Characterization of Detergent-Solubilized Sarcoplasmic Reticulum Ca^{2+}-ATPase by High-Performance Liquid Chromatography†

Jens P. Andersen,*,‡ Bente Vilsen,‡ Henning Nielsen,§ and Jesper V. Møller§

Institutes of Physiology and Medical Biochemistry, University of Aarhus, 8000 Aarhus C, Denmark

Received February 11, 1986; Revised Manuscript Received May 29, 1986

ABSTRACT: Sarcoplasmic reticulum Ca^{2+}-ATPase solubilized by the nonionic detergent octaethylene glycol monododecyl ether was studied by molecular sieve high-performance liquid chromatography (HPLC) and analytical ultracentrifugation. Significant irreversible aggregation of soluble Ca^{2+}-ATPase occurred within a few hours in the presence of ≤ 50 μM Ca^{2+}. The aggregates were inactive and were primarily held together by hydrophobic forces. In the absence of reducing agent, secondary formation of disulfide bonds occurred. The stability of the inactive dimer upon dilution permitted unambiguous assignment of its elution position and sedimentation coefficient. At high Ca^{2+} concentration (500 μM), monomeric Ca^{2+}-ATPase was stable for several hours. Reversible self-association induced by variation in protein, detergent, and lipid concentrations was studied by large-zone HPLC. The association constant for dimerization of active Ca^{2+}-ATPase was found to be 10^5–10^6 M^{-1} depending on the detergent concentration. More detergent was bound to monomeric than to dimeric Ca^{2+}-ATPase, even above the critical micellar concentration of the detergent. Binding of Ca^{2+} and vanadate as well as ATP-dependent phosphorylation was studied in monomeric and in reversibly associated dimeric preparations. In both forms, two high-affinity Ca^{2+} binding sites per phosphorylation site existed. The delipidated monomer purified by HPLC was able to form ADP-insensitive phosphoenzyme and to bind ATP and vanadate simultaneously. These results suggest that formation of Ca^{2+}-ATPase oligomers in the membrane is governed by nonspecific forces (low affinity) and that each polypeptide chain constitutes a functional unit.

The self-associated state of sarcoplasmic reticulum Ca^{2+}-ATPase is a fundamental issue in understanding the mechanism of active Ca^{2+} transport. A number of structural studies have suggested that the Ca^{2+}-ATPase polypeptide chains form oligomers in the membrane (Wang et al., 1979; Andersen et al., 1981; Napolitano et al., 1983; Taylor et al., 1984; Franzini-Armstrong & Ferguson, 1985). However, with the aid of efficient detergents like octaethylene glycol monododecyl ether ($C_{12}E_8$)[1] and deoxycholate, this protein can be solubilized in monomeric form (M_r 110 000) with retention of full enzymatic activity (Jørgensen et al., 1978; Dean & Tanford, 1978; Møller et al., 1980; Martin, 1983; Silva & Verjovski-Almeida, 1985). It has been demonstrated that monomeric Ca^{2+}-ATPase in the phosphorylated state is capable of undergoing the transition between high and low Ca^{2+} affinity forms (Andersen et al., 1983, 1985a,b; Martin et al., 1984). The tryptic digestion patterns of the two phospho forms of soluble monomeric enzyme resemble those of the two major conformational states of membrane-bound Ca^{2+}-ATPase (Andersen et al., 1985b). These studies suggest that the minimal functional unit in energy interconversion is a single Ca^{2+}-ATPase polypeptide chain. However, they do not exclude that an oligomeric organization of the Ca^{2+}-ATPase peptides is obligatory for passage of Ca^{2+} through the membrane.

Studies of equilibria between monomers and oligomers of Ca^{2+}-ATPase in solution may provide information which is important for understanding the equilibria existing in the membranous state. In our previous experiments, the assessment of the monomeric state of the soluble enzyme was based on its sedimentation behavior in the analytical ultracentrifuge. Only at detergent to protein concentration ratios below 2:1 (w/w) did the soluble protein show a pronounced tendency for reversible formation of oligomer. Silva and Verjovski-Almeida (1983, 1985), on the other hand, reached a conclusion very different from ours. By employing gel filtration, they found that most of the soluble Ca^{2+}-ATPase was self-associated even at a $C_{12}E_8$ to protein concentration ratio as high as 5000:1 (w/w). Furthermore, in their studies the dimeric enzyme showed "half-of-the-sites reactivity" with respect to Ca^{2+} binding (Silva & Verjovski-Almeida, 1983).

A major problem with traditional gel filtration techniques is that time-dependent aggregation may occur during a chromatographic run. In addition, the separation of Ca^{2+}-ATPase monomers and dimers is poor in the widely used agarose columns (Møller et al., 1986). The purpose of the present study has been to explore the possibility of using molecular sieve HPLC for preparation and characterization of monomers and well-defined oligomers of Ca^{2+}-ATPase. Combination of this fast and efficient separation technique with hydrodynamic studies has permitted us to obtain unequivocal information about the elution positions of monomeric and stable (inactive) dimeric Ca^{2+}-ATPase and to study the monomer–oligomer interconversion and ligand binding properties of active enzyme.

† Supported by the Danish Medical Research Council, the P. Carl Petersen Foundation, and the Foundation for the Advancement of Medical Science.

‡ Institute of Physiology.

§ Institute of Medical Biochemistry.

[1] Abbreviations: HPLC, high-performance liquid chromatography; $C_{12}E_8$, octaethylene glycol monododecyl ether; $NaDodSO_4$, sodium dodecyl sulfate; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Tes, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; EP, phosphorylated Ca^{2+}-ATPase; E_1 and E_2, major conformational states of Ca^{2+}-ATPase; cmc, critical micellar concentration; K_D, partition coefficient; V_0, void volume; R_s, Stokes radius.

0006-2960/86/0425-6439$01.50/0

Experimental Procedures

Materials. Sarcoplasmic reticulum vesicles were prepared from rabbit skeletal muscle and purified by extraction with a low concentration of deoxycholate [see Andersen et al. (1985a)]. $C_{12}E_8$ was obtained from Nikko Chemicals, Tokyo. ^{14}C-Labeled $C_{12}E_8$ was from C.E.A. Saclay. $^{45}Ca^{2+}$ was a product of Risø, Roskilde. [γ-^{32}P]ATP and [^{48}V]vanadyl chloride were from Amersham, Bucks., U.K. [γ-^{32}P]ATP was purified before use as previously described (Andersen et al., 1985a). Vanadate solutions were prepared as described in Andersen and Møller (1985). A gel filtration calibration kit containing aldolase, catalase, ferritin, and thyroglobulin was obtained from Pharmacia. Aspartate transcarbamylase was prepared according to Gerhart and Holoubek (1967).

High-Performance Liquid Chromatography (HPLC). Small-zone molecular sieve HPLC was performed at 20 °C in TSK G 3000 SW columns (7.5 mm × 300 mm or 7.5 mm × 600 mm) (Toyo Soda, Japan). An LKB 2150 HPLC pump was used. In most experiments, flow rates were 0.4 mL/min for the 300-mm column and 0.8 mL/min for the 600-mm column.[2] Soluble Ca^{2+}-ATPase (10–500 μL) was injected into a 500-μL injection loop. Absorbance in eluted fractions was read at 226 or 280 nm. In most experiments, the eluant contained 20 mM Tes (pH 7.0), 0.1 M NaCl or 0.1 M KCl, 10 mM $MgCl_2$, 1.5 mM $CaCl_2$, and 1.0 mM EGTA ± 5 mM DTT together with the indicated $C_{12}E_8$ concentration. All samples were centrifuged or filtered (0.22-μm Millipore filter) before injection into the column. Protein recovery from the column was >90% in these conditions but decreased at higher pH.

Large-zone experiments were conducted in a TSK GSWP column (7.5 mm × 75 mm) at 20 °C and a flow rate of 0.1 mL/min.[2] Delipidated sample (2–5 mL) was injected through a 10-mL loop. The eluant was the same as described previously. Centroid boundary positions were determined by planimetry as suggested by Valdes and Ackers (1979).

