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Self-Association and Modification of Calcium Binding in Solubilized Sarcoplasmic Reticulum Adenosinetriphosphatase†

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ABSTRACT: Solubilized sarcoplasmic reticulum adenosinetriphosphatase (ATPase) was fractionated by elution gel chromatography, in the presence of high concentrations of nonionic detergent dodecyl octaethylene glycol monoether ($C_{12}E_8$) up to 74.4 mM (40 mg of $C_{12}E_8$/mL), using a wide range of ATPase concentrations. At 10 μg of ATPase/mL in the presence of 3.72 mM $C_{12}E_8$ (2 mg/mL) the enzyme eluted predominantly in the monomeric state, with a Stokes radius (R_s) of 45 Å. The ATPase in $C_{12}E_8$ had a tendency to self-associate when the protein was increased to 50 μg/mL or higher. The resulting aggregated particle had a R_s = 60 Å, compatible with a dimeric ATPase. The dimeric state was predominant at protein concentrations as high as 6.5 mg/mL, and larger aggregates were not present at a considerable proportion. Rechromatography indicated that monomeric and dimeric forms were in thermodynamic equilibrium. ATP (8 mM), when included in the elution buffer, shifted the equilibrium toward the monomer. In the presence of 50 μM $CaCl_2$, and in the absence of ATP, monomeric ATPase did bind 17.6 ± 1.5 nmol of Ca/mg of protein while the dimeric form did bind 9.4 ± 0.7 nmol of Ca/mg of protein. In vesicular ATPase, the maximal high-affinity binding was 9.5 nmol of Ca/mg of protein. Ca^{2+}-binding cooperativity was lost in the presence of 74.4 mM $C_{12}E_8$ in dimeric soluble ATPase. Ca^{2+} activation of ATP hydrolysis with soluble ATPase was noncooperative in the presence of 1 μM ATP, and cooperative in the presence of 5 mM ATP. It is apparent that a single polypeptide chain has two high-affinity Ca sites and that upon dimerization, half of the sites are not titrated. It is concluded that $C_{12}E_8$ promotes a loss in equilibrium binding cooperativity but does not prevent the Ca sites from interacting during the catalytic cycle upon addition of millimolar ATP. It is suggested that in vesicular ATPase the enzyme might predominantly be in a dimeric state and exhibit half-of-the-sites reactivity.

Sarcoplasmic reticulum adenosinetriphosphatase (ATPase) is a membrane-bound transport enzyme isolated from skeletal muscle in the microsomal fraction (Hasselbach, 1981). The enzyme can be solubilized by the nonionic detergent dodecyl octaethylene glycol monoether ($C_{12}E_8$)[1] and retain its enzymatic activity (Dean & Tanford, 1978; LeMaire et al., 1978). The detergent replaces the membrane phospholipids and the ATPase becomes dispersed in the solution. Ultracentrifugation methods have been used to determine the molecular weight of the protein in solution. It was shown that with the use of $C_{12}E_8$ a monomeric from of the 117 000-dalton ATPase can be obtained (Dean & Tanford, 1978; LeMaire et al., 1978). However, the presence of higher molecular weight aggregates such as dimers, trimers, or tetramers simultaneously with the monomeric ATPase could not be ruled out by these authors. Dean & Tanford (1978) estimated that during sedimentation equilibrium measurements, up to 33% of the soluble ATPase could be in dimeric form and a concentration-dependent aggregation could be present. Another analytical method which has been applied to the characterization of the soluble ATPase is gel filtration chromatography (Dean & Tanford, 1978; LeMaire et al., 1978; Murphy et al., 1982). The Stokes radius of the soluble particle obtained by chromatographic measurements was 55–59 Å (Dean & Tandord, 1978; Murphy et al., 1982), a value which is higher than the 47 Å obtained by ultracentrifugation (Dean & Tanford, 1978). However, the protein concentrations used in gel filtration were 20–60 times higher than the ones used in ultracentrifugation experiments.

An important characteristic of the ATPase transport enzyme is its specificity for Ca^{2+}. The Ca^{2+}-transport ability of the enzyme cannot obviously be studied in the soluble ATPase because there are no compartments; however, Ca^{2+} binding and Ca^{2+} activation of ATP hydrolysis can be measured in the soluble preparation. It has been recently shown by Ca^{2+} titration of tryptophan fluorescence changes that upon solubilization of the ATPase by $C_{12}E_8$ the Ca^{2+} binding cooperativity is lost (Verjovski-Almeida & Silva, 1981). The stoi-

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[1] Abbreviations: $C_{12}E_8$, dodecyl octaethylene glycol monoether; Mops, 3-(*N*-morpholino)propanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; SR, sarcoplasmic reticulum; $NaDodSO_4$, sodium dodecyl sulfate.

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chiometry of Ca^{2+} binding which is 1 mol of Ca/mol of ATPase in vesicular ATPase (Inesi et al., 1978, 1980) is maintained in the solubilized ATPase (Verjovski-Almeida & Silva, 1981). The Ca^{2+} dependence of activation of hydrolysis was studied in soluble ATPase under different conditions, and both a cooperative pattern (Moller et al., 1980; Murphy et al., 1982) and a noncooperative pattern (Watanabe et al., 1981) were reported.

In the present report the degree of self-association of soluble ATPase was studied by gel chromatography in the presence of $C_{12}E_8$ and with a broad range of ATPase protein concentrations. It is shown that only at low protein concentrations (10 μg/mL) the ATPase is predominantly monomeric, and the Stokes radius obtained by chromatography (45 Å) is comparable to the reported value obtained by ultracentrifugation. The Ca^{2+}-binding stoichiometry in the monomer is 2 mol of Ca/mol of ATPase, and cooperativity is present. At higher protein concentrations in the presence of $C_{12}E_8$, the ATPase tends to self-aggregate into a dimeric form, and the Stokes radius increases to 60 Å. Upon dimerization, half of the Ca^{2+}-binding sites are not titrated, the stoichiometry being 1 mol of Ca/mol of ATPase. Ca^{2+} binding cooperativity is lost in the soluble dimeric ATPase.

Materials and Methods

Sarcoplasmic reticulum vesicles were isolated from rabbit skeletal muscle (de Meis & Hasselbach, 1971), and purified vesicular ATPase was prepared according to method 2 of Meissner et al. (1973). They were stored at −20 °C in sucrose with sodium azide. The purified vesicles are freely permeable to calcium. The specific activity of purified ATPase was 3.5–4.5 μmol of P_i mg^{-1} min^{-1}, when assayed at 25 °C in the presence of 5 mM ATP and pH 7.5. The detergent $C_{12}E_8$ was obtained from NIKKOL, through the Kouyoh Trading Co., 4-1, 2-chome, Iwamoto-cho, Chiyoda-Ku, Tokyo, Japan. The marker proteins for calibration of the gel column were from Sigma Chemical Co., with the exception of IgG which was a gift from Dr. A. Campos from the Institute of Microbiology of our university. All other reagents were of analytical grade. The water was glass distilled, deionized, and kept in plastic bottles, as well as all other solutions.

Gel chromatography and calcium binding experiments were performed in a medium containing 80 mM KCl, 10 mM $MgCl_2$, 20 mM Mops, pH 7.5, 50 μM $CaCl_2$ (^{45}Ca for binding experiments, at 20000 cpm/nmol of $CaCl_2$), 1 mM dithiothreitol, and 0, 3.72, or 74.4 mM $C_{12}E_8$ as indicated in the figures. The experiments of Figures 4 and 5 were in the absence of dithiothreitol. The protein concentrations varied from 10 μg/mL to 6.5 mg/mL as indicated in the figures. For soluble protein concentrations above 50 μg/mL, the solubilization with $C_{12}E_8$ was followed by centrifugation at 195000*g* for 30 min at 25 °C to remove the nonsolubilized protein pellet; the concentration of soluble protein in the supernatant was determined by the Folin method, and it was used immediately. The protein concentrations were referred to a standard of bovine serum albumin. The standard curve and the ATPase samples for the assay of protein concentration by the Folin method contained 2–10% $NaDodSO_4$, to avoid the interference of the nonionic detergent $C_{12}E_8$.

