Biochimica et Biophysica Acta, 731 (1983) 290–303
Elsevier

BBA 71690

SOLUBLE AND ENZYMATICALLY STABLE ($Na^+ + K^+$)-ATPase FROM MAMMALIAN KIDNEY CONSISTING PREDOMINANTLY OF PROTOMER $\alpha\beta$-UNITS

PREPARATION, ASSAY AND RECONSTITUTION OF ACTIVE Na^+, K^+ TRANSPORT

JAAKKO R. BROTHERUS [a,†], LENE JACOBSEN [b] and PETER L. JØRGENSEN [b,*]

[a] *Department of Medical Chemistry, University of Helsinki, Helsinki (Finland) and* [b] *Institute of Physiology, Aarhus University, 8000 Aarhus C (Denmark)*

(Received December 7th, 1982)

Key words: ($Na^+ + K^+$)-ATPase; Enzyme reconstitution; Phospholipid vesicle; Detergent

Soluble ($Na^+ + K^+$)-ATPase consisting predominantly of $\alpha\beta$-units with M_r below 170000 was prepared by incubating pure membrane-bound ($Na^+ + K^+$)-ATPase (35–48 μmol P_i/min per mg protein) from the outer renal medulla with the non-ionic detergent dodecyloctaethyleneglycol monoether ($C_{12}E_8$). ($Na^+ + K^+$)-ATPase and potassium phosphatase remained fully active in the detergent solution at $C_{12}E_8$/protein ratios of 2.5–3, at which 50–70% of the membrane protein was solubilized. The soluble protomeric ($Na^+ + K^+$)-ATPase was reconstituted to Na^+, K^+ pumps in phospholipid vesicles by the freeze-thaw sonication procedure. Protein solubilization was complete at $C_{12}E_8$/protein ratios of 5–6, at the expense of partial inactivation, but ($Na^+ + K^+$)-ATPase and potassium phosphatase could be reactivated after binding of $C_{12}E_8$ to Bio-Beads SM2. At $C_{12}E_8$/protein ratios higher than 6 the activities were irreversibly lost. Inactivation could be explained by delipidation. It was not due to subunit dissociation since only small changes in sedimentation velocities were seen when the $C_{12}E_8$/protein ratio was increased from 2.9 to 46. As determined immediately after solubilization, $S_{20,w}$ was 7.4 S for the fully active ($Na^+ + K^+$)-ATPase, 7.3 S for the partially active particle, and 6.5 S for the inactive particle at high $C_{12}E_8$/protein ratios. The maximum molecular masses determined by analytical ultracentrifugation were 141000–170000 dalton for these protein particles. Secondary aggregation occurred during column chromatography, with formation of enzymatically active $(\alpha\beta)_2$-dimers or $(\alpha\beta)_3$-trimers with $S_{20,w}$ = 10–12 S and apparent molecular masses in the range 273000–386000 daltons. This may reflect non-specific time-dependent aggregation of the detergent micelles.

Introduction

The important catalytic functions of the Na^+, K^+ pump can be related to the α-subunit with M_r 94000–106000. This protein forms binding sites for nucleotides and cardiac glycosides, it is phosphorylated from ATP and it undergoes conformational transitions accompanying binding and translocation of cations [1–5]. The β-subunit, a sialoglycoprotein with protein M_r 32000–38000, forms a part of the extracellular binding area for cardiac glycosides [6], but other catalytic functions have not been associated with this protein. The molar ratio of α-subunit to β-subunit is 1 : 1, but it

† Deceased.

* To whom correspondence should be addressed.

Abbreviations: $C_{12}E_8$, dodecyloctaethyleneglycol monoether; Tes, 2-((2-hydroxy-1,1-bis(hydroxymethyl)ethyl)amino)ethanesulphonic acid.

is uncertain whether the minimum active protein unit consists of a protomer $\alpha\beta$-unit or if association to oligomeric $(\alpha\beta)_2$-units is required for active cation translocation.

Determination of the subunit structure and molecular weight of the minimum functional protein unit of $(Na^+ + K^+)$-ATPase requires soluble and active preparations of $(Na^+ + K^+)$-ATPase in homogeneous and stable form. Soluble $(Na^+ + K^+)$-ATPase has been purified after extraction of crude membrane fractions from shark rectal gland with Lubrol-WX or dodecyloctaethyleneglycol monoether ($C_{12}E_8$), followed by chromatography on aminoethyl cellulose [3] or Sepharose columns [5]. The molecular mass of the soluble protein varied from 257000 to 380000 dalton [3,5], suggesting that association between $\alpha\beta$-units are required for $(Na^+ + K^+)$-ATPase activity. However, the purification involved prolonged periods of contact with non-ionic detergent and the possibility of non-functional aggregtion of mixed micelles should be considered. Polyether-derived detergents are known to form oxidizing degradation products [7] that may catalyse artefactual time-dependent aggregation of the soluble protein [8].

The basis for the present study is a new approach at preparation of pure, soluble $(Na^+ + K^+)$-ATPase involving only brief exposure to the non-ionic detergent $C_{12}E_8$. $(Na^+ + K^+)$-ATPase was purified in membrane-bound form and the pure enzyme was solubilized in non-ionic detergent immediately prior to enzyme assay and analytical ultracentrifugation [9]. Hydrodynamic studies show that this procedure yields a solution of predominantly protomeric $\alpha\beta$-units with maximum M_r 141000–170000. Procedures were developed for enzyme assay in the detergent solution and for controlled removal of detergent with polystyrene beads. The maximal specific activities of the soluble $(Na^+ + K^+)$-ATPase are equal to those of the pure membrane-bound $(Na^+ + K^+)$-ATPase and the soluble enzyme reconstitutes to active Na^+, K^+ pumps in phospholipid vesicles. Secondary aggregation to dimers or trimers during chromatography of the soluble $(Na^+ + K^+)$-ATPase suggests that the previous higher molecular weights can be explained by time-dependent aggregation of detergent micelles.

