bar{v}_{\text{lip}} = 0.975 \text{ cm}^3/\text{g}$ (le Maire *et al.*, 1976a). Reasonable values of δ_{DOC} and δ_{lip} , obtained as discussed in the Results section, were inserted into eqn. (3) for estimation of M_p and $\bar{v}$. An experimental estimation of $\bar{v}$ was made on the basis of the decrease in the sedimentation coefficient in a mixture of $^2\text{H}_2\text{O}$ and H_2O (80% $^2\text{H}_2\text{O}$ by volume). The sedimentation coefficient in H_2O is given by:

$$s = \frac{M(1 - \bar{v}\rho)}{N6\pi\eta R_s} \quad (4a)$$

where N is Avogadro's number, η the viscosity of the solution, and R_s the Stokes radius of the sedimenting particle. The corresponding equation for the sedimentation in the mixture of $^2\text{H}_2\text{O}$ and H_2O may be represented by:

$$s' = \frac{M(1 - \bar{v}'\rho)}{N6\pi\eta' R_s} \quad (4b)$$

where the primed symbols refer to changes brought about by the addition of $^2\text{H}_2\text{O}$. From eqns. (4a) and (4b) one obtains:

$$\frac{s'}{s} = \frac{1 - \bar{v}'\rho}{1 - \bar{v}\rho} \cdot \frac{\eta}{\eta'} \quad (5)$$

which can be used for estimation of $\bar{v}$ after determination in H_2O and $^2\text{H}_2\text{O}/\text{H}_2\text{O}$ of the sedimentation coefficient of the sedimenting particle, and of the viscosity and density of the solutions. Eqn. (4b) ignores the small increase in molecular weight resulting from deuterium exchange of labile hydrogen in the particle (probably approx. 1%), and further presupposes that the hydrodynamic properties of the particle are not changed in the presence of $^2\text{H}_2\text{O}$, i.e. that R_s of eqns. (4a) and (4b) is the same.

Gel-chromatography experiments

The size heterogeneity and binding properties of deoxycholate-solubilized ATPase was examined in two experiments by gel chromatography. A sample of vesicular ATPase (1 ml containing 11 mg of protein) was solubilized by addition of 0.17 ml of

0.205 M-deoxycholate containing [*carboxyl- ^{14}C*]deoxycholate (The Radiochemical Centre, Amersham, Bucks., U.K.), added to a specific radioactivity of $0.027 \mu\text{Ci}/\mu\text{mol}$. Soon after solubilization (5 min) the sample was applied to a Sepharose 6B column ($1.5 \text{ cm} \times 85 \text{ cm}$), which had been equilibrated with 5 mM-deoxycholate, 1 mM-phosphatidylcholine, 0.1 mM- Ca^{2+} , 0.01 M-Taps (pH 8.0), 0.1 M-KCl and 1 mM- NaN_3 , [^{14}C]deoxycholate also being present at a specific radioactivity of $0.027 \mu\text{Ci}/\mu\text{mol}$. The column was eluted with medium of the same composition at a rate of 6 ml/h at 20°C . The fractions (1.4 ml) were analysed for protein, phospholipid and radioactivity for determination of binding of phospholipid and deoxycholate by ATPase by the method of Hummel & Dryer (1962).

Measurement of ATPase activity

ATP hydrolysis by deoxycholate-solubilized ATPase in a detergent-free incubation medium was measured by addition of 0.015 ml of deoxycholate-solubilized ATPase preparation (approx. 2.5 mg of protein/ml) to 1 ml of 5 mM-MgATP, 0.1 mM- Ca^{2+} , 0.01 M-imidazole (pH 7.5) and 0.1 M-KCl, thermostatically controlled at 20°C . The reaction was stopped after 10 min by addition of 0.5 ml of 1 M- HClO_4 , and liberated P_i was measured by the method of Fiske & SubbaRow (1925).