Gel chromatography was performed at 25 °C in a column of Bio-Gel A-1.5 m (0.9 × 60 cm) from Bio-Rad operated with a Mariotte flask at a constant flow rate of 8 mL/h unless otherwise specified. The void volume (V_0) was measured with Blue Dextran 2000, and the internal volume (V_i) was measured in each run by the addition of trace amounts of tritiated water to the 1-mL samples applied to the column. Calibration of the column was done with alkaline phosphatase, aldolase, immunoglobulin G, and thyroglobulin. The partition coefficients were measured as $K_d = (V_e - V_0)/(V_i - V_0)$, where V_e is the elution volume of the protein. The data were plotted with $(K_d)^{1/3}$ as a function of the Stokes radii of the proteins, as suggested by Porath (1963). The calibration curve was linear with a correlation coefficient of 0.998. The column outlet was connected to the reservoir syringe port of a Durrum stopped-flow machine equipped with a fluorescence cuvette accessory. The eluted medium was continuously flown through the cuvette and directed to a fraction collector. The tryptophan fluorescence in the eluted medium was continuously monitored with excitation at 290 nm. The emitted light was measured through a 320-nm cutoff filter with the photomultiplier operated at 0.9–1 kV. The amplifier output was fed into a paper recorder. The fluorescence intensity was linearly proportional to the ATPase protein concentration in the range 1–100 μg of protein/mL in the presence of $C_{12}E_8$. When the eluted protein concentration was higher than 100 μg/mL, the direct fluorescence recording was not used. Instead, the fractionated samples were diluted, and the protein concentration was then measured by fluorescence or by the Folin method. A sample of each fraction was counted for determination of ^{45}Ca. When gel chromatography was done in the presence of 8 mM ATP, the fluorescence excitation light was shifted to 297 nm due to the UV absorbing effect of ATP at 290 nm.

Calcium binding as a function of free Ca^{2+} concentrations was measured by equilibrating the ATPase with a medium containing 80 mM KCl, 10 mM $MgCl_2$, 20 mM Mops, pH 7.5, 50 μM $^{45}CaCl_2$ (20000 cpm/nmol), 0 or 74.4 mM $C_{12}E_8$, and different amounts of EGTA to give the free Ca^{2+} concentrations in the range 10^{-7}–5 × 10^{-5} M (Schwarzenbach, 1957). The binding of Mg^{2+} and Ca^{2+} to EGTA and to ATP (for the ATPase activities below) was taken into consideration, and the different protonated forms of the chelators at pH 7.5 were calculated, using the constants compiled by Vianna (1975). The ATPase protein was equilibrated with the medium, when a 1-mL sample (5–6 mg of protein/mL) was passed through a gel column of Bio-Gel P-10 (0.9 × 20 cm) which was previously equilibrated and eluted with the same buffer medium. When soluble ATPase was used (5–6 mg of ATPase/mL in the presence of 74.4 mM $C_{12}E_8$), the sample was dissolved in a medium from which EGTA was omitted and was centrifuged for 30 min at 195000*g* at 25 °C, immediately followed by application of 1 mL of the supernatant to the column. The protein eluted within 5 min in the void volume. The excess radioactivity present in the protein-containing fractions was a measure of Ca^{2+} binding. Protein concentration in the eluted fractions was measured by the Folin method in the presence of 10% $NaDodSO_4$.

ATPase activity was assayed in a medium containing 80 mM KCl, 10 mM $MgCl_2$, 20 mM Mops, pH 7.5, 0.5 mM EGTA, different amounts of $CaCl_2$ to give the free Ca^{2+} concentrations in the range 10^{-7}–5 × 10^{-5}, and 0 or 3.72 mM $C_{12}E_8$. The ATPase protein concentration (2 or 50 μg/mL) and the ATP concentrations are indicated in the figures. At the low ATP concentrations (1–200 μM ATP) the medium also contained 5 mM phosphoenolpyruvate and 0.1 mg/mL pyruvate kinase. The reaction was started by addition of ATP, and five aliquots (0.75 mL) were drawn at different time interval (15 s to 18 min). P_i production was always linear as a function of time, confirming that pyruvate kinase was active in the presence of $C_{12}E_8$, as observed by Dean & Tanford (1978) and Moller et al. (1980). The concentration of pyruvate kinase used was in excess of the optimal concentration

determined by Vianna (1975). Pyruvate kinase was unstable only when left in a medium containing $C_{12}E_8$ for prolonged times (over 1 h). The samples were mixed with an equal volume of the reagent which was used both to quench the reaction and to measure the inorganic phosphate concentration by complexation with molybdovanadate (Lin & Morales, 1977). The absorbance at 350 nm was measured 30 s after the reaction was quenched.

Fluorescence measurements performed in individual samples were carried out with an Aminco-Bowman spectrofluorometer by using a 0.3-mL cuvette; excitation was at 290 nm and emission at 335 nm.

Polyacrylamide gel electrophoresis in a buffer containing 7.5% $NaDodSO_4$ was carried out in slab gels 1 mm thick, using the running buffer described by Laemmli (1970). Samples were prepared by pooling a number of fractions eluted from the chromatography either in the position of monomeric or dimeric ATPase. The protein concentration was adjusted to 0.1 mg/mL by diluting the samples at a constant $C_{12}E_8$ concentration into a medium to give the final concentrations of 7.5% $NaDodSO_4$, 3% glycerol, and 1 mg/mL bromophenol blue as tracking dye. Aliquots of 50–100 μL were applied to the top of the slab gels.

Results

Fractionation of Soluble ATPase by Gel Chromatography. Purified sarcoplasmic reticulum ATPase was solubilized by the nonionic detergent $C_{12}E_8$, and the soluble ATPase was applied to a chromatography column equilibrated with buffer containing 3.72 mM $C_{12}E_8$ (2 mg of detergent/mL). When the protein concentration in the applied sample was low (10 μg of ATPase/mL), the main ATPase peak eluted in fraction 54 (Figure 1A), corresponding to a partition coefficient K_d = 0.438. A negligible amount of protein eluted in the void volume (fractions 34–35), and there was a small protein peak at fractions 43–47, corresponding to a K_d = 0.244.

From the elution position in the column (K_d), it is possible to estimate the Stokes radius (R_s) of the particle by referring to the calibration curve for the column (not shown), which was obtained with proteins of known Stokes' radii. The Stokes radius is a property of the macromolecule, including the protein–detergent complex and the solvation shell (Tanford et al., 1974). The main peak in Figure 1A (K_d = 0.438) corresponds to a macromolecule of R_s = 45 Å, and it is compatible with an ATPase monomer as seen in the Discussion. The mean ± SD of three runs was 45 ± 0.8 Å. This peak would correspond to a protein of approximately 126 000 daltons, if the calibration curve is plotted as a function of the molecular weight, instead of Stokes' radius. It can be seen that at low protein concentrations (10 μg of ATPase/mL) the soluble ATPase was predominantly in the monomeric form (Figure 1A).

Increasing the protein concentration to 50 μg of ATPase/mL in the presence of 3.72 mM $C_{12}E_8$ (2 mg of detergent/mL) shifted the relative proportions of the two peaks (Figure 1B). The protein eluted predominantly in fraction 43, a K_d = 0.244. It represents a macromolecule with R_s = 60 ± 1.8 Å (four runs) and would correspond to a protein of approximately 298 000 daltons. The size of this particle is compatible with a dimer of ATPase.