Experimental procedures

Materials. $C_{12}E_8$ was obtained from Nikko Chemicals Co., Tokyo, Japan, and ^{14}C-labelled $C_{12}E_8$ from C.E.A., Saclay, France. Recrystallization of $C_{12}E_8$ did not affect its properties in analytical ultracentrifugation. Bovine serum albumin was obtained from Behring Werke, Marburg, F.R.G., Bio-Beads SM-2 (styrene-divinylbenzene copolymer beads) from Bio-Rad, Richmond, CA and L-α-phosphatidylcholine type IIS from Sigma, Saint Louis, MO. Standard proteins for the calibration of the gel filtration columns were aldolase, catalase, ferritin and thyroglobulin (Pharmacia, Uppsala, Sweden), β-galactosidase from *Escherichia coli* (Boehringer, Mannheim, F.R.G.) and bovine serum albumin. Sepharose 6B and Blue dextran were from Pharmacia.

Enzyme preparations. $(Na^+ + K^+)$-ATPase was purified in membrane-bound form from the microsomal fraction of pig kidney outer medulla by selective solubilization of contaminating proteins with SDS followed by isolation of the pure $(Na^+ + K^+)$-ATPase membranes by isopycnic-zonal gradient centrifugation in the Ti-14 zonal rotor [10]. The enzyme was stored at −70°C in 10% (w/v) sucrose/25 mM imidazole-HCl/1 mM Tris-EDTA, pH 7.5, at 2–3 mg protein per ml. The $(Na^+ + K^+)$-ATPase and potassium phosphatase activities of the preparations at 37°C were 35–48 and 5.2–7 μmol P_i/min per mg protein, respectively.

Solubilization. Prior to solubilization $(Na^+ + K^+)$-ATPase was sedimented at 100000 rpm for 10 min in the Beckman Airfuge and resuspended in 40 mM KCl/260 mM NaCl or 300 mM KCl in 20 mM Tes/2 mM dithiothreitol/2 mM Tris-EDTA, pH 7.5, at 0.3–2 mg protein per ml. After homogenization this suspension was mixed with equal volumes of $C_{12}E_8$ in water to final concentrations of 20 mM KCl and 130 mM NaCl or 150 mM KCl in $C_{12}E_8$(0–5 mg/ml)/10 mM Tes/1 mM dithiothreitol/1 mM Tris-EDTA, pH 7.5. After incubation for 30 min at 20°C, the non-solubilized residue was removed by centrifugation for 10 min at 100000 rpm in a Beckman Airfuge.

Enzyme assays. The soluble enzyme was assayed either in the detergent medium or in detergent-free medium after removal of $C_{12}E_8$ by incubation

with Bio-Beads SM-2. For $(Na^+ + K^+)$-ATPase assay in detergent medium, 40 μl of the soluble protein in 130 mM NaCl/20 mM KCl/10 Tes/1 mM dithiothreitol/1 mM Tris-EDTA, pH 7.5, and varying concentrations of $C_{12}E_8$ were mixed with 4 μl 100 mM ATP/100 mM $MgCl_2$. After 20, 40 or 60 s at 20°C the reaction was stopped with 1 ml ice-cold 0.5 N HCl containing 30 mg ascorbic acid/5 mg ammonium heptamolybdate/10 mg SDS. The tubes were transferred to an ice-bath. For colour development, 1.5 ml containing 30 mg sodium metaarsenite/30 mg sodium citrate/30 μl acetic acid were added. The tubes were heated for 10 min at 37°C and absorbance was read at 850 nm. For potassium phosphatase assay, 40 μl of the protein solution with 150 mM KCl in the buffer described above were mixed with 4 μl 200 mM $MgCl_2$/100 mM *p*-nitrophenyl phosphate. After 20, 60 or 120 s at 20°C, 2 ml 0.2 M NaOH/0.05 M Na_2 EDTA were added and absorbance was read at 410 nm [11,12].

For removal of $C_{12}E_8$, the protein solution was diluted to a concentration of 25–100 μg protein/ml in 150 mM KCl/10 mM Tes/1 mM Tris-EDTA, pH 7.5, containing 30 mg Bio-Beads SM-2 per ml in a stirred or shaken tube. 25-μl aliquots were transferred to test tubes equilibrated at 37°C and containing 1 ml of 130 mM NaCl/20 mM KCl/3 mM $MgCl_2$/3 mM ATP/25 mM imidazole, pH 7.5. After incubation for 1, 3 and 5 min at 37°C the reaction was stopped with ascorbic acid/HCl/SDS solution and the colour was developed as above. For potassium phosphatase assay, the 25-μl aliquots were transferred to 1 ml of 150 mM KCl/20 mM $MgCl_2$/10 mM *p*-nitrophenyl phosphatase/25 mM imidazole, pH 7.5. The reaction was stopped with 2 ml 0.2 M NaOH/0.05 M Na_2 EDTA and absorbance was read at 410 nm.

Na^+, K^+ transport. For reconstitution of soluble $(Na^+ + K^+)$-ATPase into phospholipid vesicles with KCl inside and NaCl outside, 10–75-μl aliquots containing 10–50 μg soluble $(Na^+ + K^+)$-ATPase in 150 mM KCl/10 mM Tes/1 mM Tris-EDTA/1 mM dithiothreitol/2.5 mg/ml $C_{12}E_8$, pH 7.0, were mixed with 3.25 mg sonicated L-α-phosphatidylcholine in 175–240 μl of the same buffer without $C_{12}E_8$. The mixture was frozen in liquid nitrogen, thawed at room temperature and sonicated for 30 s in a Bransonic 12 sonicator [13]. For exchange of the external medium, the vesicles were centrifuged at 1000 rpm for 5 min through syringes containing 2 ml Sephadex G50 equilibrated with 10 mM NaCl/140 mM Tris-HCl/10 mM Tes/1 mM Tris-EDTA, pH 7.0 [14,15]. The concentration of phospholipid in the vesicle suspensions was determined by fluorescence of diphenylhexatriene [16]. For transport assay 40 μl of the vesicle suspension were mixed with 40 μl $^{22}Na^+$ $(2 \cdot 10^6$ cpm)/10 mM NaCl/6 mM $MgCl_2$/10 mM Tes/1 mM Tris-EDTA, pH 7.0, with or without 6 mM Tris-ATP. After incubation for 20 or 40 s at 24°C the vesicles were passed over columns with Dowex 50 XS [14] and $^{22}Na^+$ inside the vesicles was counted.