The effect of MgATP concentration on ATP hydrolysis by deoxycholate-solubilized ATPase (approx. 2.5 mg of protein/ml) or ATPase vesicles (3.5 mg/ml) was performed in the presence of an ATP-generating system. The ATPase preparations contained 0.1 mM- Ca^{2+} , 1 mM- Mg^{2+} , 5 mM-phosphoenolpyruvate (Boehringer, Mannheim, W. Germany), 0.01 M-Taps (pH 8.0), 0.4 M-KCl and 0.3 M-sucrose. The reaction was started by addition of $2 \mu\text{l}$ of 30 mg of pyruvate kinase/ml (Sigma Chemical Co., St. Louis, MO, U.S.A.; 530 units/mg) and $5 \mu\text{l}$ of various MgATP solutions to produce final concentrations of the nucleotide varying from 2 to $600 \mu\text{M}$ in the reaction mixture. The reaction was terminated after 0.5–1 min at 20°C by addition of 0.5 ml of 1 M- HClO_4 .

The effect of free Ca^{2+} concentration on enzyme activity of deoxycholate-solubilized ATPase (approx. 2.5 mg of protein/ml) and vesicular ATPase (3.5 mg of protein/ml) was performed in the presence of 0.2 mM-EGTA, 0.01 M-Taps (pH 8.0), 0.4 M-KCl and 0.3 M-sucrose. The reaction was started by addition of $5 \mu\text{l}$ containing 0.15 M-MgATP and various concentrations of added Ca^{2+} (0–0.187 mM) to $50 \mu\text{l}$ of ATPase preparation. Enzymic hydrolysis of ATP was stopped after $\frac{1}{2}$ –1 min by addition of 0.5 ml of 1 M- HClO_4 . Free concentrations of Ca^{2+} were calculated from stability constants for formation of complexes of these two cations with EGTA and ATP presented in Table 1 of Vianna (1975). The calcula-

tions took into account Ca^{2+} present in the ATPase preparation, which was $10 \pm 2 \text{ nmol/mg}$ of protein as determined by atomic-absorption spectrophotometry.

Phosphorylation

Incorporation of ^{32}P in protein was measured in the presence of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (10 Ci/mmol, The Radiochemical Centre, Amersham, Bucks., U.K.) at nucleotide and Ca^{2+} concentrations of 0.1 mM. The reaction was stopped after 6 s by addition of ice-cold 4% (w/v) trichloroacetic acid containing 0.5 mM-ATP and 1 mM-potassium phosphate. The denatured protein was filtered on to a Millipore filter (pore size $0.65 \mu\text{m}$) and washed thoroughly with the same trichloroacetic acid solution. Control samples were treated in exactly the same way, except that 1 mM-EGTA was substituted for 0.1 mM- Ca^{2+} in the incubation medium. Radioactivity after solubilization of the filters in 10 ml of Bray's (1960) mixture was measured by liquid-scintillation counting. Ca^{2+} -dependent phosphorylation was calculated after subtraction of radioactivity in the control filters.

Other methods

Protein was measured by the method of Lowry *et al.* (1951) as previously described (le Maire *et al.*, 1976b), and phospholipid by the method of Bartlett (1959). The viscosity of solutions used for solubiliza-

tion of ATPase was measured at $20 \pm 0.1^\circ\text{C}$ in an Ubbelohde viscometer equipped with photoelectric cells and an electronic timer for registration of flow time. The free Ca^{2+} concentration of $\text{Ca}^{2+}/\text{EGTA}$ media used for some of the enzyme assays (in the absence of Mg^{2+} and ATP) was estimated with a Ca^{2+} -sensitive electrode (Ca-Selectrode, Radiometer, Copenhagen, Denmark), with the Ca^{2+} buffers described by Ruzicka *et al.* (1973) as standards.

Results

Characterization of solubilized ATPase prepared by gel chromatography

Fig. 1 shows the elution profile of ATPase solubilized in deoxycholate and chromatographed on a column of Sepharose 6B equilibrated with 5 mM-deoxycholate and 1 mM-phosphatidylcholine. The elution pattern of solubilized ATPase indicates size heterogeneity and the chromatogram contains some non-solubilized ATPase eluted at the void volume. The solubilized ATPase binds around 60 mol of deoxycholate and 7 mol of phospholipid per mol of protein (mol.wt. 115000; le Maire *et al.*, 1976b). The broken lines labelled I, II and III in Fig. 1 indicate an attempted resolution of the elution profile of solubilized ATPase into Gaussian curves. Molecular weights and sedimentation coefficients determined on fractions corresponding to the peaks