The addition of 8 mM ATP to the column running buffer (Figure 1C) promoted monomerization of the ATPase. The protein concentration of the applied sample in the experiment of Figure 1C was 50 μg of ATPase/mL, a protein concentration identical with that used in Figure 1B. In the absence of ATP the ATPase was predominantly in the dimeric form

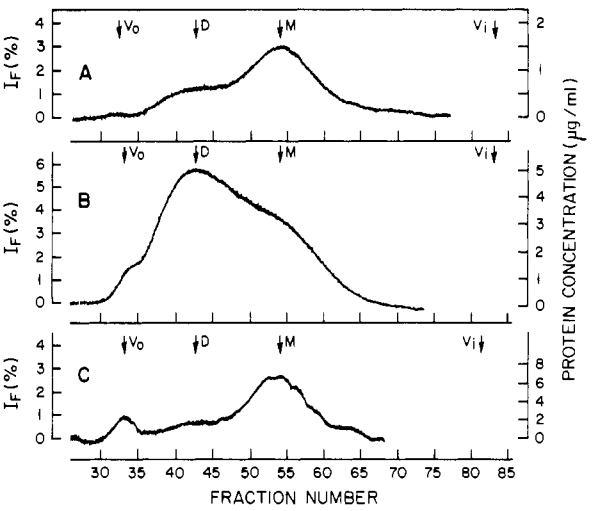

FIGURE 1: Effect of protein concentration and of ATP on the state of aggregation of $C_{12}E_8$-solubilized ATPase. Gel chromatography was performed with a column of Bio-Gel A-1.5 m (0.9 × 60 cm) equilibrated and eluted with a buffer containing the nonionic detergent $C_{12}E_8$ at a concentration of 3.72 mM (2 mg/mL), as described under Materials and Methods. Tryptophan fluorescence intensity (I_f) was continuously monitored in the eluted samples, and the recording is marked in arbitrary fluorescence units. The fluorescence intensities were referred to calibration curves, and the corresponding protein concentrations are indicated on the right. (A) The applied sample contained 10 μg of ATPase/mL. (B) The applied sample contained 50 μg of ATPase/mL. (C) The elution buffer contained 8 mM ATP in addition to all other reagents, as described under Materials and Methods, and the applied sample contained 50 μg of ATPase/mL. In recording (C) the fluorescence gain is decreased, because of the UV absorbing effect of ATP on the fluorescence excitation light.

(Figure 1B), and in the presence of 8 mM ATP the ATPase was predominantly monomeric (Figure 1C). Under the conditions of Figure 1C, an estimated maximum of 25% of the ATP has been hydrolyzed, considering the elution time, the total amount of protein, the elution volume, and the measured maximal velocity of P_i production (6 μmol of P_i mg^{-1} min^{-1}).

The protein and the detergent concentrations are interdependent factors affecting monomerization of the ATPase. In Figure 1A the ATPase eluted predominantly as a monomer at an applied protein concentration of 10 μg of ATPase/mL which gives a detergent to protein ratio of 200:1 by weight. An increase in the protein concentration to 50 μg of ATPase/mL (Figure 1B) reduced the detergent to protein ratio to 40:1, and the ATPase eluted predominantly as a dimer. In the experiment of Figure 2A a protein concentration of 50 μg of ATPase/mL was used (similar to Figure 1B), but the detergent to protein ratio was increased 20-fold to 800:1. It can be seen that the monomeric form increased with the increase in detergent concentration from 3.72 (Figure 1B) to 74.4 mM $C_{12}E_8$ (40 mg of $C_{12}E_8$/mL) (Figure 2A), at the same protein concentration of 50 μg of ATPase/mL. However, due to the protein concentration used, approximately 40% of the ATPase eluted in the dimeric form (Figure 2A) in spite of the large ratio of detergent to protein (800:1).

It should be noted that all the chromatographic profiles were highly reproducible under the experimental conditions used, and a minimum of two to three identical runs were obtained for each result reported.

For characterization of the preferential formation of dimeric ATPase over larger oligomeric forms, a high detergent concentration of 74.4 mM was used, and the ATPase protein concentration was varied over a wide range. An increase in the protein concentration by 1 order of magnitude from 50 (Figure 2A) to 500 μg of ATPase/mL (Figure 2B) shifted the

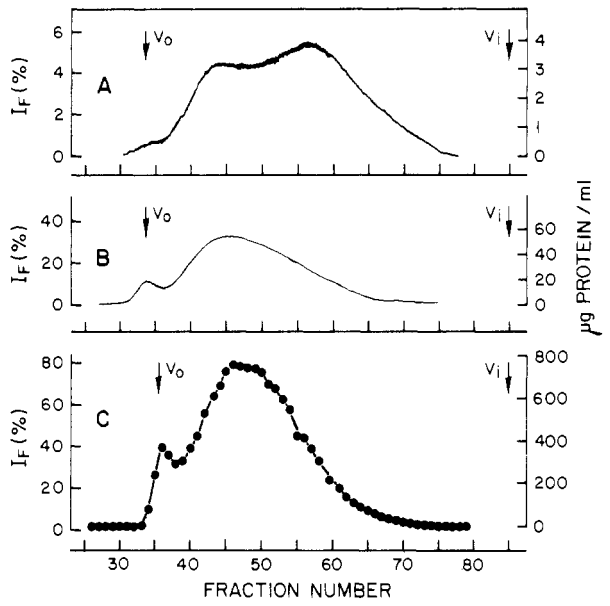

FIGURE 2: Gel chromatography of solubilized ATPase in the presence of 74.4 mM $C_{12}E_8$. Gel chromatography was performed under conditions identical with Figure 1, with the exception that the nonionic detergent concentration in the elution buffer was increased to 74.4 mM $C_{12}E_8$ (40 mg/mL). (A) The applied sample contained 50 μg of ATPase/mL. (B) The applied sample contained 500 μg of ATPase/mL. (C) The applied sample contained 5.9 mg of ATPase/mL; continuous fluorescence recording of (C) was not done, because of the high protein concentrations in the eluted medium. The fractions were collected and diluted 4 times, and the fluorescence intensity was measured. The protein concentrations on the right are final concentrations in the collected fractions, after correcting for the dilution.

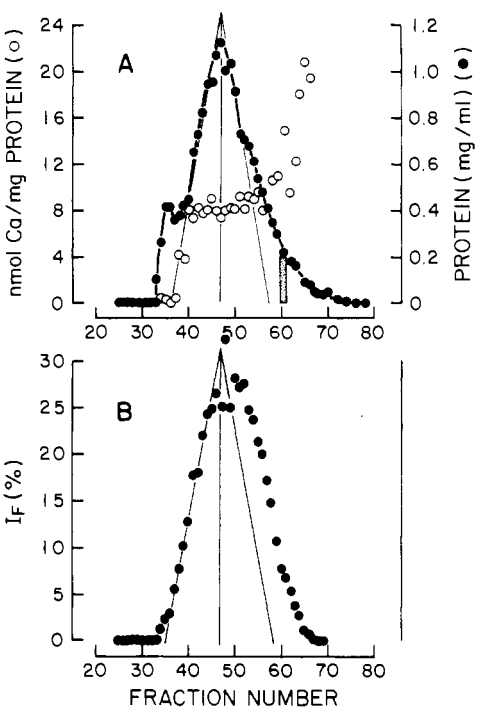

FIGURE 3: Calcium binding to monomeric and dimeric soluble ATPase and interconversion between the two forms. Gel chromatography was performed with a column of Bio-Gel A-1.5 m (0.9 × 60 cm) equilibrated and eluted with a buffer containing 74.4 mM $C_{12}E_8$ (40 mg/mL) and 50 μM $^{45}CaCl_2$ in addition to all other reagents, as described under Materials and Methods. (A) A 1-mL sample containing 6.5 mg of soluble ATPase was applied to the column, and the protein concentration of the eluted fractions was measured by the Folin method (●). The amount of Ca in the fractions was measured by radioactivity and is expressed as nmol of Ca/mg of protein in each collected fraction (○). (B) A sample which eluted in fraction number 61 in the experiment of panel A (marked with the shaded rectangle) was collected and reapplied to the column. The chromatographed material was fractionated, and the protein in the eluted samples was measured by the tryptophan fluorescence intensity (I_f) of each fraction.

equilibrium toward the dimeric form. With a further increase by 1 order of magnitude in the protein concentration to 5.9 mg of ATPase/mL (Figure 2C), the predominant form continued to be the dimer. Higher aggregates were not significantly present at the high protein concentrations tested (0.5–5.9 mg of ATPase/mL). Due to the large amount of 60-Å dimeric ATPase (Figure 2B,C) the peak of monomeric ATPase became much less evident, showing up only as a minor skewness of the profiles to the right (Figure 2B,C). The high protein concentrations (2–8 mg of ATPase/mL) used in the column chromatography experiments with solubilized ATPase in the literature (LeMaire et al., 1978; Dean & Tanford, 1978; Murphy et al., 1982) have yielded a Stokes radius of 55–59 Å, which probably correlates to the ATPase dimer of Figures 1 and 2.