Solubilization and Delipidation of Ca^{2+}-ATPase. Ca^{2+}-ATPase membrane (4–8 mg of protein/mL) was solubilized by 10–20 mg of $C_{12}E_8$/mL in the presence of 20 mM Tes (pH 7.0), 0.1 M KCl (0.1 M NaCl when $NaDodSO_4$ was to be added later), 0.5 mM $CaCl_2$, and 10 mM $MgCl_2$ ± 5 mM DTT. Insoluble residue (20–30% of the protein) was removed by centrifugation in a Beckman Airfuge at 130000*g* for 15 min. Delipidated Ca^{2+}-ATPase was prepared by HPLC of this sample in the long TSK G 3000 SW column. The major protein peak was collected, discarding the inactive void volume material and the fractions containing mixed micelles of lipid and detergent. In most cases, the detergent concentration was the same as used later in the corresponding large-zone experiment. Above 0.25 mg of $C_{12}E_8$/mL, delipidation was virtually complete (remaining phospholipid less than 0.002 g/g of protein) whereas some phospholipid (0.005–0.08 g/g of protein) was associated with the protein peak at 0.1–0.25 mg of $C_{12}E_8$/mL.

Irreversibly Aggregated Ca^{2+}-ATPase. Ca^{2+}-ATPase was solubilized and centrifuged as described above and was then diluted to obtain a final medium composition of 20 mM Tes (pH 7.0), 0.1 M KCl, 10 mM $MgCl_2$, 0.5 mg of protein/mL, 5 mg of $C_{12}E_8$/mL, and various Ca^{2+} and EGTA concentrations together with 5 mM DTT when indicated. The samples were incubated at 20 °C and aliquots drawn at various times for HPLC in TSK G 3000 SW. A similar protocol was followed in studies of aggregation of Ca^{2+}-ATPase delipidated as described above. In some experiments, HPLC was performed in the presence of 1% $NaDodSO_4$, and 0.1 M sodium acetate, pH 4.5, in a TSK G 4000 SW column.

Analytical Ultracentrifugation. Sedimentation equilibrium studies were performed in a Beckman Model E analytical ultracentrifuge at 9000 rpm at 20 °C. Buoyant molecular weights, $M(1 - \bar{v}\rho)$, were obtained from the slopes of equilibrium plots of ln C vs. r^2. The partial specific volume ($\bar{v}$) and protein molecular weight (M_p) were calculated as

$$\bar{v} = (\bar{v}_p + \delta_D\bar{v}_D + \delta_L\bar{v}_L)/(1 + \delta_D + \delta_L) \quad (1)$$

and

$$M_p = M/(1 + \delta_D + \delta_L) \quad (2)$$

where $\bar{v}_p$, $\bar{v}_D$, and $\bar{v}_L$ are the respective partial specific volumes of protein, detergent, and phospholipid (le Maire et al., 1976; Tanford et al., 1977) and δ_D and δ_L are the amounts of bound detergent and lipid in grams per gram of protein. The numbers used in the calculation were $\delta_D = 0.7$ g/g and $\delta_L = 0.002$ g/g for monomeric Ca^{2+}-ATPase and $\delta_D = 0.5$ g/g and $\delta_L = 0.002$ g/g for dimeric Ca^{2+}-ATPase (cf. Figure 5 inset). A 50% variation in the values assumed for δ_D had only a negligible influence on the resulting molecular weights (less than 10% change). Thus, our conclusions are not critically dependent on the measurement of detergent binding.

Sedimentation velocity studies were performed at 44000 rpm, 20 °C. The homogeneity of the sample was examined by simulation of the sedimenting boundary according to the diffusion equation:

$$C = \frac{C_0}{2}\left[1 - \frac{2}{\sqrt{\pi}}\int_0^{x/2\sqrt{Dt}} e^{-y^2}\,dy\right] \quad (3)$$

where D is the diffusion coefficient, C_0 is the initial protein concentration of the sample, and C is the concentration of protein during the run as a function of effective diffusion time (t) and distance (x) from the boundary centroid position. The start of the first scan at operating speed was defined as zero time ($t' = 0$). Solute dispersion taking place before $t' = 0$ was included as a correction term (t_{cor}) to give the effective diffusion time ($t = t' + t_{cor}$). The boundaries obtained at various t' values were used for measuring $x/2(Dt)^{1/2}$ from the integral in eq 3 as a function of x. From these measurements, $2(Dt)^{1/2}$ at various t' values was evaluated, and t_{cor} was estimated from plotting $4Dt$ vs. t'. Diffusion coefficients (4.0×10^{-7} and 3.0×10^{-7} cm^2/s for Ca^{2+}-ATPase monomer and dimer, respectively) were calculated from buoyant molecular weights and sedimentation coefficients according to the Svedberg equation:

$$D = \frac{RTS}{M(1 - \bar{v}\rho)} \quad (4)$$

A protomer molecular weight of 110000 (Brandl et al., 1986) was assumed.

Vanadate Binding. For studies of [^{48}V]vanadate binding, the Ca^{2+}-ATPase vesicles (4 mg/mL) were incubated for 1 h with 50 μM [^{48}V]vanadate in the presence of 20 mM Tes (pH 7.0), 0.1 M KCl, 1.0 mM EGTA, and 10 mM $MgCl_2$. The vanadate-reacted Ca^{2+}-ATPase was solubilized by addition of 10 mg of $C_{12}E_8$/mL and injected into the HPLC column after prior centrifugation, as described above, to remove insoluble material. The eluant contained 20 mM Tes

[2] These flow rates were chosen to make chromatograms obtained with different column lengths as comparable as possible. Note that in an earlier study (Andersen & Vilsen, 1985) a precolumn (TSK GSWP) was inserted before the long TSK G 3000 SW column. Therefore, the previous retention times cannot be compared directly with the present data. The TSK GSWP column also has a larger average pore size than TSK G 3000.

(pH 7.0), 0.1 M KCl, and 1 mM EGTA together with $C_{12}E_8$, EDTA, $MgCl_2$, and ATP as described in the tables. Radioactivity in eluted fractions was measured by γ scintillation counting. Radioactivity associated with the protein peak was well separated from radioactivity in the total volume, when a flow rate of 0.8 mL/min was used. In the experiments run at 0.16 mL/min, where significant dissociation of bound vanadate occurred during chromatography, the separation was poorer. In this case, the base line used for calculation of binding was that observed immediately before the protein peak.

Ca^{2+} Binding. High-affinity Ca^{2+} binding was measured by the method of Hummel and Dreyer (1962) after equilibration of the column with eluant containing 50 μM $^{45}CaCl_2$, 30 μM EGTA, 5 mM $MgCl_2$, 0.1 M KCl, 20 mM Tes (pH 7.0), 5 mM DTT, and $C_{12}E_8$ as indicated. The pump rate was varied (0.05–0.8 mL/min) to test that complete equilibration between $^{45}Ca^{2+}$ in sample and eluant occurred. Radioactivity in eluted fractions was measured by scintillation counting in Lumagel (Lumac/3M bv). Total Ca^{2+} concentration in the eluant was tested by atomic absorption spectrometry.

Phosphorylation. Phosphorylation from [γ-^{32}P]ATP (25 μM) in eluted fractions was measured in 2.0-mL samples as previously described (Andersen et al., 1985a). For examination of the ability to form ADP-insensitive phosphoenzyme, the pH was increased to 8.1 by addition of 3 volumes of 100 mM Tes/Tris, pH 8.2, buffer. At this high pH, a significant amount of ADP-insensitive phosphoenzyme accumulates even at low temperature [cf. Takisawa & Tonomura (1979)], and its dependence on Ca^{2+} and Mg^{2+} concentrations can be studied. The ADP-insensitive fraction of total EP was assumed identical with the fraction of phosphoenzyme remaining after 5-s dephosphorylation in the presence of 1 mM ADP [cf. Andersen et al. (1985a,b)].

Other Methods. Ca^{2+}-ATPase activity was measured in the presence of 0.1 mM $CaCl_2$, 10 mM $MgCl_2$, 5 mM ATP, 0.1 M KCl, and 20 mM Tes (pH 7.0) by the procedure of Brotherus et al. (1981), which avoids significant dilution of protein and detergent.

Protein concentration was measured by the method of Lowry et al. (1951) after precipitation of the protein in trichloroacetic acid and resuspension in $NaDodSO_4$ as suggested by Peterson (1977). Human serum albumin (KABI) was used as standard. The correction factor for the difference in color yield between albumin and Ca^{2+}-ATPase, $E_{750}(Ca^{2+}\text{-ATPase})/E_{750}(\text{albumin})$, was found to be 1.1 by quantitative determination of the content of glutamic acid, aspartic acid, glycine, alanine, and leucine after acid hydrolysis of test samples of membrane-bound and soluble HPLC-purified Ca^{2+}-ATPase. The sequence data (Brandl et al., 1986) were used to calculate the total amount of Ca^{2+}-ATPase protein in these samples.