Fig. 1. Gel chromatography of ATPase in the presence of deoxycholate and phosphatidylcholine. ATPase (11 mg/ml) was solubilized by deoxycholate (14 mg) as described in the Methods section and gel-chromatographed on Sepharose 6B in the presence of 5 mM-deoxycholate, containing $[\text{C}^{14}]\text{deoxycholate}$, 1 mM-phosphatidylcholine, 0.1 mM Ca^{2+} , 0.01 M-Taps (pH 8.0), 0.1 M-KCl and 1 mM- NaN_3 . The fractions (1.4 ml) were analysed for protein ($\circ$), phospholipid and radioactivity, and from these analyses binding of phospholipid and deoxycholate was estimated to be 0.044 and 0.21 g/g of solubilized ATPase respectively. The broken lines on the Figure indicate an attempted resolution of the elution profile into its constituent components. Peak fractions of the components labelled I, II and III were examined in the analytical ultracentrifuge by sedimentation equilibrium and sedimentation velocity. The molecular weights and sedimentation coefficients ($s_{20,w}$) obtained were: peak I: 136000, 5.3S; peak II: 218000, 8.7S; peak III: 525000, 14.3S. V_0 indicates void volume.

of these curves are given in the legend. The molecular weights obtained indicate that peaks I, II and III are probably derived from a monomer, dimer and tetramer of ATPase respectively. In comparison with gel-chromatography experiments performed in the presence of deoxycholate alone (le Maire *et al.*, 1976b), there is more tendency for oligomer formation. The eluted ATPase was found to be enzymically inactive. An elution profile similar to that of Fig. 1 was obtained in another experiment in which 0.3M-sucrose was added to the eluent. The sedimentation coefficients shown in Fig. 1 are low in relation to the molecular weight, probably indicating a rather asymmetric shape of the complexes. Thus the frictional ratio, $f/f_{min.}$, is around 1.7, whereas a value of around 1.25 is usually considered to be the upper limit for a typical globular protein (Tanford *et al.*, 1974).

Solubilization of ATPase by deoxycholate in an enzymically active form

To examine properties of enzymically active ATPase, solubilized by deoxycholate, it was necessary to find conditions for maintaining activity of the solubilized protein, at least for some hours. Fig. 2 shows the enzymic activity of vesicular ATPase solubilized by deoxycholate at pH8.0 and 20°C in various media at a deoxycholate/protein (w/w) ratio of 0.8. In the presence of 0.3M-sucrose specific activity is decreased to 50% in about 3h (curve a). A similar decay of ATPase activity is noted in the presence of 0.3M-sucrose and 0.1M-KCl (curve b). But when the medium contains 0.3M-sucrose and 0.4M-KCl (curve c) activity remains virtually constant for 4h, followed by a slow decline. When solubilized ATPase was stored in this medium at 0°C for 24h the decline in activity was only 10%. As shown in Fig. 2, removal of non-solubilized vesicular material before the enzymic assay did not appear to affect specific activity. The initial specific activity for the curves shown in Fig. 2 is the same as that of the original vesicular ATPase used for their preparation. It should be noted that in these experiments, activity is measured by addition of a small volume of the preparation to an incubation medium not containing deoxycholate, and this procedure results in formation of ATPase lipid complexes (see below). We are thus not measuring the activity of solubilized ATPase, but rather the results of Fig. 2 may be considered to indicate the time-dependent irreversible inactivation of ATPase by deoxycholate. As demonstrated below, the enzyme also hydrolyses ATP in the solubilized state. It must be assumed that maintenance of activity in these experiments is dependent on the presence of sarcoplasmic-reticulum lipid, which counteracts the inactivating effect of deoxycholate on enzyme function (Martonosi, 1968). In the previous

Fig. 2. Retention of activity of deoxycholate-solubilized ATPase in the presence of 0.3M-sucrose and different concentrations of KCl at 20°C