A small amount of protein eluted in the void volume in the experiments of Figures 1 and 2. This protein is probably denatured ATPase present in the original preparation, since it did not bind calcium and did not appear in rechromatographed samples, as will be shown below.

Interconversion between Monomeric and Dimeric Soluble ATPase. The soluble ATPase at a protein concentration of 6.5 mg/mL and a detergent concentration of 74.4 mM $C_{12}E_8$ was fractionated by gel chromatography, and the predominant peak was of dimeric ATPase (Figure 3A). A sample which eluted in fraction number 61 at a protein concentration of 0.22 mg of ATPase/mL was collected and reapplied to a column. The profile of the rechromatographed material (Figure 3B) showed that the predominant peak was of dimeric ATPase. The result of Figure 3B indicates that the ATPase which eluted in the first run in the region of monomer (fraction number 61) (Figure 3A) had already dimerized when rechromatography was performed. A fraction from the dimer peak, from an experiment similar to Figure 3A, was diluted to 10 μg of protein/mL at a constant detergent concentration and rechromatographed to establish reversibility. As a result of the protein dilution the ATPase dissociated and eluted as a monomer (not shown).

Calcium Binding to Monomeric and Dimeric Soluble ATPase. The chromatography column in the experiment of Figure 3A was equilibrated with an elution buffer which contained 50 μM $^{45}CaCl_2$. The radioactivity of the collected fractions was counted and the amount of calcium bound to the ATPase measured by the excess radioactivity present in the protein-containing fractions. Figure 3A shows the binding of calcium to monomeric and dimeric ATPase forms. The small amount of protein which eluted in the void volume did not bind calcium and probably represents denatured protein. The dimeric ATPase (fractions 40–50) did bind 7.5–8.0 nmol of Ca^{2+}/mg of protein, which represents approximately 1 mol of Ca^{2+}/mol of ATPase, taking the molecular weight of 117 000 daltons. The binding of calcium to the monomeric ATPase form (fractions 60–65) was 15–20 nmol of Ca/mg of protein, representing approximately 2 mol of Ca^{2+}/mol of ATPase. Due to the high protein concentration used (6.5 mg of ATPase/mL) only a small fraction of the protein eluted as a monomer; the high protein concentration is necessary in order to obtain resolution in the radioactive ^{45}Ca measurements of the eluted samples. The binding measurements are therefore limited to the experimental conditions where the dimer is predominant (Figure 3A), i.e., to high protein concentrations (5–6.5 mg of ATPase/mL).

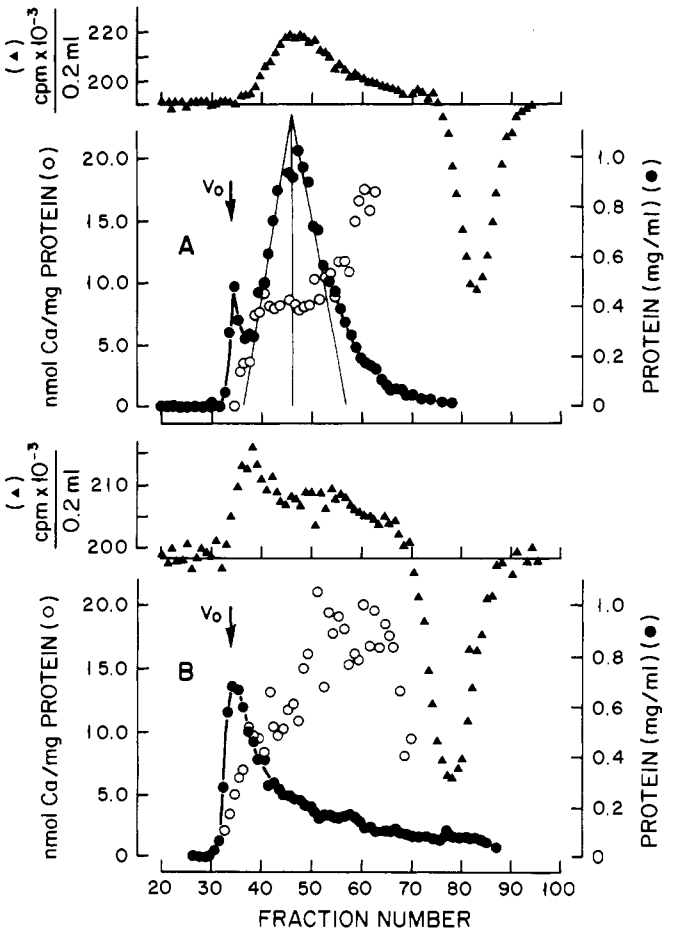

FIGURE 4: Calcium binding to monomeric and dimeric soluble ATPase separated by an increase in the column flow rate. Gel chromatography was performed under conditions identical with Figure 3A. The elution flow rate was either 8 (A) or 60 mL/h (B). Protein concentration determined by the Folin method (●). The amount of Ca in each fraction was measured by radioactivity (▲), and the binding is expressed as nmol of Ca/mg of protein (○).

The column flow rate was increased in order to increase the resolution of Ca^{2+} binding to monomeric ATPase without having to decrease the protein concentration. In an interconverting system such as monomer $\rightleftharpoons$ dimer, a better resolution is obtained if the rate of separation is as fast as or faster than the rate of interconversion itself (Kegeles & Cann, 1978). Figure 4 shows an experiment where the rate of flow of the elution buffer was increased from 8 (Figure 4A) to 63 mL/h (Figure 4B). As a result of the increase in the flow, equilibrium conditions for gel filtration were not reached with the type of gel used, and the large more excluded macromolecules were pushed downstream faster than the smaller ones. The dimeric ATPase eluted near the void volume (Figure 4B), and a long tail of protein was obtained in the region expected to be of monomeric ATPase. Figure 4B shows that in fractions 39–45 the calcium binding was 9.4 ± 0.7 nmol of Ca^{2+}/mg of protein and in fractions 54–66 the amount of calcium binding was 17.6 ± 1.5 nmol of Ca^{2+}/mg of protein. It can be seen in Figure 4 that the ATPase monomer binds 2 mol of Ca/mol of ATPase and that dimerization eliminates half of the high-affinity binding sites.

$NaDodSO_4$ gel electrophoresis of the protein which eluted as a dimer (fractions 37–45) or as a monomer (fractions 55–67) was performed as described under Materials and Methods. A single protein band was present in both cases, corresponding to the 117 000-dalton ATPase (not shown). There was no detectable amount of other proteins such as calsequestrin or calcium binding protein in the electrophoresis gels of the samples from either the dimeric or monomeric peak. The electrophoresis data further ascertain that the two elution peaks correspond to different states of aggregation of the same ATPase protein.

The ATPase eluted within 3 h from the column in the experiments of Figures 1–3 and within 30 min in the experiment of Figure 4B. In all cases both monomeric and dimeric ATPases were active, and the rate of ATP hydrolysis catalyzed by the eluted ATPase was approximately 80% of that of a freshly solubilized ATPase sample. A necessary condition for the stability of the soluble ATPase was the presence of calcium (e.g., 50 μM). In the presence of 0.5 mM EGTA and low protein concentrations (10–50 μg/mL) the soluble ATPase lost 50% of its activity within 10 min, as already reported (Moller et al., 1980). A sample kept in 0.15 mM EGTA for 300 min and eluted in a buffer similar to that of Figure 3A showed that over 80% of the ATPase eluted in the void volume and did not bind calcium, representing denatured ATPase (not shown).