Phospholipid content was determined according to Bartlett (1959).

Detergent binding was calculated from the increase in radioactivity from [^{14}C]$C_{12}E_8$ associated with the protein peak in both small-zone and large-zone HPLC experiments. In large-zone experiments, the protein had been equilibrated 2 times with the desired concentration of radioactive detergent since the latter was present also in the eluant used for delipidation. There was no difference between the amount of bound detergent measured before and after reequilibration.

Results

Irreversible Aggregation of $C_{12}E_8$-Solubilized Ca^{2+}-ATPase. Previous studies have shown that Ca^{2+}-ATPase solubilized in monomeric form by $C_{12}E_8$ at 0.1 mM Ca^{2+}, pH 7.5, in the presence of glycerol retains high enzymatic activity for several hours (Dean & Tanford, 1978; Møller et al., 1980). The activity of soluble Ca^{2+}-ATPase is much less stable in the absence of glycerol, especially at low pH and Ca^{2+} concentration (Dean & Tanford, 1978; Møller et al., 1980; Andersen et al., 1982; Kosk-Kosicka et al., 1983; Gafni & Boyer, 1984; Martins & de Meis, 1985).

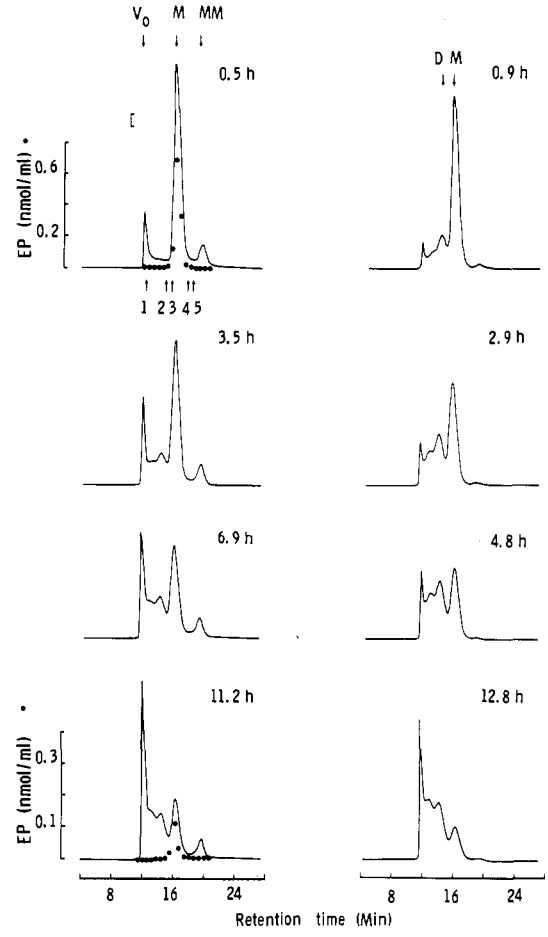

FIGURE 1: Time-dependent aggregation of soluble Ca^{2+}-ATPase. (Left side) Ca^{2+}-ATPase was solubilized in $C_{12}E_8$ as described under Experimental Procedures and diluted to obtain final concentrations of 20 mM Tes (pH 7.0), 0.1 M KCl, 50 μM Ca^{2+}, 10 mM Mg^{2+}, 0.5 mg of protein/mL, and 5 mg of $C_{12}E_8$/mL. After incubation at 20 °C for various time periods, 100-μL aliquots were subjected to HPLC in a TSK G 3000 SW column with 20 mM Tes (pH 7.0), 0.1 M KCl, 1 mM EGTA, 1.5 mM $CaCl_2$, 10 mM $MgCl_2$, and 5 mg of $C_{12}E_8$/mL as eluant. Time after solubilization is indicated on the chromatograms. (Right side) Soluble Ca^{2+}-ATPase was delipidated as described under Experimental Procedures by HPLC in the presence of 20 mM Tes (pH 7.0), 0.1 M KCl, 50 μM Ca^{2+}, 10 mM $MgCl_2$, and 5 mg of $C_{12}E_8$/mL and was then incubated at 0.5 mg of protein/mL and rechromatographed as described above. Time after delipidation is indicated on the chromatograms. Line shows absorbance at 226 nm. Vertical bar indicates ΔE_{226} corresponding to 10 μg of Ca^{2+}-ATPase protein/mL. Phosphorylation capacity measured in the eluted fractions with [γ-^{32}P]ATP as described under Experimental Procedures is shown as dots. M and D indicate the major Ca^{2+}-ATPase peaks discussed in this paper. MM indicates mixed micelles of phospholipid and detergent. Elution positions 1, 2, 3, 4, and 5 represent thyroglobulin (R_s = 8.6 nm), ferritin (R_s = 6.3 nm), aspartate transcarbamylase (R_s = 6.0 nm), catalase (R_s = 5.2 nm), and aldolase (R_s = 4.6 nm), respectively.

Figure 1 shows that the instability of Ca^{2+}-ATPase solubilized at 5 mg of $C_{12}E_8$/mL (9.30 mM) without glycerol is accompanied by aggregation of the protein as evidenced by molecular sieve HPLC in a TSK G 3000 SW column. In this experiment, soluble Ca^{2+}-ATPase was incubated with 50 μM Ca^{2+} and 10 mM Mg^{2+}, pH 7.0 at 20 °C, in the presence and

absence of endogenous lipid (~0.5 g of phospholipid/g of protein). After various time periods, aliquots were taken for HPLC in the presence of 500 μM Ca^{2+}. Thirty minutes after solubilization (i.e., the time necessary to perform precentrifugation and HPLC), the major part of the Ca^{2+}-ATPase protein elutes as a distinct peak (M) with high enzymatic activity. As will be shown later, this peak represents monomeric Ca^{2+}-ATPase. After some time (30–60 min for the delipidated sample, up to 2–3 h for the sample without separation of endogenous lipid), another peak (D) elutes between peak M and the void volume. After longer incubation times, a considerable fraction of the protein has formed larger aggregates eluting close to the void volume. Neither peak D nor the larger aggregates possess activity as indicated by the lack of phosphorylation from ATP (Figure 1). Tryptic digestion patterns [cf. Andersen et al. (1985b)] document that the aggregated protein consists of Ca^{2+}-ATPase. The aggregation process as monitored by the disappearance of peak M takes place most rapidly in the delipidated sample. However, more void volume material is formed in the presence of lipid, suggesting a role of lipid in formation of larger Ca^{2+}-ATPase oligomers [cf. le Maire et al. (1978) and below].

Inclusion of 5 mM DTT in the buffer medium did not protect against aggregation, but when more Ca^{2+} was added, the rate of aggregation declined. Thus, after 10 h there was still 90% of the monomer left in the presence of 500 μM Ca^{2+} (results not shown). After addition of EGTA to totally remove free Ca^{2+}, aggregation proceeded very rapidly (less than 10% monomer present after 30 min). The rate of aggregation further increased when soluble Ca^{2+}-ATPase was incubated with EGTA at a higher protein concentration. Above 4 mg of protein/mL, the rate became constant and identical with the rate of irreversible inactivation. These results suggest that time-dependent aggregation is a secondary phenomenon, which follows irreversible inactivation of the Ca^{2+}-depleted enzyme form (E_2). Similar secondary aggregation has been observed for the (Na^+, K^+)-ATPase (Jørgensen & Andersen, 1986).

To study the stability of inactive aggregates, HPLC elution patterns were examined after dilution of EGTA-treated sample in various media. When aggregates had been formed in the absence of reducing agent, dissociation could neither be induced by dilution to 1 μg of protein/mL in 10 mg of $C_{12}E_8$/mL nor be induced by dilution in 1% $NaDodSO_4$. However, when the aggregates had been formed in the presence of 5 mM DTT, they were dissociated by dilution in $NaDodSO_4$ but not in $C_{12}E_8$. From these results, we conclude that aggregation of inactivated Ca^{2+}-ATPase primarily involves strong hydrophobic interactions which are supplemented by the formation of disulfide bonds in the absence of reducing agent. Previous results have also indicated an increased reactivity of thiol groups in soluble Ca^{2+}-ATPase after irreversible inactivation (Andersen et al., 1980).