ATPase vesicles (3.5 mg/ml) were treated with deoxycholate (final concn. 2.8 mg/ml) in a medium containing 0.1M- Ca^{2+} , 0.01M-Taps (pH8.0), 0.3M-sucrose and different concentrations of KCl: (a) zero ($\Delta, \triangle$); (b) 0.1M ($\blacksquare, \square$); (c) 0.4M ($\bullet, \circ$). The samples were divided in two parts, one of which was centrifuged at 200000g for 30min to remove non-solubilized ATPase. Filled symbols refer to mean values of specific activities of solubilized ATPase in three experiments (centrifuged samples), and unfilled symbols refer to the corresponding activities of the non-centrifuged samples, measured in detergent-free incubation medium. The broken parts of curve (a), (b) and (c) represent an extrapolation of the data on solubilized ATPase to zero time, assuming that the decline in specific activity of the centrifuged samples equals that of the non-centrifuged ATPase.

study (le Maire *et al.*, 1976b), activity of deoxycholate-solubilized ATPase was maintained at a low protein concentration (0.2–0.3 mg/ml) in the presence of 0.3M-sucrose, 0.1M-KCl and phosphatidylcholine. Under these conditions inactivation followed a similar time course to that in Fig. 2 (curve b). Attempts to solubilize ATPase in a stable and active state at low protein concentrations in similar mixtures of deoxycholate and phosphatidylcholine, by increasing the KCl concentration to 0.4M, were unsuccessful. Therefore the molecular characterization of ATPase described below only reports results obtained on the deoxycholate-solubilized preparation in the presence of 0.3M-sucrose and 0.4M-KCl at a rather high protein concentration (approx. 2.5 mg/ml).

Molecular weight of enzymically active deoxycholate-solubilized ATPase

Fig. 3(a) shows sedimentation of solubilized vesicular ATPase in the analytical ultracentrifuge. One clear boundary with a minor slower-moving component is observed after centrifugation for 40 min at 44000 rev./min. The shape and sedimentation coefficient of the dominating part of the boundary corresponds to that of a monomer of ATPase, prepared by gel chromatography in the presence of deoxycholate, as depicted by Fig. 3(b), which is taken from an experiment in connection with the study of le Maire *et al.* (1976b). In a total of 11 sedimentation experiments, $s_{20,w}$ of the fast-moving boundary of solubilized vesicular material was determined to be 6.0 ± 0.3 S.

The slowly sedimenting component characteristic of the sedimentation pattern of deoxycholate-solubilized vesicles is presumably caused by the sedimentation of mixed micelles of lipid and deoxycholate, which exhibit a detectable light-scattering at the wavelength of the scan (294 nm). If we assume that the amount of bound deoxycholate is 0.2 g/g of protein (i.e. the same value as for ATPase prepared in gel-chromatographic experiments in the presence of phospholipid and deoxycholate), then the concentration of non-protein-bound deoxycholate in the

Fig. 3. Sedimentation of (a) enzymically active ATPase, solubilized by deoxycholate, and (b) delipidated monomer of deoxycholate-solubilized ATPase

(a) Soluble ATPase (approx. 2.5 mg/ml) was prepared by solubilization of ATPase vesicles (final concn. 3.5 mg/ml) in a medium containing 2.8 mg of deoxycholate/ml, 0.1 mM- Ca^{2+} , 0.01 M-Taps (pH 8.0), 0.4 M-KCl and 0.3 M-sucrose, with subsequent removal of non-solubilized ATPase by centrifugation at 200000 g for 30 min. Sedimentation was measured at 44000 rev./min in an An-D rotor at 20°C. (b) The monomer of ATPase was prepared by gel chromatography in the presence of deoxycholate as previously described (le Maire *et al.*, 1976a). Sedimentation was measured under the same conditions as in (a). r_m and r_b are distance of meniscus and bottom of cell from centre of rotation respectively.

sample is around 5–5.5 mM. Adding this concentration to the solvent side of the double sector cell does not affect the appearance of the scan, since deoxycholate does not absorb light at 294 nm. However, the slowly sedimenting boundary of Fig. 3(a) disappears after a further addition of 1–1.5 mM-phosphatidylcholine to the solvent. This concentration of phosphatidylcholine may indicate the concentration of lipid existing as mixed micelles with deoxycholate in the solubilized ATPase sample. On the basis of the phospholipid content of the sample (0.45 mg/mg of protein) and assuming 30 mol of phospholipid bound per mol of ATPase (Warren *et al.*, 1974a,b), a concentration of non-protein-bound phospholipid of 1.15 mM is calculated.