Calcium Binding as a Function of Free Ca^{2+}. Calcium binding to soluble ATPase was measured as a function of free Ca^{2+} concentrations in the range 10^{-7}–5×10^{-5} M (Figure 5A). Soluble ATPase at a protein concentration of 5–6 mg/mL was used, in the presence of 74.4 mM $C_{12}E_8$. The ATPase was predominantly in the dimeric form (see Figures 2–4), due to the protein concentration used. Ca^{2+} binding to solubilized dimeric ATPase was noncooperative (Figure 5A) with a Hill number $n_H = 0.93$ (Figure 5B) and $K_{0.5} = 1.3$ μM. The maximal amount of calcium bound to solubilized dimeric ATPase was 0.85 mol of Ca/10^5 g of protein (Figure 5C) which corresponds to 1.02 mol of Ca/mol of ATPase, taking the molecular weight of the enzyme as 117 000 daltons (LaMaire et al., 1976a; Rizzolo et al., 1976) and having approximately 95% of ATPase protein in the purified preparation (Verjovski-Almeida, 1981). The small amount of monomeric ATPase which is present when the ATPase is dissolved at 5–6 mg of protein/mL in 74.4 mM $C_{12}E_8$ (see Figures 2–4) did not contribute significantly to increase the binding over 1 mol of Ca/mol of ATPase.

The binding of Ca^{2+} to vesicular ATPase was measured in the Ca^{2+} concentration range 10^{-7}–5×10^{-5} M (Figure 5A). In the vesicular ATPase the Ca^{2+} binding was cooperative, with a Hill $n_H = 1.96$ and $K_{0.5} = 0.3$ μM (Figure 5B). The maximal amount of binding was 0.95 mol of Ca/10^5 g of protein (Figure 5C), corresponding to 1.15 mol of Ca/mol of ATPase.

The ATPase activity was measured in the column effluents in order to check that the ATPase in the experiments of Figure 5 did retain its activity after passage through the column with exposure to different Ca^{2+} concentrations. For that purpose in the experiments of Figure 5 the fractions were collected into test tubes containing microliter amounts of concentrated nonradioactive $CaCl_2$ to give an additional 100 μM $CaCl_2$ in the collected sample. As a result, the ATPase which was exposed in the column to lower free radioactive Ca^{2+} concentrations necessary for the titration became saturated with cold Ca^{2+} as soon as it came out of the column (~5 min). In that way, possible denaturation due to the presence of $C_{12}E_8$ and nonsaturating Ca^{2+} was reduced to a minimum. The ATPase activity of solubilized ATPase in the presence of 50 μM Ca^{2+} was 4.5–4.8 μmol of P_i mg^{-1} min^{-1} before passage through the column. The soluble ATPase which was exposed to Ca^{2+} concentrations as low as 2 μM (pCa 5.7) which gives

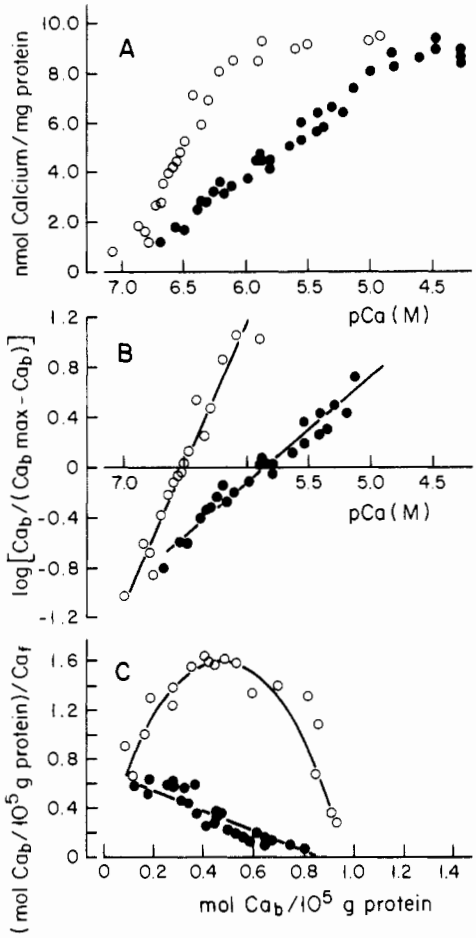

FIGURE 5: Calcium binding as a function of free Ca^{2+} concentrations in vesicular and solubilized dimeric ATPase. (A) Calcium binding was measured in samples containing 5–6 mg/mL purified ATPase, in the absence of detergent [vesicular ATPase (O)] or in the presence of 74.4 mM $C_{12}E_8$ [solubilized dimeric ATPase (●)]. The protein eluted in the void volume of a low molecular weight exclusion gel which was equilibrated with media containing the different radioactive free calcium concentrations indicated in the figure. The excess radioactivity in the protein-containing fractions was measured along with the protein concentrations, and the amount of calcium bound to the proteins is shown. (B) Hill plot of the data from (A). (C) Scatchard plot of the data from (A). The abbreviation Ca_b is for calcium bound to the protein and Ca_f for free Ca^{2+} in μM.

50% saturation of the Ca^{2+} sites (see Figure 5A) retained 100% of the activity in the eluted fractions. The ATPase which was exposed to 0.3 μM Ca^{2+} (pCa 6.55), corresponding to 15% saturation of the sites (Figure 5A), retained 65% of the original activity.

The short times (5 min) and the high protein concentrations used in the experiments of Figure 5 permitted the study of direct Ca^{2+} binding titration in solubilized active dimeric ATPase. Both the loss in cooperativity and the decrease in the affinity for Ca^{2+} upon solubilization (Figure 5) are similar to the results obtained indirectly with Ca^{2+} titration of tryptophan fluorescence changes in soluble (dimeric) ATPase (Verjovski-Almeida & Silva, 1981).

Ca^{2+} Dependence of Enzymatic Activity in Dimeric and Monomeric Soluble ATPase. The velocity of hydrolysis catalyzed by the enzyme was measured as a function of Ca^{2+} concentrations in the range 10^{-7}–5×10^{-5} M. The detergent concentration in the assay was 3.72 mM $C_{12}E_8$. In the experiments of Figure 6 the protein concentration was 50 μg of ATPase/mL. The ATPase was predominantly in the dimeric form (see Figure 1B) in the absence of ATP. Figure 6A shows

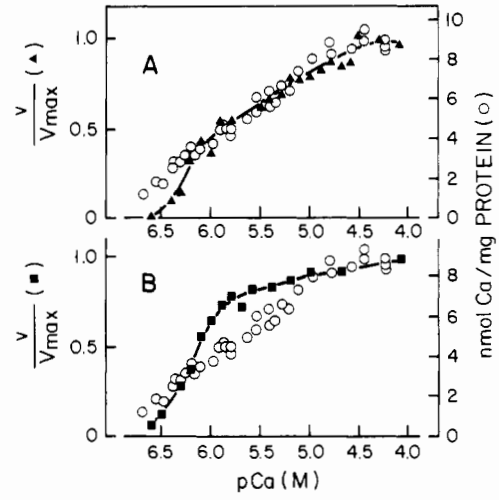

FIGURE 6: Enzyme activity in solubilized dimeric ATPase. The velocities of P_i production (v, full symbols) were measured at the different free Ca^{2+} concentrations indicated in the figure in the presence of 50 μg of ATPase/mL and 3.72 mM $C_{12}E_8$ (2 mg/mL), as described under Materials and Methods. (A) The ATP concentration was 1 μM. The maximal velocity (V_{max}) was 0.57 μmol of P_i mg^{-1} min^{-1}. (B) The ATP concentration was 50 μM, and V_{max} was 4.15 μmol of P_i mg^{-1} min^{-1}. The open symbols show the calcium binding data of Figure 5, with solubilized ATPase in the absence of ATP.

the velocity of ATP hydrolysis as a function of Ca^{2+} concentration in the presence of 1 μM ATP. At this low ATP concentration and in the presence of 50 μg of ATPase/mL, Ca^{2+} activation of hydrolysis paralleled the noncooperative Ca^{2+} binding to dimeric soluble ATPase (Figure 6A). Only a small portion of the activation curve deviated from the binding curve, showing a tendency to a cooperative pattern in the first 25–30% of the maximal activation.