Assignment of Molecular Size. To obtain information about the molecular size of the Ca^{2+}-ATPase–protein complexes separated by HPLC, various standard proteins were applied to the columns as suggested by le Maire et al. (1980). The peak of active Ca^{2+}-ATPase (M) in Figure 1 elutes between catalase (R_s = 5.2 nm) and aspartate transcarbamylase (R_s = 6.0 nm). Interpolation by use of Ackers plot of R_s as a function of $\mathrm{erf}^{-1}(1 - K_D)$ or by plotting R_s as a function of $K_D^{1/3}$ gives an apparent Stokes radius of 5.6 ± 0.2 nm for peak M Ca^{2+}-ATPase protein. As shown previously (le Maire et al., 1980), the true Stokes radius of monomeric Ca^{2+}-ATPase solubilized in $C_{12}E_8$ as determined by hydrodynamic studies is 5.1–5.5 nm. Therefore, the elution position in Figure 1

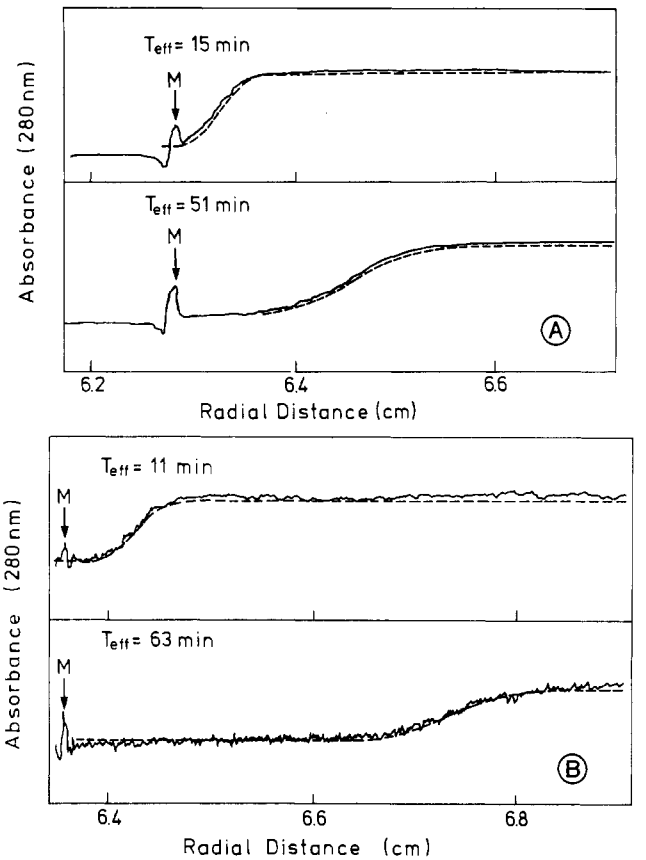

FIGURE 2: Sedimentation velocity profiles of peaks' M and D protein. Peak M (A) was isolated by HPLC in the presence of 20 mM Tes (pH 7.0), 0.1 M NaCl, 1.0 mM EGTA, 1.5 mM $CaCl_2$, 10 mM $MgCl_2$, and 5 mg of $C_{12}E_8$/mL. Peak D (B) was isolated by HPLC with the same eluant as for peak M, but after treatment of soluble enzyme with 1 mM excess EGTA for 8 min. Effective sedimentation times are indicated together with the meniscus position (M). Dashed lines are theoretical curves simulated as described under Experimental Procedures on the basis of diffusion coefficients for monomeric (4.0×10^{-7} cm^2/s) (A) and dimeric (3.0×10^{-7} cm^2/s) (B) Ca^{2+}-ATPase.

suggests that peak M consists of Ca^{2+}-ATPase monomer. Peak D has an apparent Stokes radius of 7.0 ± 0.2 nm. In $NaDodSO_4$, peak M protein elutes close to the position of erythrocyte membrane band III. Peak D protein elutes later than both thyroglobulin subunit (M_r 330 000) and spectrin monomer (M_r 220 000–240 000), consistent with a Ca^{2+}-ATPase dimer. Similar results were obtained by $NaDodSO_4$ gel electrophoresis in the absence of reducing agent.

The assignment of peaks M and D as monomeric and dimeric Ca^{2+}-ATPase has been confirmed by analytical ultracentrifugation. Stable peak M protein was isolated by HPLC in the presence of 5 mg of $C_{12}E_8$/mL and 500 μM Ca^{2+}. Peak D protein was isolated by HPLC after incubation with excess EGTA for 8 min. Sedimentation equilibrium analysis of these samples resulted in linear plots with slopes corresponding to molecular weights of 114 000 (peak M) and 194 000 (peak D). Sedimentation velocity profiles are shown in Figure 2A,B. Sedimentation coefficients for peak M and peak D protein were 5.0 ± 0.3 and 7.2 ± 0.2 S, respectively. For both preparations, only a single boundary formed. It can be seen from Figure 2 that the spreading of the boundary as a function of time can be accounted for entirely by diffusion of a homogeneous species with the diffusion coefficients calculated for monomeric Ca^{2+}-ATPase (4.0×10^{-7} cm^2/s) and dimeric Ca^{2+}-ATPase (3.0×10^{-7} cm^2/s).

The Stokes radii of monomeric and dimeric Ca^{2+}-ATPase, calculated from the sedimentation equilibrium and sedimen-

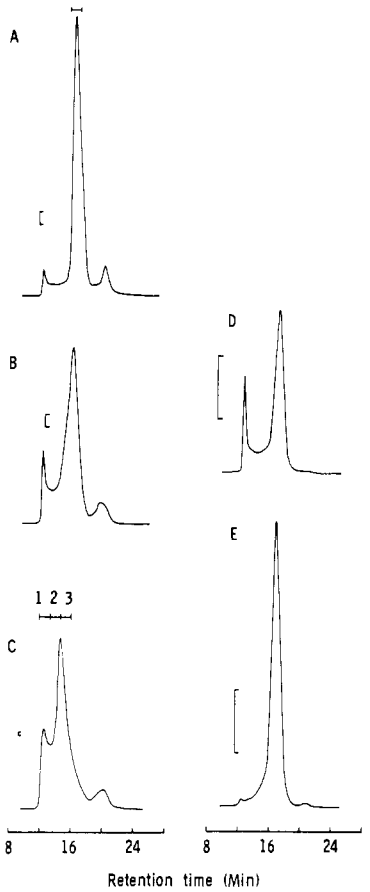

FIGURE 3: Effect of detergent and protein concentration on small-zone HPLC of soluble Ca^{2+}-ATPase in a TSK G 3000 SW column. The eluant composition was 20 mM Tes (pH 7.0), 0.1 M KCl, 10 mM $MgCl_2$, 1.5 mM $CaCl_2$, 1.0 mM EGTA, and 5 mM DTT with the following concentrations of $C_{12}E_8$: 2.0 (A), 0.15 (B), and 0.15 mg/mL (C). In (A) and (B), 400 μg of soluble protein was injected, whereas 2.0 mg of Ca^{2+}-ATPase was applied in (C). Rechromatographies of fraction 2 of (C) and fraction 3 of (C) are shown in (D) and (E), respectively. The eluant composition was the same as in (C). The vertical bar indicates ΔE_{226} corresponding to 10 μg of Ca^{2+}-ATPase protein/mL. In (A) and (C), the fractions selected for rechromatography and ligand binding measurements (Table I) are indicated.

tation velocity data, are 56 and 65 Å, respectively, in fair agreement with previous analytical ultracentrifuge results and with the column calibration data.

Reversible Oligomerization of $C_{12}E_8$-Solubilized Ca^{2+}-ATPase. In addition to irreversible aggregation of inactive Ca^{2+}-ATPase, we also obtained evidence for reversible self-association of active Ca^{2+}-ATPase under appropriate conditions. This phenomenon was studied in media containing 500 μM Ca^{2+} and 5 mM DTT to provide optimal protection against irreversible aggregation.

Figure 3 shows effects of variation of detergent and protein concentrations on the HPLC elution pattern. When the concentration of $C_{12}E_8$ is reduced to 0.15 mg/mL (0.28 mM), the major peak broadens and a distinct shoulder appears (Figure 3B). Furthermore, the amount of material eluting at the void volume position increases. When in addition the protein concentration is increased (ratio of detergent to protein at the peak <1 mg of $C_{12}E_8$/mg of protein), the major part of the peak moves to an elution position closer to the void volume (Figure 3C). This new position is almost identical with the elution position of the dimer shown in Figure 1. However, in contrast to the latter, most of the self-associated Ca^{2+}-ATPase polypeptide in Figure 3C is enzymatically active and readily dissociates on dilution. This dissociation can be demonstrated by rechromatography of fractions 2 and 3 of Figure 3C (Figure 3D,E) which results in 10–20 times dilution. For both fractions, the major part of the rechromatographed protein elutes as a monomer. In addition, there is a small amount of irreversibly aggregated material present in fraction 2.