The estimation of non-protein-bound deoxycholate and phospholipid is a case for consideration in the experiments in which the diffusion of ATPase was measured after layering of solvent over the preparation at low rotor speeds (see the Methods section). The composition of the solvent in these experiments should be similar to that of the solubilized ATPase sample, apart from the protein component. Changing the phospholipid concentration of the solvent from 1.0 to 1.5 mM, and the deoxycholate concentration from 5 to 6 mM, did not affect the diffusion of the protein. Consistent values for the diffusion coefficient at 6000 rev./min were obtained at different times after formation of the boundary (Fig. 4). The diffusion coefficient ($D_{20,w}$) estimated from a total of seven experiments was $(3.55 \pm 0.25) \times 10^{-7} \text{ cm}^2/\text{s}$.

The partial specific volume ($\bar{v}$) of solubilized protein calculated from eqn. (3) on the basis of 0.2 g of deoxycholate and 0.2 g of lipid bound per g of protein is 0.779. It was found in five experiments that the sedimentation rate of solubilized ATPase in 80% $^2\text{H}_2\text{O}$ /20% H_2O in 0.3 M-sucrose/0.4 M-KCl was 57% of that obtained in 0.3 M-sucrose/0.4 M-KCl in H_2O . Allowing for the viscosity increase in presence of $^2\text{H}_2\text{O}$ ($\eta_{^2\text{H}_2\text{O}}/\eta_{\text{H}_2\text{O}} = 1.11$), this leads to a buoyancy of deoxycholate-solubilized ATPase of 0.773 (eqn. 5), in fair agreement with the estimated value. These experiments suggest that the estimate of bound lipid is reasonable. They do not, on the other hand, allow any conclusion to be made about the amount of bound deoxycholate, since the partial specific volume of deoxycholate (0.778) is not significantly different from that of the whole particle.

On the basis of the sedimentation and diffusion coefficients and the estimated values for bound deoxycholate and lipid, an apparent protein mol.wt. of 133000 for solubilized ATPase is calculated, which is to be compared with the previously determined value of 115000 for the monomer of ATPase prepared by gel chromatography (le Maire *et al.*, 1976b). The use of sedimentation and diffusion coefficients that are not extrapolated to zero protein concentration could lead to some under-

EXPLANATION OF PLATE I

Formation of ATPase-lipid complexes by dilution of deoxycholate-solubilized ATPase with a detergent-free electrolyte medium

(a) Solubilized ATPase was prepared as described in Fig. 3, and a small volume (0.015 ml) was added to 1 ml of a deoxycholate-free medium containing 0.1 mM- Ca^{2+} , 0.01 M-imidazole (pH 7.5) and 0.1 M-KCl. Samples of the resultant dilution were negatively stained with uranyl acetate. The samples were examined in an electron microscope (Siemens Elmiskop 101). Magnification $\times 120000$. (b) ATPase vesicles (0.5 mg of protein/ml) were negatively stained with uranyl acetate for comparison with (a). Magnification $\times 120000$.

estimation of molecular weight. If, on the other hand, the assumed amount of bound deoxycholate is too low, this would lead to an overestimate of molecular weight (higher values than 0.2 g of deoxycholate/g of protein were found for binding of deoxycholate to delipidated ATPase; (le Maire *et al.*, 1976b). We conclude that the solubilized sample contains ATPase in monomeric form, with perhaps some admixture of oligomer. Evidence in favour of the presence of some oligomer was obtained when diffusion was allowed to take place at a rotor speed of 20000 rev./min rather than 6000 rev./min. In this case the boundary became asymmetric after a long time interval, as shown by the filled circles of Fig. 4. This phenomenon is consonant with the presence of some faster-sedimenting material in the sample, which would add to the flattening of the protein profile at the higher speed. On the other hand, the resemblance of the sedimentation boundary for the monomer prepared by gel chromatography and the rather well-defined upper part of the boundary in the sedimentation-velocity experiments strong-

ly suggests that oligomeric protein only can be present in minor amounts.