Gel filtration. Soluble $(Na^+ + K^+)$-ATPase was characterized by gel filtration at 15°C on 1.5 × 90 cm columns of Sepharose 6B eluted downwards at a flow rate of 5 ml/h. Fraction volumes were determined by weighing. The peak position is expressed as $erf^{-1}(1 - K_d)$ [17], where the partition coefficient K_d is defined as $K = (V_e - V_o)/(V_t - V_o)$ and V_e, V_o, and V_t are the elution volume of the sample, void volume and total volume, respectively. The columns were calibrated using marker proteins with known Stokes radii [17]. Dithiothreitol (measured by ultraviolet absorption) or NaCl (analyzed by flame photometry) and Blue dextran were used as total and void volume markers.

Protein analysis. Protein was measured by the method of Lowry et al. [44] after precipitation with trichloroacetic acid. As standard we used membrane-bound $(Na^+ + K^+)$-ATPase in which the protein concentration had been determined by quantitative amino acid analysis as described previously [9].

Amino acid analysis. A sample of purified $(Na^+ + K^+)$-ATPase was depleted of lipids by extraction with chloroform/methanol [18], and dissolved in 0.5% SDS/100 mM $(NH_4)_2CO_3$ in order to eliminate the contribution of phosphatidylserine and phosphatidylethanolamine.

Other determinations. Lipids were extracted by a scaled-down version of the method of Bligh and Dyer [18]. Phospholipids were analyzed by the method of Bartlett [19], assuming an average molecular weight of 775. Cholesterol was assayed using the Tsugaeff reaction in the modification of Hanel and Dam [20]. Hexoses were determined by

the anthrone reaction [21] after precipitation with 5% trichloracetic acid, using 0.5 mg albumin as a carrier. Standards and blanks contained the same amount of trichloroacetic acid-precipitated albumin. Hexosamines were measured by the Elson-Morgan reaction [22] and sialic acid by the thiobarbituric acid method after hydrolysis in 0.01 M H_2SO_4 for 1 h at 80°C [23]. The carbohydrate composition was also assayed by gas chromatography of the trimethylsilyl ethers using inositol as an internal standard [24]. ^{14}C-labeled $C_{12}E_8$ was measured by liquid scintillation counting.

Analytical ultracentrifugation. Analytical ultracentrifugation was performed in a Beckman model E centrifuge equipped with a photoelectric scanner. The absorbance of the sample was scanned at 280 nm in a double-sector cell at 20°C against a protein-free reference sample, using an Ad-D rotor for sedimentation velocity and an An-H rotor for sedimentation equilibrium studies.

Sedimentation coefficients were measured at 44770 rpm on the basis of the movement of the midpoint between the baseline and the plateau region of the A_{280} scan of the sample. The scans had an elevated baseline (5–20% of the protein absorbance) as a result of light scattering from lipid-detergent mixed micelles present after solubilization of $(Na^+ + K^+)$-ATPase. Sedimentation of these mixed micelles was very slow because the buoyant density of both lipid and $C_{12}E_8$ are close to that of the solvent. Graphs of radial distance of the protein boundary from the rotor axis ($\ln r$) versus time were linear from 10 min after reaching the specified speed up to at least 90 min.

Sedimentation equilibrium experiments were performed at 7905 and 9924 rpm using a solution column height of approximately 3.5 mm and an equilibration time of 28–30 h. The elevated baseline originating from the $C_{12}E_8$-lipid micelles gave a very small contribution to the A_{280} profile. This was shown by centrifuging $C_{12}E_8$-solubilized samples of $(Na^+ + K^+)$-ATPase for 3–24 h at 44770 rpm to sediment the protein to the bottom of the cell. The resulting A_{280} profile yielded a slope corresponding to 430 for $M(1 - \phi\rho)$. This is about 1% of that of the protein-containing particles and it did not contribute significantly to the curvature of the A_{280} scan at lower speeds. The baseline to be subtracted after the low-speed sedimentation was measured after overspeeding to 44770 rpm for 1 h and reading the absorbance near the center of the solution column. The recovered protein absorbance together with the baseline accounted for 92–100% of the initial absorbance of the sample at 280 nm. The buoyant density factor and the protein molecular weight were estimated as described in detail previously [25,26]. Published values for the partial specific volume contribution of amino acid residues [27], carbohydrate residues [28], $C_{12}E_8$ [29], phospholipid and cholesterol [26,30] were used in the calculation.

Results

Activity of soluble $(Na^+ + K^+)$-ATPase. In the experiments shown in Figs. 1 and 2 we examined the solubilization of purified, membrane-bound $(Na^+ + K^+)$-ATPase at different $C_{12}E_8$/protein