When the ATP concentration was raised from 1 (Figure 6A) to 50 μM (Figure 6B), the pattern of Ca^{2+} activation of hydrolysis was biphasic. The first 70–80% of maximal activation showed a cooperative pattern (Figure 6B) followed by a noncooperative component. The appearance of the cooperative pattern of Ca^{2+} activation of the enzyme is related to the increase in ATP concentration in the assay medium (Figure 6) which also promotes monomerization of the enzyme (see Figure 1C).

In the experiments of Figure 7 the Ca^{2+} dependence of enzyme activation was determined in the soluble ATPase (3.72 mM $C_{12}E_8$) and in vesicular ATPase (no detergent in the assay). Figure 7A shows that in the presence of 2.5 μM ATP, Ca^{2+} activation of hydrolysis was cooperative in the purified *vesicular* ATPase, having a linear Hill plot with a slope of 2.87. Recently, it was reported that the slopes of Hill plots obtained with purified vesicular ATPase depend on the pH of the assay media (Watanabe et al., 1981), and at the pH used in our experiments (pH 7.5) a Hill number of 2.95 was reported (Watanabe et al., 1981). In the *soluble* ATPase the Ca^{2+}-activation pattern was biphasic, and the cooperative component was responsible for 50% of maximal activation. The Hill plot exhibited two slopes. Figure 7B shows that in the presence of 5 mM ATP the vesicular ATPase continued to exhibit only the cooperative component (n_H = 2.84). The solubilized ATPase was activated up to 80–90% of its maximal velocity in a cooperative mode (n_H = 2.49), and the remaining 10–20% of the curve was noncooperative. It should be noted that in the presence of 5 mM ATP, over 80% of the ATPase was in the monomeric form (see Figure 1C).

When the protein concentration in the assay medium was reduced from 50 (Figure 7) to 2 μg/mL (Figure 8) the Ca^{2+}

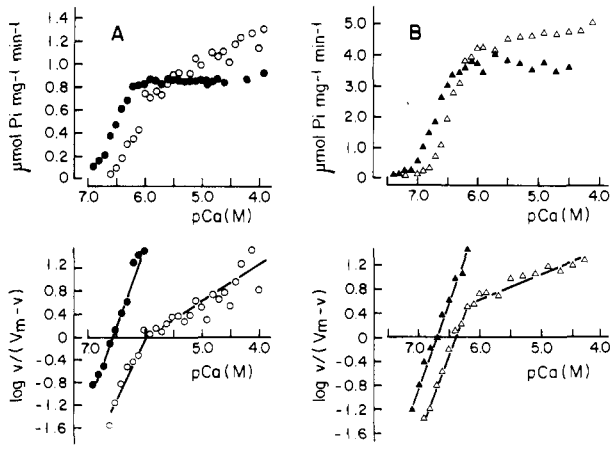

FIGURE 7: Enzyme activity in vesicular and solubilized ATPase at micro- or millimolar ATP concentrations. The velocities of P_i production were measured at the free Ca^{2+} concentrations indicated in the figure, with 50 μg/mL of purified ATPase in the absence (●, ▲) or in the presence (○, △) of 3.72 mM $C_{12}E_8$ (2 mg/mL). In (A) the ATP concentration was 2.5 μM (○, ●), and in (B) it was 5 mM (△, ▲). The lower graphs are Hill plots of the data from the corresponding upper panels.

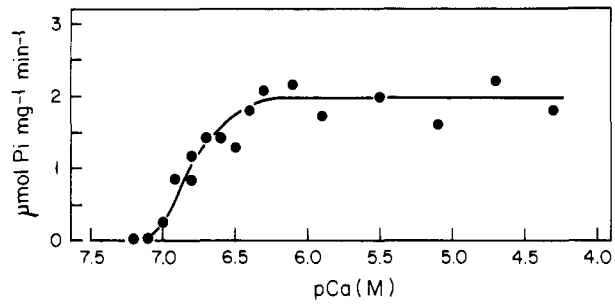

FIGURE 8: Enzyme activity in solubilized monomeric ATPase. The velocities of ATP hydrolysis were measured at the free Ca^{2+} concentrations indicated, with 2 μg of ATPase/mL in the presence of 3.72 mM $C_{12}E_8$ (2 mg of $C_{12}E_8$). The ATP concentration was 5 mM.

dependence of enzyme activation was cooperative, and the maximal velocity was reached with 5×10^{-7} M Ca^{2+}. The noncooperative component was not present, and the velocity of ATP hydrolysis remained constant up to 5×10^{-5} M Ca^{2+} (Figure 8). At 2 μg/mL in the presence of 3.72 mM $C_{12}E_8$ the ATPase was in the monomeric form independent of the presence of ATP (see Figure 1A). It is apparent that Ca^{2+} cooperativity for activation of the enzyme is a property of the monomeric ATPase (Figure 8). The interaction between the Ca^{2+} sites is of an intramolecular nature and probably involved the two Ca^{2+} binding sites/mol of ATPase monomer which were evidenced in the experiments of Figures 3 and 4.

The maximal velocity of ATP hydrolysis obtained with different solubilized preparations in the presence of 5 mM ATP and optimal Ca^{2+} concentration (50 μM) was 2–2.5 μmol of P_i mg^{-1} min^{-1} when assayed at 2 μg of ATPase/mL (see Figure 8) and 4.5–5.0 μmol of P_i mg^{-1} min^{-1} when assayed at 50 μg of ATPase/mL (see Figure 7B).

ATP Dependence of Enzymatic Activity in Soluble and Vesicular ATPase. The catalytic activity of the enzyme was measured as a function of ATP concentrations. Soluble ATPase at 50 μg/mL in the presence of 3.72 mM $C_{12}E_8$ was used, and the hydrolytic activity was compared to the activity of vesicular ATPase (with the omission of detergent). It was observed that the velocity of ATP hydrolysis was similar in both soluble ATPase and vesicular ATPase, in the ATP concentration range 10^{-7}–~10^{-5} M (not shown) reaching 1.5 μmol of P_i mg^{-1} min^{-1}. The activity in the soluble preparation raised steeply to reach saturation at approximately 2×10^{-4} M ATP with a maximal velocity of 5.5 μmol of P_i mg^{-1} min^{-1}. In the vesicular ATPase saturation occurred at 2×10^{-3} M ATP and a maximal velocity of 3.5 μmol P_i mg^{-1} min^{-1} (not shown). The enzyme activation observed at millimolar ATP concentrations is probably related to a regulatory low-affinity site for ATP (Inesi et al., 1967; Yamamoto & Tonomura, 1967; de Meis & de Mello, 1973; Verjovski-Almeida & Inesi, 1979). It is possible that the detergent affects the low-affinity ATP sites, which might be related to the modification in intermolecular interactions between ATPase polypeptide chains.

Discussion

State of Aggregation of Solubilized ATPase. This paper has compared the gel chromatography elution positions of the ATPase solubilized in $C_{12}E_8$ over a wide range of protein concentrations and with $C_{12}E_8$:protein ratios up to 800:1 by weight. It was shown that the smaller protein–detergent particle obtained by chromatography in the presence of $C_{12}E_8$ has a Stokes radius (R_s) of 45 Å (Figure 1). At 10 μg of ATPase/mL the elution profile showed a fairly homogeneous population of such particles (Figure 1A), and higher protein concentrations gave rise to the appearance of a larger, 60-Å macromolecular particle (Figures 1B and 2). The Stokes radius obtained by chromatography is a property of the whole macromolecule, including ligands to the protein such as lipids and detergent, and is affected by hydration and molecular asymmetry (Tanford et al., 1974). Although it is extremely difficult to evaluate unambiguously the individual contributions of all these factors, the observed Stokes radius can give some indication as to the state of aggregation of the ATPase polypeptide chains, if some of the parameters are measured by other independent methods. Then, the minimal radius (R_{min}) of a dry spherical particle can be calculated and compared to the observed radius (R_s), as in (Tanford et al., 1974)

$$R_{min} = [3M_p(\bar{\nu}_p + \delta_L\bar{\nu}_L + \delta_D\bar{\nu}_D)/(4\pi N)]^{1/3}$$

where N is the Avogadro constant, M_p is the protein molecular weight, $\bar{\nu}_p$, $\bar{\nu}_L$ and $\bar{\nu}_D$ are the partial specific volumes of the protein, lipid, and detergent, respectively, and δ_L and δ_D are the amounts of bound lipid and detergent, expressed in grams per gram of protein.