Formation of ATPase-lipid complexes by removal of deoxycholate

Plate 1 shows the electron-microscopic appearance of a negatively stained preparation obtained by dilution of solubilized ATPase with a deoxycholate-free solution as used in the activity experiments of Fig. 2. Apart from a few vesicular structures, most of the preparation is characterized by the presence of compact rod-shaped particles. The width of the particles is 12–16 nm, but the length is variable. Some preliminary characterization of such preparations was made after removal of deoxycholate by gel chromatography on sepharose 2B. Most of the protein and phospholipid (approx. 90%) entered the gel and was eluted as a broad peak with K_D 0.55. The peak only contained particles which in the analytical ultracentrifuge moved with a rate corresponding to $s_{20,w}$ = approx. 30S. We conclude that the particles consist of complexes of several ATPase molecules and lipid.

Enzymic properties of deoxycholate-solubilized ATPase

The object of these experiments was to characterize enzymic activity of ATPase in the deoxycholate-solubilized state. Therefore measurements were performed by addition of minimal volumes of substrates to the solubilized preparations, to avoid formation of ATPase-lipid complexes. For comparative purposes ATP hydrolysis by the preparation in the vesicular state (no addition of deoxycholate) was simultaneously measured.

Fig. 5 shows a Lineweaver-Burk plot of the dependence of ATP hydrolysis on the concentration of MgATP at a fixed Ca^{2+} concentration (0.1 mM). The plot is linear for solubilized ATPase, giving a K_m value of 16 μM , corresponding to high-affinity binding of MgATP to the phosphorylation site (Meissner, 1973). For vesicular ATPase a linear part is observed at nucleotide concentrations below 100 μM from which an apparent K_m value of 16 μM is calculated. However, the curve bends downwards at high nucleotide concentrations. This behaviour contrasts with that of solubilized ATPase, but is in agreement with the previously demonstrated stimulatory effect of the nucleotide on ATP hydrolysis at high concentrations (Inesi *et al.*, 1967; Yamamoto & Tonomura, 1967; Vianna, 1975). ATPase activity is lower in the deoxycholate-solubilized sample than in the vesicular preparation. Thus there is a 50% decrease in ATP hydrolysis at MgATP concentrations below 100 μM , and a difference of 65% at the highest nucleotide concentrations.

Fig. 6 shows that the dependence of deoxycholate-

Fig. 4. Diffusion of enzymically active ATPase, solubilized by deoxycholate

The solution side of a double-sector cell (synthetic-boundary capillary-type) was filled with deoxycholate-solubilized ATPase, prepared as described in Fig. 3(a), to the level of the lower capillary. The solvent side was filled to just beneath the level of the upper capillary with 1.5 mM-phosphatidylcholine and 5.7 mM-deoxycholate dissolved in the same sucrose and electrolyte medium used for the solubilization of ATPase. At the start of the centrifugation, fluid from the solvent side was layered on to the protein sample. The lines shown in the Figure are calculated according to eqn. (1), assuming $D = 2.6 \times 10^{-7} \text{ cm}^2/\text{s}$. $\Delta, \circ$, Points taken from the scan 20 and 88 min respectively, after the start of the centrifugation in an experiment performed at 6000 rev./min. $\bullet$, Points taken from the scan 88 min after the start of the centrifugation in an experiment performed at 20000 rev./min. On the ordinate the A_{294} of the sample is in relative units, 0% indicating absorption at meniscus, 100% indicating absorption in the plateau region of the scan.

Fig. 5. Effect of MgATP concentration on enzymic activity of deoxycholate-solubilized and vesicular ATPase

Deoxycholate-solubilized (2.5 mg of protein/ml) and vesicular ATPase (3.5 mg of protein/ml) was prepared in a medium containing 0.1 mM- Ca^{2+} , 5 mM-phosphoenolpyruvate, 0.01 M-Taps (pH 8.0), 0.4 M-KCl and 0.3 M-sucrose. The enzymic activity was measured in the presence of an ATP-generating system as described in the Methods section. The concentrations of MgATP were varied to produce final concentrations of the nucleotide of 2–600 μM . The ATP hydrolysis at 2 μM is outside the Figure but lies on the lines of the Lineweaver-Burk plots. ●, Deoxycholate-solubilized ATPase; ○, vesicular ATPase.