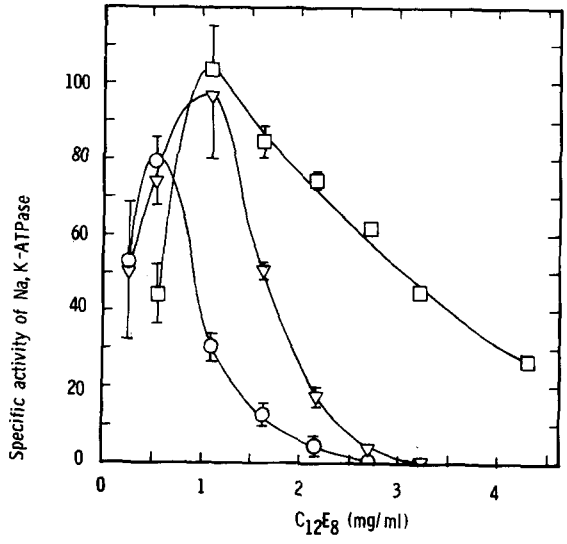

Fig. 1. Specific activity of soluble $(Na^+ + K^+)$-ATPase. Solubilization of purified membrane-bound $(Na^+ + K^+)$-ATPase at three different protein concentrations (○, 0.18 mg/ml; ▽, 0.34 mg/ml; □, 0.62 mg/ml). The enzyme was mixed at 20°C with increasing concentrations of $C_{12}E_8$ in 130 mM NaCl/20 mM KCl/10 mM Tes/1 mM Tris-EDTA/1 mM dithiothreitol, pH 7.5. $(Na^+ + K^+)$-ATPase was assayed at 20°C in detergent solution by adding 4 μl MgATP to 40-μl detergent solution (see Experimental procedures). Activities are expressed in percent of the specific activity of untreated membrane-bound $(Na^+ + K^+)$-ATPase which was assayed in parallel. The points are mean of 1–6 individual experiments and the error bars denote standard error of the mean.

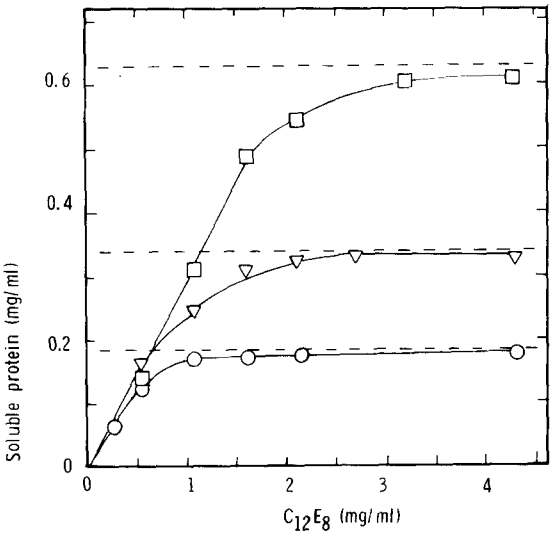

Fig. 2. Solubilization of the protein of purified membrane-bound $(Na^+ + K^+)$-ATPase as in Fig. 1 with 0.18 (○), 0.34 (▽) or 0.62 (□) mg protein per ml. The dashed lines denote the protein concentrations before sedimentation of the non-solubilized residue.

weight ratios. The maximum specific activity of soluble $(Na^+ + K^+)$-ATPase was close to 100% of that of the membrane-bound enzyme (Fig. 1) at protein concentrations of 0.34 and 0.62 mg/ml. At the lower protein concentration the specific activity reached a maximum at 70–90%. The maximum was reached at $C_{12}E_8$/protein ratios of 2–3, but complete protein solubilization required ratios of 5–6 (Fig. 2). The yield of soluble $(Na^+ + K^+)$-ATPase activity with specific activity equal to that of pure membrane-bound $(Na^+ + K^+)$-ATPase was, therefore, limited to 50–60%. The efficiency of solubilization was the same in 150 mM KCl as in 130 NaCl/20 mM KCl. The use of these two media allowed for direct assay in the detergent medium of the potassium phosphatase and $(Na^+ + K^+)$-ATPase with a minimum change (under 10%) in the composition of the solution due to the addition of ATP or *p*-nitrophenyl phosphate and $MgCl_2$. The $(Na^+ + K^+)$-ATPase activity of the soluble enzyme was fairly stable, declining with a rate constant of $-0.01\ h^{-1}$ to $-0.05\ h^{-1}$ at 20°C, but inactivation was much faster at 37°C, with rate constants in the range $-0.02\ min^{-1}$ to $-0.04\ min^{-1}$.

Using membrane-bound $(Na^+ + K^+)$-ATPase with specific activity 35–48 μmol P_i/min per mg protein as starting material, the relative content of α- and β-subunits in the soluble $(Na^+ + K^+)$-ATPase determined by polyacrylamide gel electrophoresis in SDS was identical to that of the membrane-bound enzyme (not shown). At low $C_{12}E_8$ concentrations, 10–30% of the protein was solubilized with a specific $(Na^+ + K^+)$-ATPase activity that was only 50–70% of the maximum (Fig. 1). The peptide composition of this material with low specific activity was also similar to that of the membrane-bound enzyme. The reduced specific activity may, therefore, be explained by preferential solubilization at low $C_{12}E_8$ concentrations of a small fraction of partially inactivated molecules of $(Na^+ + K^+)$-ATPase.

At $C_{12}E_8$/protein ratios above 3, the directly measured activity of the soluble enzyme was decreased, $(Na^+ + K^+)$-ATPase being more sensitive than potassium phosphatase [9]. This inactivation was reversible at $C_{12}E_8$/protein ratios of 3–5 when excess detergent was absorbed to the hydrophobic resin Bio-Beads SM-2 and activity was assayed in detergent-free medium. Fig. 3 shows that the isotherms of detergent-binding to the resin

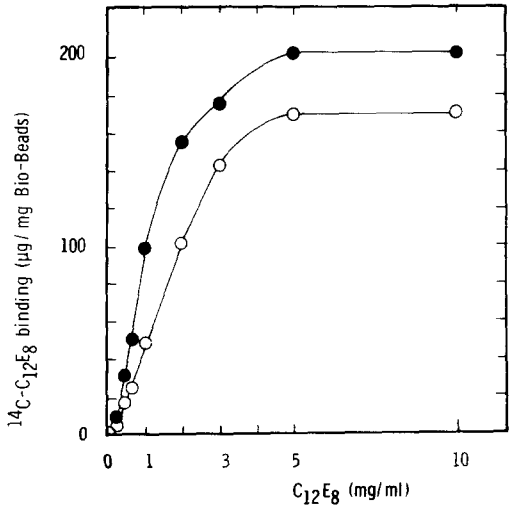

Fig. 3. Langmuir isotherm of the binding of ^{14}C-labeled $C_{12}E_8$ to Bio-Beads SM2. Incubation with 10 mg (●) or 20 mg (○) Bio-Beads per ml in 3 ml 10 mM Tes/1 mM Tris-EDTA/1 mM dithiothreitol/150 mM KCl, pH 7.5, at 20°C for 180 min in a shaker. After incubation the beads were allowed to sediment and aliquots were taken out for counting of unbound ^{14}C-labeled $C_{12}E_8$.