The Ca^{2+}-ATPase protein molecular weight (M_p) has been rigorously measured by sedimentation equilibrium and found to be 117 000 daltons (LeMaire et al., 1976a,b; Rizzolo et al., 1976). The partial specific volume of the Ca^{2+}-ATPase has been calculated on the basis of its amino acid composition to be $\bar{\nu}_p$ = 0.740 cm^3/g (LaMaire et al., 1976b). The value of $\bar{\nu}_L$ = 0.975 cm^3/g has been calculated on the basis of the lipid composition of the SR ATPase preparation (LeMaire et al., 1976b), and the value of $\bar{\nu}_D$ for $C_{12}E_8$ is 0.973 cm^3/g (Tanford et al., 1977). The amount of phospholipids remaining bound to the ATPase in the presence of an excess of nonionic detergent was measured to be δ_L = 0.23 g/g (LeMaire et al., 1976b, 1978). The amount of bound detergent is the most difficult parameter to access because of the low protein concentrations necessary to give a monomeric form of the enzyme, a conditions where most of the detergent is free in solution in comparison to the amount bound. The bound δ_D was measured at high protein concentrations by Dean & Tanford (1978) to be 1.1 ± 0.5 g/g and was estimated by LeMaire et al. (1978) and Moller et al. (1980) as being 0.3–0.4 g/g in their calculations of M_p. Estimating one micelle of bound detergent per mol ATPase would give δ_D = 0.55 g/g. Using this value, the other parameters above, and taking integer multiples of the protein molecular weight, one can calculate

the minimal radius of the corresponding dry spherical monomer, dimer, etc. For the monomer R_{min} = 41 Å. The ratio R_s/R_{min} = 45/41 = 1.10. This ratio is in the range found for globular proteins (Tanford et al., 1974) and suggests that the monomeric soluble ATPase is a fairly symmetric protein–detergent macromolecule. Hydration can probably account for the 10% higher Stokes radius observed over the calculated value. For a dimeric, detergent-solubilized ATPase R_{min} = 52 Å. The ratio R_s/R_{min} = 60/52 = 1.15, which indicates that the dimeric soluble ATPase is probably also a globular protein–detergent macromolecule. The uncertainty in the amount of bound detergent does not impair the above calculations and conclusions. Binding of detergent higher than that estimated would reinforce the conclusions. In the extreme situation of a soluble ATPase with *no* bound detergent the minimal radius of a dry spherical particle with $M_p = 1.17 \times 10^5$ having only the residual lipids bound to the protein (δ_L = 0.23 g/g and δ_D = 0) would be 36 Å and R_s/R_{min} would be 1.25, which is still within the value for globular proteins.

To check whether the observed 45-Å particle could be compatible with a dimeric soluble ATPase, we used the extreme situation of a soluble dimeric ATPase with no bound detergent to calculate an R_{min}. When $M_p = 2.34 \times 10^5$ is used and residual-bound lipids (δ_L = 0.23 g/g and δ_D = 0) are only allowed for, the calculated minimal radius still does not fall below 45 Å, which clearly suggests that the observed 45-Å macromolecule (Figures 1 and 2) cannot accommodate more than one detergent-solubilized polypeptide chain. It is the 60-Å particle, observed when the protein concentration is increased in the medium (Figures 1 and 2), which has the size compatible with a solubilized dimer (R_{min} = 52 Å). Dean & Tanford (1978) have obtained a Stokes radius of 47 Å for the monomeric ATPase in $C_{12}E_8$ at low protein concentrations, using ultracentrifugation techniques. Our value of 45 Å for the monomeric ATPase (Figures 1 and 2) agrees quite well with their reported data. The chromatography data of Dean & Tanford (1978) gave a larger 55-Å particle for the ATPase solubilized in $C_{12}E_8$. The ATPase concentration used in the chromatography was 2.7 mg/mL (Dean & Tanford, 1978).

Recently, fluorescence anisotropy decay was used to measure the Stokes radius of solubilized ATPase (Murphy et al., 1982) in combination with gel chromatography. High protein concentrations were used, and the Stokes radius obtained was 55–59 Å (Murphy et al., 1982). The authors assumed that it was the smallest particle obtainable and assigned it to a monomer, leading to R_s/R_{min} = 1.35 (Murphy et al., 1982) and to the conclusion that the large R_s observed was due to an asymmetric monomer (Murphy et al., 1982). The smallest R_s obtainable was 45–47 Å both by ultracentrifugation (Dean & Tanford, 1978) and by gel chromatography (Figures 1 and 2) and R_s/R_{min} = 1.10. The monomeric ATPase detergent particle seems therefore to be globular. From the present data, it should not be concluded that the detergent-free native ATPase protein molecule is globular. Rhodopsin, which is an asymmetric cylindrical molecule, also showed a globular detergent–protein particle when solubilized with $C_{12}E_8$ (McCaslin & Tanford, 1981). Recently, studies with small-angle X-ray diffraction suggest that the vesicular native SR ATPase is a cylinder 142 Å long and 35 Å in diameter (Brady et al., 1981) and that the inactive monomeric DOC–ATPase complex is a double cylinder 100 Å long and 40–75 Å in diameter (LeMaire et al., 1981).

Self-Association of Solubilized ATPase. Our data indicate that the solubilized ATPase has a tendency to self-associate into a dimeric form even in the presence of an excess $C_{12}E_8$ up to 800:1 by weight (Figure 2), as long as the protein concentration is 50 μg of protein/mL or higher. At 50 μg of protein/mL monomeric and dimeric forms coexist (Figures 1B and 2A). At higher protein concentrations, dimeric ATPase predominates (Figure 2). Dean & Tanford (1978) have already suggested that in their sedimentation equilibrium experiments (at 100 μg of ATPase/mL) there was a possibility of some concentration-dependent aggregation, and care was taken to obtain the molecular weight of the monomeric species from the upper two-thirds of the centrifuge cell (Dean & Tanford, 1978). LeMaire et al. (1978) have also characterized the monomeric ATPase in $C_{12}E_8$ by sedimentation velocity, presumably at low protein concentrations. More recent studies of these authors (Moller et al., 1980) indicate that the best conditions for monomerization involve a rather low protein: detergent ratio and a protein concentration in the range 50–200 μg of ATPase/mL (Moller et al., 1980).

Although the studies with detergent-solubilized ATPase indicate that the protein has a tendency to aggregate especially in the presence of residual lipids, it was not documented in the literature the tendency of association of the ATPase in the presence of excess $C_{12}E_8$. In fact, a number of studies have recently used $C_{12}E_8$ assuming that it would be sufficient to have a weight ratio of detergent to protein in the range 2–5 to have monomeric ATPase, irrespective of the high protein concentrations used. The tendency of the ATPase in $C_{12}E_8$ to form dimeric and higher aggregated forms may preclude the study of monomeric ATPase in $C_{12}E_8$ by the techniques of protein chemistry which require high protein concentrations and may restrict experimentation to the aggregated ATPase.

The self-association is not a characteristic exclusively found in the presence of $C_{12}E_8$. In the original experiments using nonionic detergents (LeMaire et al., 1976b, 1978), $C_{12}E_8$ was used only for the initial solubilization, followed by complete removal and replacement with Tween 80 (LeMaire et al., 1976b, 1978). The chromatographic data reported were always obtained in the presence of Tween 80 and show that also in the presence of this nonionic detergent the ATPase eluted in two peaks, representing oligomeric and monomeric ATPase (LeMaire et al., 1976b, 1978). The main difference reported is that according to LeMaire et al. (1978) there was no evidence for reversible equilibrium between the different species of ATPase in the presence of Tween 80. Our data indicate that monomeric and dimeric ATPases are in a thermodynamic reversible equilibrium in the presence of $C_{12}E_8$. The data of Figures 1–4 suggest that interconversion is a relatively slow process, permitting separation in the column and yielding bimodal elution profiles.