solubilized ATPase on free Ca^{2+} is essentially the same as that of the vesicular preparation, apart from the smaller maximal rates obtained in the presence of detergent. The curves are sigmoidal, in accordance with observations by other authors on ATPase kinetics of sarcoplasmic-reticulum vesicles (Kana-zawa *et al.*, 1971; Panet *et al.*, 1971; Vianna, 1975). From the results we calculate Hill coefficients of 1.9 and 1.5 for vesicular and solubilized ATPase respectively. Maximal activity is reached at a low concentration of free Ca^{2+} . The Ca^{2+} concentration for half-maximal ATP hydrolysis is 0.01 μM , to be compared with 0.1 μM at pH 7.0 [performed as described by Vianna (1975), who obtained a value of 0.2 μM]. Measurements with a Ca^{2+} -sensitive electrode indicated that the apparent stability constant of CaEGTA used in the calculation of free Ca^{2+} (2×10^8 at pH 8.0) is essentially correct. We suggest that the phenomenon could be dependent on a tighter association of ATPase with Ca^{2+} at pH 8.0 than at pH 7.0, in agreement with the binding data of Meissner (1973).

Phosphorylation of (Ca^{2+} +Mg $^{2+}$)-dependent ATPase]

In the presence of 0.1 mM- Ca^{2+} and 0.1 mM-MgATP at 0°C and pH 8.0, phosphorylation of ATPase was 9.5 ± 0.8 nmol/mg of protein (mean $\pm$ s.d. for eight experiments), indicating quantitative phosphorylation, whereas at 20°C the phosphorylation was 6.0 ± 1.6 nmol/mg. A comparable degree of

Fig. 6. Effect of free Ca^{2+} concentration on enzymic activity of deoxycholate-solubilized and vesicular ATPase

Deoxycholate-solubilized (2.5 mg of protein/ml) and vesicular ATPase (3.5 mg of protein/ml) were prepared in 0.2 mM-EGTA, 0.01 M-Taps (pH 8.0), 0.4 M-KCl and 0.3 M-sucrose. The enzymic activity was measured after addition of 5 μl of solution, containing 150 mM-MgATP and various concentrations of Ca^{2+} (0–1.87 mM), to 50 μl of the ATPase preparations. Free concentrations of Ca^{2+} were calculated as described in the Methods section. ●, Deoxycholate-solubilized ATPase; ○, vesicular ATPase.

phosphorylation (6.4 ± 1.0 nmol/mg of protein at 20°C) was found by incubation in the medium used for solubilization of enzymically active ATPase (Fig. 2c), but incorporation of ^{32}P into the protein content was only 1.8 ± 0.4 nmol in the medium used in Fig. 2(b) 2 h after solubilization. Thus the extent of phosphorylation appears to be correlated with the decrease in activity occurring after solubilization (cf. Fig. 2).

Discussion

The results of the present study indicate that it is possible to obtain enzymically active preparations of deoxycholate-solubilized ATPase which predominantly consist of ATPase in monomeric form. This result agrees with that of a previous study in which monomer ATPase, prepared by solubilization of vesicular ATPase in a mixture of deoxycholate and phosphatidylcholine at a low protein concentration, was found to be enzymically active (le Maire *et al.*, 1976b). However, the present study resulted in enzymically more stable preparations of solubilized ATPase, and it may be surmised that practically no inactivation occurred during the time needed for characterization by analytical ultracentrifugation. Thus under optimal conditions activity remained constant for up to 4 h at 20°C, and during this period the degree of phosphorylation was about the same in the solubilized and vesicular preparations of ATPase. Optimal solubilization conditions included addition of sucrose and a high salt concentration

(0.4M-KCl) to the deoxycholate-solubilization medium. Meissner & Fleischer (1974) have reported that a similar deoxycholate solubilization medium can be used for reconstitution of ATPase vesicles capable of transporting Ca^{2+} . Furthermore, it is noteworthy that the procedure of MacLennan (1970) for purification of enzymically active ATPase in a deoxycholate-solubilized state uses sucrose and a high salt concentration (in this case ammonium acetate). The retention of activity in our preparations is comparable with that of MacLennan (1970), since 90% activity could be recovered when the solubilized preparation was stored for 24 h at 0°C.