were hyperbolic, with saturation at the level of 170–200 μg $C_{12}E_8$ bound per mg resin. Using ^{14}C-labeled $C_{12}E_8$, the time course of detergent removal from the protein solution was examined (Fig. 4). The reduction in detergent concentration was accompanied by a sharp increase in activity of both $(Na^+ + K^+)$-ATPase and potassium phosphatase. Binding of protein to the beads explains the moderate loss of enzyme activity after 20–30 min. Reactivation was essentially complete, with recovery to 75–100% of the specific activity in the starting material at a $C_{12}E_8$/protein ratio of 5, while at ratios higher than about 6 the inactivation became irreversible.

Reconstitution of active Na^+, K^+ transport. The soluble $(Na^+ + K^+)$-ATPase with maximal specific activity in $C_{12}E_8$ solution could be reconstituted immediately into phospholipid vesicles and very high rates of active Na^+, K^+ transport were demonstrated (Fig. 5). After mixing soluble $(Na^+ + K^+)$-ATPase in $C_{12}E_8$ with sonicated phospholipid in buffer without detergent, active Na^+, K^+ transport was reconstituted by the freeze-thaw sonication method [13]. The vesicles were formed with 150 mM KCl inside and 10 mM NaCl/140 mM Tris-HCl in the outside medium at very low protein/lipid ratios. At the optimum protein concentration given in Fig. 5 this corresponds to about one functioning pump per vesicle (for calculation see Ref. 1). The rate of active uptake of $^{22}Na^+$, 0.95 μmol Na^+/min per mg protein was equal to the highest rates reported before (For references, see Ref. 1). With a stoichiometry of 3Na/ATP and random insertion of the pumps in the vesicles this rate is 40–50% of the turnover of pure membrane bound $(Na^+ + K^+)$-ATPase in similar conditions (pH 7.0, 24°C).

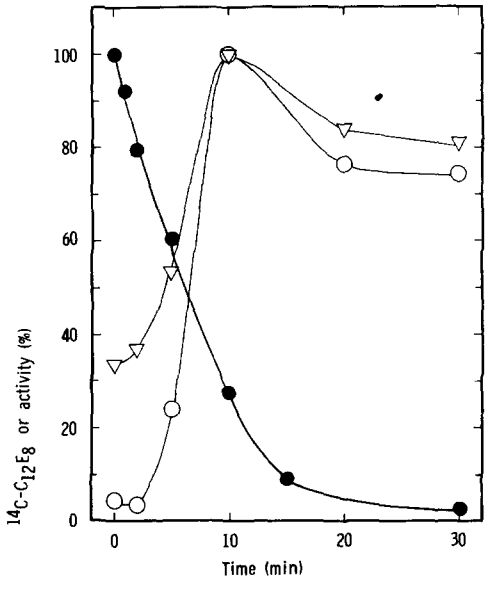

Fig. 4. Time course of reactivation of soluble $(Na^+ + K^+)$-ATPase (○) and potassium phosphatase (▽) on removal of $C_{12}E_8$ (●) with Bio-Beads SM-2. $(Na^+ + K^+)$-ATPase (1.6 mg/ml) was solubilized with 5.4 mg/ml ^{14}C-labeled $C_{12}E_8$ ($2 \cdot 10^4$ cpm/mg) in 10 mM Tes/1 mM Tris-EDTA, pH 7.5. After 30 min at 30°C the sample was diluted to a protein concentration of 95 μg/ml in the same buffer (without detergent), containing 30 mg Bio-Beads SM-2 per ml. The solution was shaken at room temperature and 25-μl aliquots were removed at different time intervals for enzyme assays and for counting of free ^{14}C-labeled $C_{12}E_8$.

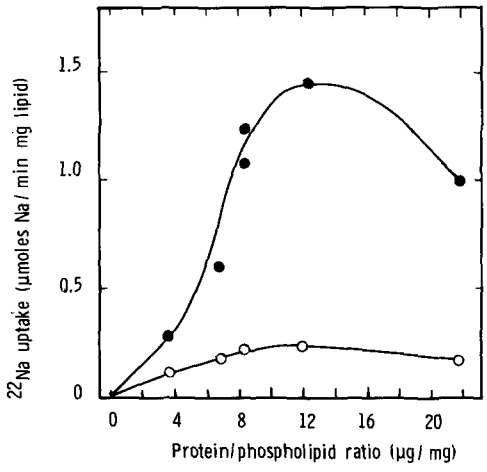

Fig. 5. Reconstitution of soluble $(Na^+ + K^+)$-ATPase into phospholipid vesicles with 150 mM KCl inside and 10 mM NaCl/140 mM Tris-HCl/3 mM $MgCl_2$/3 mM ATP in the outside medium. Procedure as described in Experimental procedures. After incubation for 20 or 40 s uptake of ^{22}Na in presence (●) or absence ATP (○) was calculated from initial velocity curves.

Gel filtration. The soluble $(Na^+ + K^+)$-ATPase was applied to Sepharose 6B columns equilibrated with $C_{12}E_8$ to determine the Stokes radius and the chemical composition of the soluble enzyme. After solubilization at a $C_{12}E_8$/protein weight ratio of 5 and elution with a high concentration of 1 mg/ml $C_{12}E_8$, protein and phospholipid were separated. The inactive protein appeared as a slender peak with a Stokes radius of 68 Å, which represents a

minimum R_S value for the soluble $(Na^+ + K^+)$-ATPase (Fig. 6).