In the analytical ultracentrifuge, fraction 3 of Figure 3C gave rise to a broad, drawn-out boundary, distinctly different from that of irreversibly associated dimer, and with an average sedimentation coefficient of 6 S, i.e., intermediate between those of monomeric and dimeric Ca^{2+}-ATPase.

In a few experiments, otherwise similar to those of Figure 3A,C, the pump pressure was varied between 5 and 40 bar (resulting in flow rates between 0.25 and 2.0 mL/min in the long TSK G 3000 SW column). The upper limit of 40 bar corresponds to the pressure at the bottom of the ultracentrifuge cell during a sedimentation velocity run. We did not observe any effect of this pressure variation on the elution patterns.

The dependence of the reversible equilibrium between Ca^{2+}-ATPase complexes on protein and detergent concentration has also been examined by the large-zone chromatographic procedure of Valdes and Ackers (1979) as suggested by Silva and Verjovski-Almeida (1985). Soluble Ca^{2+}-ATPase was delipidated on a long TSK G 3000 SW column (duration at maximum 15 min) at the $C_{12}E_8$ concentration to be studied. The sample was then applied to a short TSK GSWP column after appropriate dilution in the same buffer. Representative chromatograms obtained at three different protein concentrations and at $C_{12}E_8$ concentrations of 0.10 mg/mL (0.186 mM) and 10 mg/mL (18.6 mM) are shown in Figure 4A,B. It is seen that the front centroid position changes little when protein concentration is varied at the higher detergent concentration, whereas a considerable change in position is induced by variation of protein concentration at 0.1 mg of $C_{12}E_8$/mL. The shape of the leading boundary is unimodal, in accordance with a reversible equilibrium which is rapid on the time scale of HPLC. By contrast, when samples containing a large fraction of irreversibly aggregated protein (cf. Figure 1) were subjected to large-zone chromatography, several components could easily be distinguished (not shown). As can be seen in Figure 4, the appearance of the trailing boundary varies between unimodal and bimodal depending on protein and detergent concentration. A bimodal trailing boundary indicates the presence of higher oligomers than dimer (Valdes & Ackers, 1979).

Large-zone chromatograms were quantitatively evaluated in a number of conditions by measuring the front centroid positions. Results are shown in Figure 5 in which elution positions corresponding to monomeric and stable dimeric Ca^{2+}-ATPase are also indicated. The data define conditions in which soluble active Ca^{2+}-ATPase predominantly is in monomeric form (e.g., detergent concentration 10 mg/mL, protein concentration <1 mg/mL; detergent concentration 2 mg/mL, protein concentration <0.4 mg/mL; detergent concentration 0.5 mg/mL, protein concentration <0.1 mg/mL). It is seen that even at quite high protein concentrations it is possible to maintain enzymatically active Ca^{2+}-ATPase in a monomeric state, provided that an adequately high $C_{12}E_8$ concentration is present. Association constants for dimerization range from about 10^5 to 10^6 M^{-1} depending on $C_{12}E_8$ concentration. It should be noted that variation of detergent concentration affects association of Ca^{2+}-ATPase even above the cmc (0.05 mg/mL), suggesting that micellar detergent is involved in the association process (see Discussion). In accordance with this view, we found that binding of $C_{12}E_8$ to Ca^{2+}-ATPase differed in various aggregational states, as in-

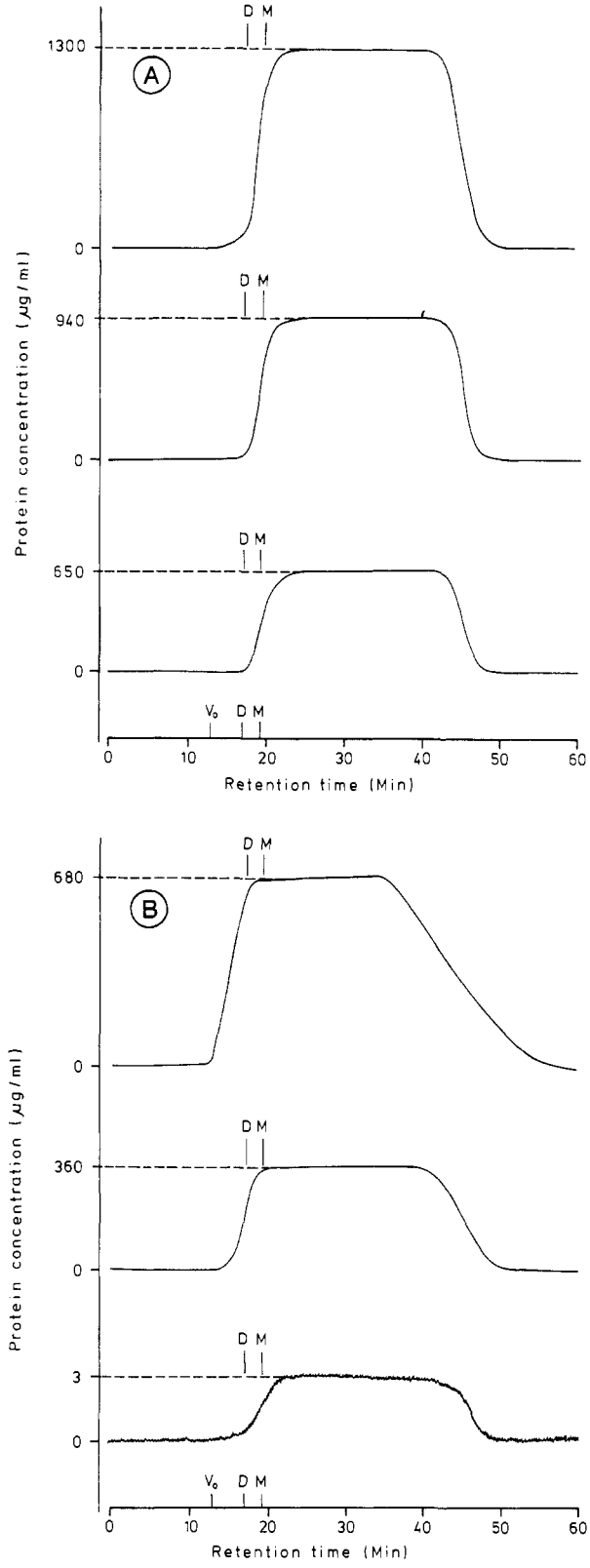

FIGURE 4: Large-zone HPLC of soluble Ca^{2+}-ATPase. Ca^{2+}-ATPase protein was delipidated by small-zone HPLC using the same eluant as for large-zone chromatography. Delipidated protein (2.5 mL) was subjected to large-zone chromatography in a small TSK GSWP column run at 0.1 mL/min. The eluant composition was 20 mM Tes (pH 7.0), 0.1 M NaCl, 10 mM $MgCl_2$, 1.5 mM $CaCl_2$, 1.0 mM EGTA, 5 mM DTT, and 10 mg of $C_{12}E_8$/mL (A) or 0.1 mg of $C_{12}E_8$/mL (B). The protein concentrations are indicated by the plateau values. Boundary centroid positions of pure monomeric (M) and dimeric (D) Ca^{2+}-ATPase are indicated together with the void volume position (V_0).