Gel chromatography of ATPase in the presence of deoxycholate and phospholipid (Fig. 1) confirms the tendency of solubilized ATPase to exist in oligomeric forms, as was previously demonstrated after solubilization with non-ionic detergents (le Maire *et al.*, 1976a). The amount of phospholipid bound after elution from the column was low (7 mol/mol of ATPase), and enzymic activity was lost. The lipid-exchange experiments of Warren *et al.* (1974a,b) have shown that a complement of at least 15 mol of phospholipid/mol of ATPase is necessary for detection of ATPase activity, and that 30 mol are required for full activity. The analytical-ultracentrifuge data of the present study are consistent with an amount of bound lipid in enzymically active preparations of deoxycholate-solubilized ATPase of this order. It was shown in our previous paper (le Maire *et al.*, 1976b) that the presence of oligomers in the gel-chromatographed fractions was contingent on a high protein concentration in the solubilization medium, and it was then speculated that the failure to observe aggregation in enzymically active preparations of deoxycholate-solubilized ATPase might be due to the low protein concentration used. The present study, in which solubilization was always done at a high protein concentration, indicates that deoxycholate-solubilized monomers of active ATPase do not have any pronounced tendency for oligomer formation. Rather it appears that aggregation is a process that occurs during delipidation and inactivation.

The maximal ATPase activities as measured directly in the deoxycholate-solubilized preparations were about 35–40% of that of vesicular ATPase. However, this apparent inactivation was of a reversible nature, since freshly solubilized preparations of ATPase after dilution with a detergent-free incubation medium exhibited the same activity as the original vesicular preparation. Electron-microscopic observations and gel-chromatographic experiments indicated that most of the ATPase under these conditions formed compact aggregates with lipid which appeared to approximate to the shape of prolate ellipsoids. Formation of non-vesicular structures after removal of deoxycholate from solubilized

ATPase has previously been described (Selinger *et al.*, 1969).

The activity of deoxycholate-solubilized ATPase exhibited a sigmoidal dependence on the free Ca^{2+} concentration identical with that of the transport protein in vesicular form (Fig. 6), suggesting that interaction of the solubilized monomer with more than one Ca^{2+} is required for ATPase activity. The deoxycholate-solubilized preparations were also activated by low concentrations of MgATP, indicating the presence of high-affinity binding of nucleotide in the deoxycholate-solubilized state. By contrast with the vesicular preparations, Lineweaver-Burk plots of solubilized preparations were linear over the whole range of MgATP concentrations (2–600 μM), indicative of high-affinity interaction only of the solubilized protein with MgATP. The lack of the modulatory effect of MgATP at high nucleotide concentrations, characteristic of vesicular ATPase (Inesi *et al.*, 1967; Yamamoto & Tonomura, 1967; Vianna, 1975), in part, accounts for the lower maximal extents of ATP hydrolysis by the deoxycholate-solubilized preparations, and raises the possibility that this phenomenon is mediated by ATPase-ATPase interactions in the membrane. It is notable in this connection that indirect evidence of a functional role of associated ATPase has been obtained by Yates & Duanne (1976), who observed negative co-operativity in the binding of MgATP by ($\text{Ca}^{2+} + \text{Mg}^{2+}$)-dependent ATPase in membranous form. Froehlich & Taylor (1976) on the basis of kinetic studies of phosphorylation of sarcoplasmic-reticulum ($\text{Ca}^{2+} + \text{Mg}^{2+}$)-dependent ATPase have implicated an oligomer in the transport process. According to the latter authors, Ca^{2+} transport into sarcoplasmic-reticulum vesicles by one ATPase molecule is linked to transport of Mg^{2+} outward by another ATPase molecule in a functional dimer unit ('flip-flop' mechanism). Structural evidence for the presence of oligomeric ATPase (trimer or tetramer) in the membrane has previously been obtained by preparation of solubilized ATPase in non-ionic detergents (le Maire *et al.*, 1976a), freeze-fracture (Scales & Inesi, 1976a,b) and cross-linking experiments (Murphy, 1976) on sarcoplasmic-reticulum membranes. The present study does not exclude the possibility that ATPase associations may be involved in the translocation of Ca^{2+} across the membrane, since we cannot assess the transport capacity of solubilized ATPase preparations. However, it may be concluded from the results obtained that most of the catalytic functions of the transport protein presumably can be performed by the transport protein in monomeric form.

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