With 0.15 mg/ml $C_{12}E_8$ in the eluent and solubilization at a $C_{12}E_8$/protein weight ratio of 2.5–3, the $(Na^+ + K^+)$-ATPase remained active and eluted in a rather broad peak (Fig. 7) with a Stokes radius (72 ± 4 Å) which is higher than that obtained in Fig. 6, presumably reflecting differences in phospholipid content or a tendency for aggregation of the particles. The specific activities of $(Na^+ + K^+)$-ATPase and potassium phosphatase at the peak were 33.8 and 5.7 μmol/min per mg, respectively, corresponding to 70–90% of those in the pure membrane-bound enzyme preparation. In the fractions from the column, phospholipid, cholesterol and ^{14}C-labeled $C_{12}E_8$ in excess of the concentration in the column eluent were distributed in two overlapping peaks (Fig. 7B). The early, smaller peak represented material associated to the soluble protein particles, while the latter, prominent peak consisted of detergent lipid mixed micelles. The micelle peak was assumed to be symmetric and the overlapping portion was subtracted in calculation of the cmpositions of the protein-containing particles. The resulting data are given in Table I. The distributions of the carbohydrate constitutents were also bimodal (Fig. 7C), probably reflecting the dissociation of membrane glycolipids from the glycoprotein particles. The carbohydrate composition of the protein peak is also given in Table I, together with results obtained for purified lipid-depleted $(Na^+ + K^+)$-ATPase by gas chromatographic analysis.

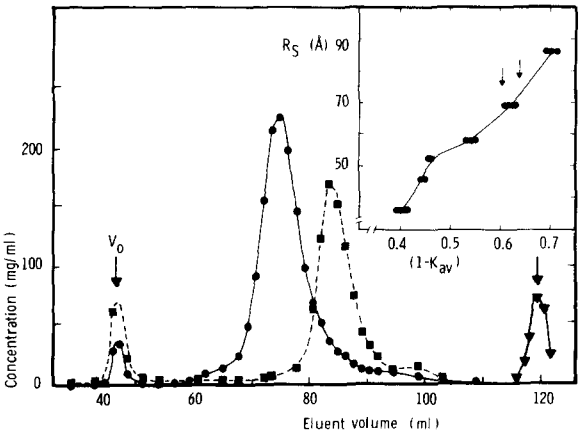

Fig. 6. Determination of the Stokes radius of soluble $(Na^+ + K^+)$-ATPase. Samples containing 2.6 mg $(Na^+ + K^+)$-ATPase were solubilized with a 5-fold excess of $C_{12}E_8$ by weight in 1 ml 150 mM KCl/10 mM Tes/1 mM NaN_3/4 mg/ml dithiothreitol, pH 7.5. The column (1.5 × 90 cm) was eluted with 1.0 mg/ml $C_{12}E_8$ in the buffer described above. Fractions of approx. 1.2 ml were weighed and assayed for protein (●), phospholipid (■) and NaCl (▼) as indicator of V_t. The inset shows the calibration of the Sepharose 6B column. Elution with 0.15 mg/ml $C_{12}E_8$ in 150 mM KCl/10 mM Tes/1 mM EDTA/1 mM dithiothreitol, pH 7.5. Calibration with thyroglobulin (86 Å), ferritin (69 Å), Ca^{2+}-ATPase from sarcoplasmic reticulum (58 Å), catalase (52 Å), aldolase (46 Å) and bovine serum albumin (36 Å). The elution positions of $(Na^+ + K^+)$-ATPase in the experiment above (left) and in the experiment of Fig. 7 (right) are shown with arrows.

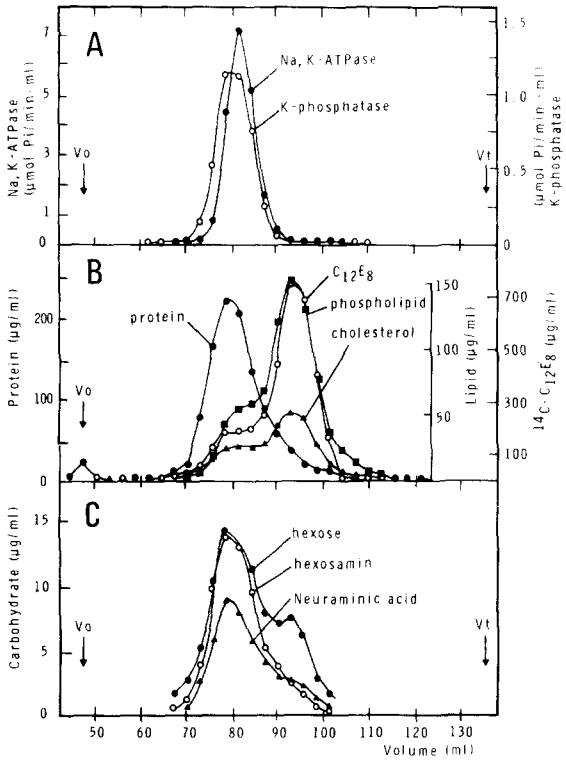

Fig. 7. Composition of solubilized $(Na^+ + K^+)$-ATPase. A sample of 3.2 mg of $(Na^+ + K^+)$-ATPase was solubilized by 10 mg of ^{14}C-labeled $C_{12}E_8$ ($4 \cdot 10^4$ cpm/mg) in 1 ml of 20% glycerol/150 mM KCl/10 mM Tes/1 mM Tris-EDTA/1 mM dithiothreitol, pH 7.5, and chromatographed on a Sepharose 6B column (1.5 × 90 cm) equilibrated with 150 μg/ml of ^{14}C-labeled $C_{12}E_8$ ($4 \cdot 10^4$ cpm/mg) in the same buffer but without dithiothreitol. Fractions of about 2.6 ml were assayed as follows. A: $(Na^+ + K^+)$-ATPase, ●; potassium phosphatase, ○. B: Protein, ●; phospholipid, ■; cholesterol, ▲; excess $C_{12}E_8$, ○. C: Hexose, ●; hexosamine, ○; sialic acid, ▲, residues.

TABLE I

CHEMICAL COMPOSITION OF SOLUBLE $(Na^+ + K^+)$-ATPase ISOLATED BY GEL FILTRATION

Data are from the experiment shown in Fig. 7. Mean values ± S.D. are given, with the number of samples analyzed in parentheses.