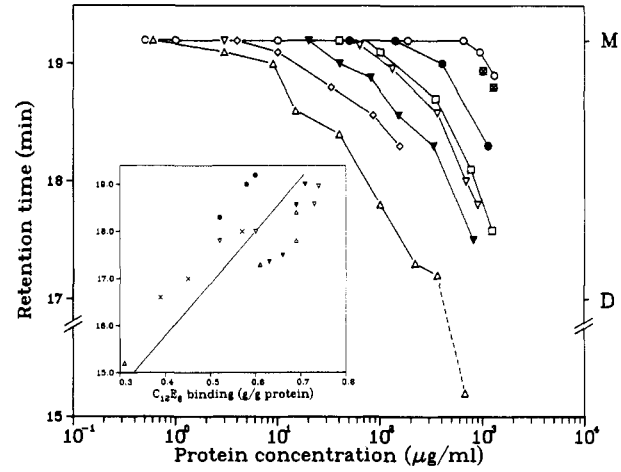

FIGURE 5: Large-zone HPLC of soluble Ca^{2+}-ATPase at various protein and detergent concentrations. The experiments were conducted as in Figure 4 at 0.10 mg of $C_{12}E_8$/mL (△,◇), 0.25 mg of $C_{12}E_8$/mL (▼,▽), 0.50 mg of $C_{12}E_8$/mL (□), 2.0 mg of $C_{12}E_8$/mL (●), and 10 mg of $C_{12}E_8$/mL (○,⊗). Delipidation was performed at the same $C_{12}E_8$ concentrations except in one experiment (◇) in which a higher concentration (0.5 mg of $C_{12}E_8$/mL) was used. The latter procedure ensured a phospholipid content less than 0.002 g/g of protein. In (▽), 1 mM ADP was present in the eluant. The abscissa indicates protein concentrations on a logarithmic scale. The ordinate shows boundary centroid positions. M and D indicate positions of pure monomeric and dimeric Ca^{2+}-ATPase, respectively. Inset: Detergent binding to Ca^{2+}-ATPase at various protein and detergent concentrations. Binding was measured by the aid of ^{14}C-labeled $C_{12}E_8$ present in the solutions used for solubilization, delipidation, and large-zone chromatography. Identical values were obtained in the plateau region of the large-zone chromatogram and in the corresponding delipidated sample before injection. The symbols represent the same experiments as in the main figure. In one set of experiments (×), irreversibly aggregated Ca^{2+}-ATPase formed by exposure to EGTA for various times was studied. The binding data are shown as a function of elution position. The regression line for all points is shown.

dicated by a positive correlation between detergent binding at the plateau and the front centroid position (Figure 5 inset). The data were somewhat scattered as a consequence of the inaccuracy in measuring a small increase in the concentration of ^{14}C-labeled detergent on top of a high base line. Nevertheless, it is clear from the results shown in Figure 5 that there is a general trend toward higher values for detergent binding to the monomer, relative to dimer and higher oligomers.

The variation of association constant with the concentration of micellar detergent cannot be accounted for by the presence of residual phospholipid in the preparations. In the range of detergent concentrations between 0.25 and 10 mg of $C_{12}E_8$/mL, delipidation was virtually complete (<1 mol of phospholipid retained/mol of Ca^{2+}-ATPase). At the lowest $C_{12}E_8$ concentration (0.1 mg/mL), approximately 8 mol of phospholipid/mol of Ca^{2+}-ATPase remained after one passage through the large HPLC column, but when the protein was delipidated at 0.5 mg of $C_{12}E_8$/mL and then diluted to obtain a detergent concentration of 0.1 mg/mL during large-zone HPLC the association constant was still found to be significantly higher than that measured in the presence of 0.5 mg of $C_{12}E_8$/mL [Figure 5, compare (◇) with (□)].

On the other hand, it is clear from Figure 5 that there is some effect of the presence of residual phospholipid on the association constant measured at 0.1 mg of $C_{12}E_8$/mL [compare (◇) with (△)]. In a few experiments, large-zone chromatography was performed also on soluble Ca^{2+}-ATPase from which no endogenous lipid had been removed. The general appearance of the leading boundaries under these conditions was very similar to the boundaries shown in Figure 4. It was

Table I: Ligand Binding Capacity and Ca^{2+}-ATPase Activity in Soluble Ca^{2+}-ATPase Fractionated by HPLC

	Ca^{2+} binding[c] (nmol/mg)	phosphorylation[d] from ATP (nmol/mg)	vanadate binding[e] (nmol/mg)	Ca^{2+}-ATPase[f] (μmol mg^{-1} min^{-1})
oligomer (Figure 3C)[a]				
fraction 1		1.0 ± 0.1		0.8
fraction 2	8.7 ± 0.4[g]	4.1 ● 0.3	5.9 ± 1.0	2.3
fraction 3	11.4 ± 0.7	5.0 ± 0.2	6.5 ± 0.9	3.8
monomer (Figure 3A)[b]	12.6 ± 1.0	5.3 ± 0.2	7.4 ± 0.8	5.4

[a] Detergent and protein concentrations were not kept constant in the different experiments, but only data from experiments in which the major peak eluted at the same position as in Figure 3C were used for calculation of the binding data. Fractions 1, 2, and 3 were selected as shown in Figure 3C. [b] The detergent and protein concentrations were as in Figure 3A. One fraction from the major peak was selected as indicated in Figure 3A. [c] $^{45}Ca^{2+}$ binding was measured by the method of Hummel and Dreyer (1962). The eluant contained 50 μM $^{45}CaCl_2$, 30 μM EGTA, 5 mM $MgCl_2$, 0.1 M KCl, 20 mM Tes (pH 7.0), and 5 mM DTT together with 0.15 mg of $C_{12}E_8$/mL (oligomer) or 2 mg of $C_{12}E_8$/mL (monomer). [d] Phosphorylation was measured after addition of 25 μM [γ-^{32}P]ATP to the eluate as described under Experimental Procedures. The eluant contained 1.5 mM $CaCl_2$, 1.0 mM EGTA, 10 mM $MgCl_2$, 0.1 M KCl, 20 mM Tes (pH 7.0), 5 mM DTT together with 0.15 mg of $C_{12}E_8$/mL (oligomer) or 2 mg of $C_{12}E_8$/mL (monomer). [e] [^{48}V]binding was measured in the eluate as described under Experimental Procedures. The eluant contained 1.0 mM EGTA, 10 mM $MgCl_2$, 0.1 M KCl, and 20 mM Tes (pH 7.0) together with 0.05 mg of $C_{12}E_8$/mL (oligomer) or 2 mg of $C_{12}E_8$/mL (monomer). [f] Ca^{2+}-ATPase activity was measured after addition of 5 mM ATP to the eluate as described under Experimental Procedures. The eluant contained 0.1 mM $CaCl_2$, 10 mM $MgCl_2$, 0.1 M KCl, and 20 mM Tes (pH 7.0) together with 0.15 mg of $C_{12}E_8$/mL (oligomer) or 2.0 mg of $C_{12}E_8$/mL. [g] When shown, SD corresponds to three experiments.

found that endogenous lipid contributes less than 10% of the total absorbance at 226 nm (cf. also Figure 1 peak MM). Figure 5 shows that the effect of the presence of this lipid on the front centroid position is negligible at 10 mg of $C_{12}E_8$/mL (⊗). This result suggests that the lipid is almost totally distributed into the detergent micelles when these are present in high concentration, in accordance with the very efficient delipidation obtained by small-zone HPLC in such conditions.

The effect of nucleotides on the monomer–dimer equilibrium has also been examined by large-zone chromatography. As seen in Figure 5 (▽) 1 mM ADP promotes dissociation of dimeric Ca^{2+}-ATPase into monomer in accordance with previous results of small-zone chromatography (Andersen & Vilsen, 1985). This effect is of interest in relation to low-affinity modulatory effects of nucleotide during the ATPase cycle (see Discussion).

Ligand Binding to Monomeric and Dimeric Ca^{2+}-ATPase. Ca^{2+} binding has been measured by the method of Hummel and Dreyer (1962) at relatively low free Ca^{2+} and high Mg^{2+} concentrations to ensure that only high-affinity sites were studied. The same detergent and protein concentrations as in Figure 3A,C were employed. Binding was calculated in the fractions indicated in these figures. The phosphorylation capacity from ATP was also measured in similar experiments (Table I). It is evident from these data that monomeric and dimeric Ca^{2+}-ATPases have the same number of high-affinity Ca^{2+} binding sites per phosphorylation site (approximately 2).