It should be noted that in our present work we always refer to the protein concentration of the sample applied to the top of the column; a sample having 50 μg of ATPase/mL (Figure 1B) is diluted approximately 10 times while being separated in the column, and the maximal concentration of the collected samples was 5 μg/mL. Therefore, the experiments of Figures 1 and 2 show only a range of protein concentrations giving rise to dimerization. Additional experiments are in progress to clarify this point.

Calcium Binding and Enzyme Activity in Monomeric and Dimeric ATPase. Our data show that monomeric soluble ATPase binds 15–19 nmol of Ca/mg of protein (Figures 3 and 4) which corresponds to approximately 2 mol of Ca sites/mol of polypeptide. Upon dimerization of the ATPase, the binding is reduced to 8–9 nmol of Ca/mg of protein (Figures 3–5), which indicates that half of the Ca sites are not titrated in the dimeric ATPase. The rechromatography data of Figure 3B

indicates that the ATPase particle which elutes as a dimer originates from the monomeric ATPase. The reduced binding capacity of the dimeric ATPase (8–9 nmol/mg) probably cannot be explained by the existence of a permanently inactive nonbinding protein, but rather by a functional property of the dimeric state. It should be noted that in the experiment of Figure 4B, the weight-average level of calcium binding is 10.4 nmol of Ca/mg of protein. This weight average is calculated by multiplying the amount of protein in each fraction by the corresponding binding level, adding up all the factors, and dividing the sum by the total amount of eluted protein. Although there was a considerable number of fractions in which the binding level was 15–19 nmol of Ca/mg of protein (Figure 4B), the monomer $\rightleftharpoons$ dimer equilibrium was greatly shifted toward the dimer, and the absolute amount of protein eluting as a monomer was small when compared to the amount of protein in dimeric form.

The maximal binding capacity of the high-affinity sites in vesicular ATPase is 8–9 nmol of Ca/mg of protein (Figure 5) which agrees with the previous values reported in the literature (Inesi et al., 1978, 1980; Dupont, 1980, 1982; Verjovski-Almeida & Silva, 1981; Watanabe et al., 1981). Since the monomeric ATPase binds 15–19 nmol of Ca/mg of protein (Figures 3 and 4) and dimeric ATPase binds 8–9 nmol of Ca/mg of protein, it is suggested that the minimal state of aggregation of the ATPase in the membrane is a dimer. Recently, Klingenberg (1981) has pointed out that proteins which transverse membranes tend to have a dimeric structure. Upon dimerization of the enzyme, half of the high-affinity Ca sites would become nontitrated in high-affinity equilibrium binding experiments. At present we do not know whether it is one calcium site out of the two in each polypeptide chain or the two calcium sites in one polypeptide chain in a dimer which would not be titrated.

The maximal number of phosphorylating sites in the ATPase is 4 nmol/mg of protein (de Meis, 1981), which corresponds to half the number of sites expected per milligram of protein, if the ATPase polypeptide chains are homogeneous. Upon phosphorylation of these sites, a rapid translocation of 8 nmol of Ca/mg of protein takes place (Verjovski-Almeida et al., 1978). Therefore, the functional unit seems to involve half of the theoretically available sites. In view that the maximal Ca^{2+} binding in the monomeric ATPase is twice that of the dimeric (Figures 3 and 4), it is possible that dimerization of the ATPase gives rise to two kinetically nonequivalent ATPase units, only one of them binding calcium with a high-affinity in equilibrium conditions, but the two of them being able to function in an alternate sequence during the enzyme cycle. Recently, the kinetics of Ca^{2+} binding to the ATPase was studied in great detail, and two kinetically distinct types of high-affinity Ca sites were identified (Ikemoto et al., 1981; Dupont, 1982). At present it seems reasonable to suggest that one polypeptide chain (monomer) contains two calcium sites and one phosphorylation site and that the functional dimeric unit exhibits half-of-the-sites reactivity.

We have observed by indirect fluorescence measurements that calcium binding cooperativity was lost and $K_{0.5}$ for Ca^{2+} was decreased upon addition of $C_{12}E_8$ (Verjovski-Almeida & Silva, 1981). This finding is confirmed by direct Ca^{2+}-binding measurements (Figure 5). Ca^{2+} activation of ATP hydrolysis is also noncooperative in solubilized ATPase (Figure 6A) when the ATP concentration is low (1 μM). We have previously interpreted that the loss in cooperativity resulted from a decrease in the interaction between two calcium sites, one in each polypeptide chain. This interpretation seems to be wrong. Our present data indicate that a single polypeptide chain has two Ca^{2+} sites (Figures 3 and 4), and probably the Ca^{2+}-binding cooperativity is a result of intramolecular interactions between these two sites within one chain.

The loss in cooperativity in the presence of $C_{12}E_8$ (Figure 5) would result from a specific effect of detergent on the binding site region of the polypeptide, promoting a decrease in the degree of intramolecular interaction between the two Ca sites. Alternatively, the detergent could promote the observed loss in Ca^{2+}-binding cooperativity in dimeric soluble ATPase (Figure 5) by decreasing the degree of polarity and orientation of the ATPase molecules in the regions of intermolecular association; as a result, dimers of ATPase with different arrangements could be formed in $C_{12}E_8$, as already suggested by LeMaire et al. (1981), originating some sterical hindrance to the cooperative Ca^{2+} binding. Ikemoto et al. (1981) have found that addition of $C_{12}E_8$ to the ATPase promotes a change in the kinetics of Ca^{2+} binding and that this was a direct effect of detergent on the ATPase polypeptide which was independent of enzyme dissociation into the monomeric state (Ikemoto et al., 1981).

The effect of detergent would decrease the degree of binding cooperativity (Figure 5), but it is proposed that the detergent would not prevent that the two sites continue to interact in the presence of millimolar ATP, when the enzyme is operating during the catalytic cycle. The Ca^{2+} dependence of enzyme activation in the presence of high ATP (5 mM) is cooperative (Figures 6B and 7). High ATP concentrations promote monomerization of the ATPase solubilized in $C_{12}E_8$ (Figure 1C), and it would also modify the Ca^{2+}-binding sites restoring the high degree of intramolecular interaction between them (see Figure 7).

The Ca^{2+} dependence of enzyme activation is cooperative when the enzyme is solubilized at very low protein concentrations (2 μg of ATPase/mL) in the presence of an excess of $C_{12}E_8$ (2 mg/mL) (Figure 8). At these protein and detergent concentrations the enzyme is monomeric, independent of the presence of ATP. The cooperative pattern (Figure 8) supports the finding that a single ATPase polypeptide chain contains two Ca^{2+} sites. The maximal velocity of ATP hydrolysis in the presence of 5 mM ATP is twice as high in ATPase assayed at 50 μg/mL (Figure 7B) as when assayed at 2 μg/mL (Figure 8). A possible explanation is that the activating effect of millimolar ATP is only present when the enzyme tends to be in the dimeric state (high protein) (Figure 7B). The activation effect of ATP would depend on intermolecular interactions, which has also been proposed by Moller et al. (1980). Additional experiments are in progress to clarify this point. At present we cannot explain the effect of millimolar ATP in promoting monomerization of the soluble ATPase (Figure 1C). The possible significance of this effect to the catalytic cycle of the enzyme should prove an interesting subject for study.

Acknowledgments

We thank Sheila Silva for the excellent technical assistance in preparing the purified sarcoplasmic reticulum ATPase. We are grateful to Dr. A. Hasson-Voloch, Institute of Biophysics of our University, for lending the Durrum stopped-flow equipment.

Registry No. ATPase, 9000-83-3; Ca, 7440-70-2; ATP, 56-65-5.

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