Component	mg/g protein
Hexose [a,b]	58 ± 3 (3)
N-Acetylhexosamine [a,c]	56 ± 4 (5)
Sialic acid [a]	35 ± 3 (4)
Total carbohydrate	149 ± 6
Phospholipid	176 ± 25 (3)
Cholesterol	95 ± 4 (4)
$C_{12}E_8$	783 ± 86 (5)

[a] In calculation of the weight contribution of the carbohydrate residues, a loss of one water molecule per glycosidic bond is accounted for.

[b] 36% mannose and 64% galactose according to gas chromatographic analysis.

[c] More than 94% *N*-acetylglucosamine according to gas chromatographic analysis.

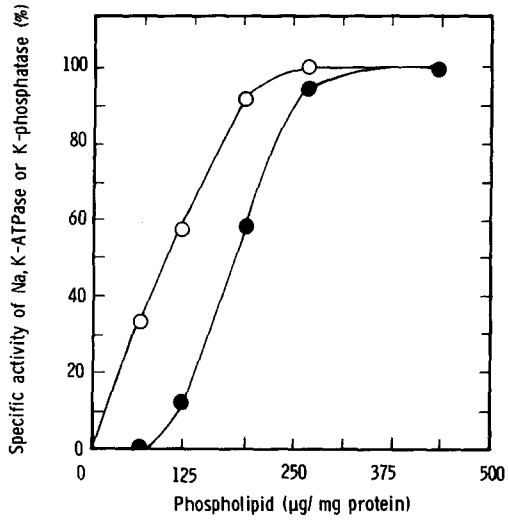

Fig. 8. $(Na^+ + K^+)$-ATPase (●) and potassium phosphatase (○) activity as a function of phospholipid/protein ration in the fractions of the leading edge of the peak of protein in Fig. 5A. 100% $(Na^+ + K^+)$-ATPase activity corresponds to 33.8 µmol P_i/min per mg protein and 100% potassium phosphatase activity to 5.7 µmol P_i/min per mg protein.

The leading edge of the protein peak in Fig. 7 eluted slightly earlier than the leading edges of the peaks of potassium phosphatase and $(Na^+ + K^+)$-ATPase activities. The explanation for this dissociation is that phospholipids are extracted from the protein particles at the leading edge during migration through the detergent solution in the column. It is seen from Fig. 8 that the levels of potassium phosphatase and $(Na^+ + K^+)$-ATPase activities are related to the amount of phospholipid remaining in association with the protein in the same manner as that demonstrated earlier in relipidation experiments [31]. More lipid was required for $(Na^+ + K^+)$-ATPase than for potassium phosphatase activity. The curve relating potassium phosphatase activity to phospholipid concentration was hyperbolic, while the curve for $(Na^+ + K^+)$-ATPase was sigmoid shaped. The minimum concentrations of phospholipid required for full activity were 240 or 360 µg phospholipid per mg protein for potassium phosphatase and $(Na^+ + K^+)$-ATPase, respectively, corresponding to 43 or 65 mol phospholipid per 140 000 dalton protein.

Knowledge of the chemical composition of the soluble particle (Table I) and the amino acid composition of the lipid-depleted $(Na^+ + K^+)$-ATPase (data not shown) allowed the calculation of the partial specific volume. The value, $v = 0.83$ cm^3/g, was relatively high due to the large content of bound detergent in the soluble particles. The data was used in Table II for calculation of the protein molecular weight of the soluble $(Na^+ + K^+)$-ATPase.

Sedimentation velocity studies. In the range of $C_{12}E_8$ concentrations where the enzyme was changed from a state of full activity to complete inactivity, there were no abrupt changes that could be taken as a sign of oligomer-protomer dissociation of the soluble $(Na^+ + K^+)$-ATPase. Inspection of the sedimentation profiles in Fig. 9 shows that the boundaries obtained at low $C_{12}E_8$ concentrations for the fully active enzyme were almost identical to those obtained at high $C_{12}E_8$ concentration for the inactive protein. The broken lines indicate the contribution of light scattering of mixed micelles of lipid and $C_{12}E_8$ contributing to the baseline absorbance. This baseline sediments at a relatively low speed, as seen from Fig. 9C and D. The sedimentation profiles of the protein consisted of a major and a minor overlapping component. From the sedimentation profiles and from

TABLE II

HYDRODYNAMIC CHARACTERISTICS OF SOLUBLE $(Na^+ + K^+)$-ATPase

M_p is the molecular mass of the protein moiety of the particle; $1 - \phi\rho$ is the boyant density factor; ϕ is the effective partial specific volume of the protein plus all bound components and ρ is the solvent density [3,9]. n.d., not determined.

	$C_{12}E_8$/protein, weight ratio		
	2.9	5–5.9	46
Stokes radius (Å)	72 ± 4 (4)	68	
$s_{20,w}$	7.4 ± 0.3 (5)	7.3 ± 0.2 (8)	6.5 ± 0.2 (4)
$1 - \phi\rho$	0.355		
M_p (velocity)	170000 ± 8600	159000 ± 7000	141000 ± 6000
M_p (equilibrium)	n.d.	168000 ± 5000 (8)	
M_p (equilibrium after gel filtration)	273000–386000 (6)	n.d.	n.d.

calculation of the secondary moment of the sedimentation boundaries (dA/dr) it was estimated that the major component comprised 80–90% of the total protein absorbance. The minor component, comprising 10–20%, consisted of more rapidly sedimenting aggregated particles.