Vanadate binding to the soluble Ca^{2+}-ATPase forms was also measured. The technique for doing this differed from the one used for Ca^{2+} binding measurements. To avoid irreversible inactivation in the absence of Ca^{2+}, the enzyme was incubated with vanadate and EGTA before solubilization. After vanadate had bound, the soluble Ca^{2+}-ATPase was stable, and it could be reactivated by addition of Ca^{2+} to promote dissociation of vanadate [cf. Andersen & Vilsen (1985)]. In the present experiments, the vanadate-reacted soluble Ca^{2+}-ATPase was chromatographed in buffer containing EGTA without vanadate. This was possible because release of vanadate is too slow to induce a significant error when the HPLC is performed within 8 min. As shown previously (Andersen & Vilsen, 1985), the binding of vanadate shifts the monomer–oligomer equilibrium in the direction of the monomer. Therefore, to obtain an elution profile similar to that in Figure 3C, the detergent concentration had to be decreased to 0.05 mg/mL. The results shown in Table I indicate that the capacity for vanadate binding is identical in monomeric and dimeric Ca^{2+}-ATPase but in both cases it is somewhat higher than the phosphorylation capacity. Several explanations are possible: (1) A significant amount of nonphosphorylated intermediate is present in the steady-state ATP hydrolysis experiment in which phosphorylation is measured (note that the ratio between Ca^{2+} bound and EP is slightly above 2). (2) The stabilization induced by binding of vanadate hinders denaturation caused by solubilization. In line with this suggestion, much less aggregated protein (V_0 and peak D) was observed in the vanadate-reacted soluble Ca^{2+}-ATPase preparation relative to the Ca^{2+} form (Figure 1), in the absence of DTT. Furthermore, the levels of vanadate binding and phosphorylation are closer before solubilization (Andersen & Møller, 1985). (3) The presence of decavanadate or other oligoanions (Varga et al., 1985) together with monovanadate increases the apparent binding capacity. We have excluded the latter possibility by performing binding measurements at various vanadate concentrations (10–100 μM) with no significant change in the apparent capacity.

Ca^{2+}-ATPase activity was also measured in dimeric as well as in monomeric Ca^{2+}-ATPase (Table I). The turnover rate is somewhat higher in the monomer relative to the dimer, suggesting that protein–protein interactions or detergent binding affects the overall hydrolysis rate.

The fraction of total phosphoenzyme which is ADP insensitive (E_2P) has also been measured on the HPLC-purified soluble monomer (Figure 3A) after adjusting the pH to 8.1 to decrease the rate of dephosphorylation of E_2P (Andersen et al., 1985a). The E_2P phosphoenzyme fraction was 91%, 49%, and 19% in media containing 0.15 mM Ca^{2+} + 10 mM Mg^{2+}, 1.0 mM Ca^{2+} + 10 mM Mg^{2+}, and 1.0 mM Ca^{2+} + 1.0 mM Mg^{2+}, respectively. These data confirm our previous conclusion that a single polypeptide chain is able to perform the $E_1P \rightarrow E_2P$ conformational transition (Andersen et al., 1985a,b) but provides the additional information that lipid is not required, neither, for this transition (the phospholipid content of the monomeric peak is less than 1 mol/mol of Ca^{2+}-ATPase). The variation in E_2P phosphoenzyme fraction with Ca^{2+} and Mg^{2+} is similar to that observed for membrane-bound enzyme (Andersen et al., 1985a).

We have shown that ATP and vanadate bind simultaneously to Ca^{2+}-ATPase (Andersen & Møller, 1985). However, previously it was not excluded that two different binding sites on interacting subunits are involved. The experiments shown in Table II were designed to answer this question. The vanadate-reacted monomeric Ca^{2+}-ATPase was passed slowly through the HPLC column to allow dissociation of approximately 50% of bound vanadate in the absence of nucleotide. The effect of ATP and Mg^{2+} on the dissociation of vanadate was examined. In the absence of Mg^{2+}, ATP (25 μM) clearly hinders release of vanadate from the monomeric protein. In

Table II: Effect of ATP and Mg^{2+} on Release of Vanadate from Soluble Monomeric Ca^{2+}-ATPase during HPLC

conditions of HPLC[a]	vanadate binding (nmol/mg)[b] 8 min	40 min
1 mM EDTA	7.6	3.8
1 mM EDTA + 25 μM ATP	7.7	6.8
1 mM EDTA + 1 mM ATP	7.6	7.8
10 mM $MgCl_2$	7.6	3.9
10 mM $MgCl_2$ + 25 μM ATP	7.5	4.5
10 mM $MgCl_2$ + 1 mM ATP	7.6	6.3

[a] Vanadate-reacted Ca^{2+}-ATPase (400 μg) was solubilized and chromatographed as described under Experimental Procedures. The eluant contained 20 mM Tes (pH 7.0), 0.1 M KCl, 1.0 mM EGTA, and 2 mg of $C_{12}E_8$/mL together with the additions indicated. [b] Vanadate binding was measured in the peak fraction (cf. Figure 3A) after HPLC in a 300-mm column at a pump rate of 0.8 mL/min (elution time 8 min) or 0.16 mL/min (elution time 40 min).

the presence of 10 mM Mg^{2+}, the ATP effect occurs with much lower apparent affinity. Thus, the soluble monomer isolated by HPLC behaves in a manner similar to that observed for membrane-bound Ca^{2+}-ATPase: ATP but not MgATP binds with high affinity to the vanadate-reacted enzyme. On the basis of these data, it can be concluded that the binding sites for ATP and vanadate are on the same polypeptide chain.

Discussion

The present study has permitted us to define conditions in which lipid-depleted and enzymatically active Ca^{2+}-ATPase, solubilized by $C_{12}E_8$, exists in monomeric and in oligomeric forms. This was achieved by the use of HPLC which in comparison with the classical gel materials permits an improved analysis of the presence of enzymatically active and inactive Ca^{2+}-ATPase species in various aggregational states within a short time period. To obtain preparations of monomeric, enzymatically active Ca^{2+}-ATPase requires that the detergent concentration is above the cmc (0.05 mg of $C_{12}E_8$/mL) and that the detergent to protein concentration ratio is well above 1:1 (w/w). Below this ratio, significant reversible formation of oligomer occurs. A situation can be achieved in which the major part of the oligomer consists of dimer (cf. Figure 3C). However, such a situation is stringently dependent on experimental conditions, since the dimer is in reversible equilibrium not only with the Ca^{2+}-ATPase monomer but also with higher oligomeric forms. In the present study, it has been possible for the first time to unequivocally identify the elution position of dimeric Ca^{2+}-ATPase. This required the use of protein, which has been prepared in a stable self-associated state by inactivation. The dimeric state was documented by sedimentation equilibrium and sedimentation velocity analysis.

Association constants for dimerization of active enzyme (10^5–10^6 M^{-1}, depending on detergent concentration) were significantly lower than previously measured by Silva and Verjovski-Almeida (1985) by large-zone chromatography on Sephacryl S-300 columns. Their studies as well as ours were conducted on virtually delipidated preparations and in the presence of reducing agent. However, we noticed a time-dependent aggregation of soluble Ca^{2+}-ATPase, which was essentially irreversible in nonionic detergent (association constant $>10^8$ M^{-1}) even without disulfide formation. The present data suggest that the longer times involved in chromatography on ordinary gel columns may lead to a similar aggregation during the separation process.

We found that the formation of inactive oligomeric complexes could be speeded up by addition of EGTA. In the absence of reducing agent, these aggregates were stabilized by spontaneous formation of intermolecular disulfide linkages. We surmise that the large tendency for aggregation after irreversible inactivation may reflect exposure of hydrophobic residues to solvent in relation to inactivation [cf. Andersen et al. (1982) and Jørgensen & Andersen (1986)]. Previous studies have indicated changes in secondary structure when the soluble Ca^{2+}-ATPase is treated with EGTA (Andersen et al., 1980).

Self-association of enzymatically active delipidated Ca^{2+}-ATPase was reduced by an increase in detergent concentration. Therefore, unbound detergent must be involved in the dissociation reaction. In agreement with this conclusion, we found a decreased binding of $C_{12}E_8$ by oligomeric Ca^{2+}-ATPase. Accordingly, the reaction scheme may be formulated:

$$nPD_x \rightleftharpoons P_nD_y + (nx - y)D$$

where PD refers to protein–detergent complex, D to detergent, and the subscripts to average numbers of molecules involved in the reaction. Since the effect of detergent on the association equilibria is observed at detergent concentrations well above the cmc, the reaction presumably involves detergent micelles.

The levels of binding of $C_{12}E_8$ measured by HPLC (0.7 g/g of protein for monomeric Ca^{2+}-ATPase and 0.5 g/g of protein for dimeric Ca^{2+}-ATPase) are higher than previously reported by equilibrium chromatography on DEAE-cellulose columns (le Maire el al., 1983) [for a discussion of the methodological problems inherent in the use of chromatographic materials for the determination of detergent binding, see Møller et al. (1986)]. By rechromatography in TSK G 3000 SW of Ca^{2+}-ATPase eluted from a DEAE-cellulose column, we have now obtained evidence that the ion-exchange procedure underestimates detergent binding by one-third (unpublished observations).

The reduced detergent binding by active oligomeric Ca^{2+}-ATPase may indicate that hydrophobic interactions are involved also in the self-association of native Ca^{2+}-ATPase. Our data on the effect of lipid on the association constant at low detergent concentration suggest a possible role of lipid in mediating such interactions in the membrane [cf. Andersen et al. (1981)].

ADP was found to shift the monomer–oligomer equilibrium towards the monomer in agreement with previous results (Andersen & Vilsen, 1985), showing that ligands which bind to the phosphorylation site destabilize protein–protein contacts. In this connection, it is of interest that nucleotide exerts a somewhat different modulatory effect on monomeric Ca^{2+}-ATPase as compared to oligomeric or membrane-bound Ca^{2+}-ATPase (Møller et al., 1980). The destabilization of interpeptide interactions induced by nucleotide binding provides a possible explanation for this if it is assumed that protein–protein contacts impose restrictions on the rate-limiting conformational change.

Some enzymatically inactive aggregates were usually present even when Ca^{2+}-ATPase was subjected to HPLC immediately after solubilization (Figure 1). The presence of denatured Ca^{2+}-ATPase protein may provide an explanation for the relatively low ligand binding capacity of unfractionated Ca^{2+}-ATPase as compared to the estimated total Ca^{2+}-ATPase polypeptide content in the preparation [7–8 nmol/mg; cf. also Barrabin et al. (1984)]. Our membranous Ca^{2+}-ATPase preparations usually bind 8–10 nmol of Ca^{2+}/mg of protein with high affinity and have phosphorylation and vanadate binding capacities of 4–5 nmol/mg. The data of Table I show that some purification of active Ca^{2+}-ATPase can be achieved by precentrifugation and HPLC but only in the case of va-

nadate does binding capacity approach the theoretical value. This may reflect the ability of vanadate to protect the soluble enzyme against irreversible inactivation. An important aspect of the data of Table I is that there is no significant difference between the number of sites in monomeric and in reversibly associated dimeric Ca^{2+}-ATPase. Thus, the data do not support the concept of "half-of-the-sites reactivity" in the dimer (Dupont, 1980; Silva & Verjovski-Almeida, 1983).

We have previously presented evidence that the soluble monomeric Ca^{2+}-ATPase is able to perform the transition from ADP-sensitive to ADP-insensitive phosphoenzyme with a resultant change in Ca^{2+} affinity (Andersen et al., 1985a,b). The present data show that this transition which is dependent on Mg^{2+} and Ca^{2+} concentrations can occur in the HPLC-purified delipidated monomeric enzyme. Furthermore, the simultaneous binding of vanadate and ATP (Andersen & Møller, 1985) also occurs to a single Ca^{2+}-ATPase polypeptide chain (Table II).

In conclusion, the present data do not support the view that active Ca^{2+}-ATPase peptides have a high affinity for self-association to form specific complexes of functional significance. However, due to the high protein concentration in the native membrane, most of the Ca^{2+}-ATPase polypeptides are likely to be in a self-associated state in this environment. The HPLC technique for preparation of defined monomers and oligomers of Ca^{2+}-ATPase is likely to be useful in future studies of the role of protein–protein interaction in Ca^{2+} translocation. In addition, the molecular size assignment of the elution positions may be of value in studies of other membrane proteins.

ACKNOWLEDGMENTS

We thank Liselotte Madsen for skillful technical assistance. Aspartate transcarbamylase was a generous gift from Dr. Marc le Maire.

Registry No. ATPase, 9000-83-3; $C_{12}E_8$, 3055-98-9.

REFERENCES

Andersen, J. P., & Møller, J. V. (1985) *Biochim. Biophys. Acta 815*, 9–15.

Andersen, J. P., & Vilsen, B. (1985) *FEBS Lett. 189*, 13–17.

Andersen, J. P., le Maire, M., & Møller, J. V. (1980) *Biochim. Biophys. Acta 603*, 84–100.

Andersen, J. P., Fellmann, P., Møller, J. V., & Devaux, P. F. (1981) *Biochemistry 20*, 4928–4936.

Andersen, J. P., Møller, J. V., & Jørgensen, P. L. (1982) *J. Biol. Chem. 257*, 8300–8307.

Andersen, J. P., Gerdes, U., & Møller, J. V. (1983) in *Structure and Function of Membrane Proteins* (Quagliariello, E., & Palmieri, F., Eds.) pp 57–61, Elsevier/North-Holland, Amsterdam.

Andersen, J. P., Lassen, K., & Møller, J. V. (1985a) *J. Biol. Chem. 260*, 371–380.

Andersen, J. P., Jørgensen, P. L., & Møller, J. V. (1985b) *Proc. Natl. Acad. Sci. U.S.A. 82*, 4573–4577.

Barrabin, H., Scofano, H. M., & Inesi, G. (1984) *Biochemistry 23*, 1542–1548.

Bartlett, G. R. (1959) *J. Biol. Chem. 234*, 466–468.

Brandl, C. J., Green, N. M., Korczak, B., & MacLennan, D. H. (1986) *Cell (Cambridge, Mass.) 44*, 597–607.

Brotherus, J. R., Møller, J. V., & Jørgensen, P. L. (1981) *Biochem. Biophys. Res. Commun. 100*, 146–154.

Dean, W. L., & Tanford, C. (1978) *Biochemistry 17*, 1683–1690.

Dupont, Y. (1977) *Eur. J. Biochem. 72*, 185–190.

Dupont, Y. (1982) *Biochim. Biophys. Acta 688*, 75–87.

Franzini-Armstrong, C., & Ferguson, D. G. (1985) *Biophys. J. 48*, 607–615.

Gerhart, J. C., & Holoubek, H. (1967) *J. Biol. Chem. 242*, 2886–2892.

Hummel, J. P., & Dreyer, W. J. (1962) *Biochim. Biophys. Acta 63*, 530–533.

Jørgensen, K. E., Lind, K. E., Røigaard-Petersen, H., & Møller, J. V. (1978) *Biochem. J. 169*, 489–498.

Jørgensen, P. L., & Andersen, J. P. (1986) *Biochemistry 25*, 2889–2897.

Kosk-Kosicka, D., Kurzmack, M., & Inesi, G. (1983) *Biochemistry 22*, 2559–2567.

le Maire, M., Møller, J. V., & Tanford, C. (1976) *Biochemistry 15*, 2336–2342.

le Maire, M., Lind, K. E., Jørgensen, K. E., Røigaard, H., & Møller, J. V. (1978) *J. Biol. Chem. 253*, 7051–7060.

le Maire, M., Rivas, E., & Møller, J. V. (1980) *Anal. Biochem. 106*, 12–21.

le Maire, M., Kwee, S., Andersen, J. P., & Møller, J. V. (1983) *Eur. J. Biochem. 129*, 525–532.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Martin, D. W. (1983) *Biochemistry 22*, 2276–2282.

Martin, D. W., Tanford, C., & Reynolds, J. A. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 6623–6626.

Martins, O. B., & de Meis, L. (1985) *J. Biol. Chem. 260*, 6776–6781.

Møller, J. V., Lind, K. E., & Andersen, J. P. (1980) *J. Biol. Chem. 255*, 1912–1920.

Møller, J. V., le Maire, M., & Andersen, J. P. (1986) in *Progress in Protein–Lipid Interactions 2* (Watts & de Pont, Eds.) pp 147–196, Elsevier, Amsterdam.

Napolitano, C. A., Cooke, P., Segalman, K., & Herbette, L. (1983) *Biophys. J. 42*, 119–125.

Peterson, G. L. (1977) *Anal. Biochem. 83*, 346–356.

Silva, J. L., & Verjovski-Almeida, S. (1983) *Biochemistry 22*, 707–716.

Silva, J. L., & Verjovski-Almeida, S. (1985) *J. Biol. Chem. 260*, 4764–4769.

Takisawa, H., & Tonomura, Y. (1979) *J. Biochem. (Tokyo) 86*, 425–441.

Tanford, C., Nozaki, Y., & Rohde, M. F. (1977) *J. Phys. Chem. 81*, 1555.

Taylor, K., Dux, L., & Martonosi, A. (1984) *J. Mol. Biol. 174*, 193–204.

Valdes, R., & Ackers, G. K. (1979) *Methods Enzymol. 61*, 125–142.

Varga, S., Csermely, P., & Martonosi, A. (1985) *Eur. J. Biochem. 148*, 119–126.

Wang, C.-T., Saito, A., & Fleischer, S. (1979) *J. Biol. Chem. 254*, 9209–9219.