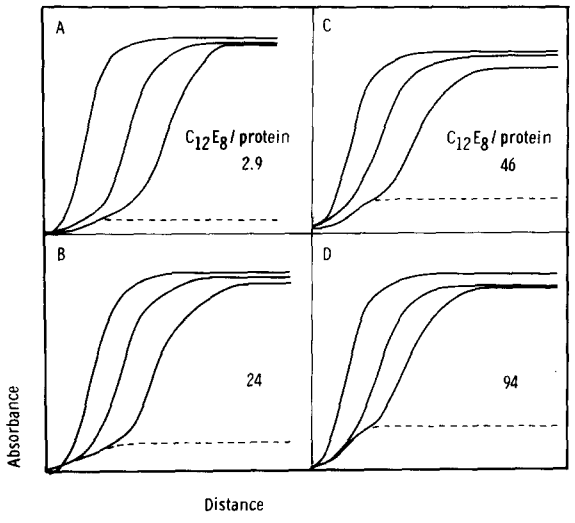

Fig. 9. Effect of $C_{12}E_8$ concentration on sedimentation of solubilized $(Na^+ + K^+)$-ATPase in the analytical ultracentrifuge. In A is shown the sedimentation boundaries after 15, 40 and 60 min and in B, C and D, the boundaries after 20, 40 and 60 min centrifugation at 44770 rpm at 20°C at $C_{12}E_8$/protein ratios varying from 2.9 to 24, 46 and 96. The protein concentration was 0.184 mg/ml in 150 mM KCl/10 mM Tes/1 mM Tris-EDTA/1 mM dithiothreitol (pH 7.5). The dashed lines indicate the baseline positions used to correct for the absorbance of the lipid-detergent micelles.

There was no significant change in sedimentation velocity over the range of the $C_{12}E_8$/protein weight ratios where the specific activity of $(Na^+ + K^+)$-ATPase was reduced from 70–100% to 0–28% (Cf. Fig. 1). It is seen from Fig. 10 that the $s_{20,w}$ at the $C_{12}E_8$/protein weight ratio of 2.8 and 6 was 7.3–7.4 S in 150 mM KCl and 7.1–7.2 S in medium containing 130 mM NaCl/20 mM KCl. On increasing the $C_{12}E_8$/protein ratio in 150 mM KCl medium to 24, 46 and 94 the $s_{20,w}$ was reduced to 6.5 and 6.2 S.

The sedimentation rate did not change when the protein concentration was increased from 0.18 to 0.62 mg/ml at a constant $C_{12}E_8$/protein weight ratio of 2.9 or 5.9 (Fig. 10). It is, therefore, unlikely that associations dependent on protein concentration took place under the present conditions. The sedimentation coefficient was also independent of the salt composition of the medium.

It can be seen from Table II that the sedimentation coefficient of the soluble $(Na^+ + K^+)$-ATPase with 75–100% of original specific activity was 7.4. This value, together with the Stokes radius of 72 Å and buoyant density factor, gives an estimated molecular weight of 170000 ± 9000 for the protein moiety of the soluble enzyme. The estimated molecular weight becomes 161000 if the minimum value for the Stokes radius of 68 Å is used in the calculation. For the partially inactivated enzyme and the inactive enzyme at $C_{12}E_8$/protein ratios of 5.9 and 54 the $s_{20,w}$ values corresponded to molecular weights of 159000 ± 5000 and 141000 ± 6000 when a Stokes radius of 68 Å was used in

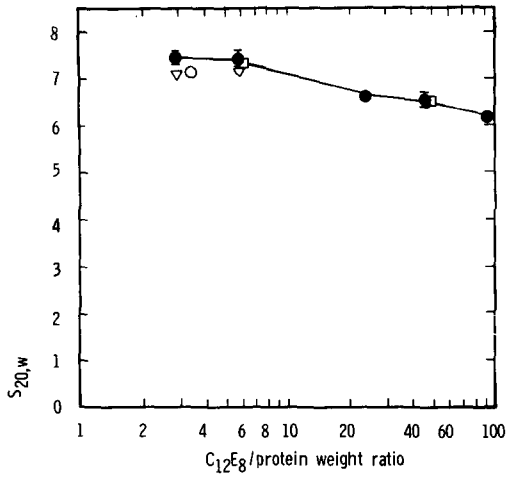

Fig. 10. Effect of $C_{12}E_8$ concentration on the sedimentation coefficient of $(Na^+ + K^+)$-ATPase solubilized at increasing detergent concentrations in 150 mM KCl at 0.184 mg/ml (●), in 130 mM NaCl/20 mM KCl at 0.184 mg protein/ml (▽), in 150 mM KCl at 0.62 mg protein/ml (○) or in 150 mM Tris-HCl at 0.18 mg protein/ml (□) in 10 mM Tes/1 mM EDTA/1 mM dithiothreitol, pH 7.5.

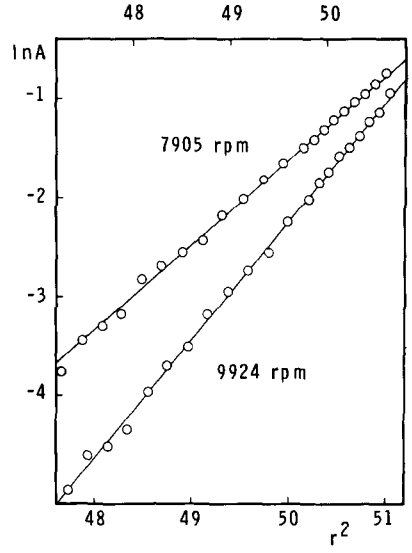

Fig. 11. Sedimentation equilibrium centrifugation of solubilized $(Na^+ + K^+)$-ATPase. Graph of $\ln A$ versus r^2 at two different speeds of centrifugation. Left, $(Na^+ + K^+)$-ATPase (0.184 mg protein/ml) solubilized in 1.08 mg/ml $C_{12}E_8$ was centrifuged to sedimentation equilibrium at 7905 rpm for 29 h. Right, $(Na^+ + K^+)$-ATPase (0.1 mg protein/ml) solubilized in 0.54 mg/ml $C_{12}E_8$ and centrifuged to equilibrium at 9924 rpm for 29 h. The buffer in both experiments was 150 mM KCl/10 mM Tes/1 mM EDTA/1 mM dithiothreitol (pH 7.5). Baseline corrections were as described in Experimental procedures.

the calculation. These values represent overestimates rather than underestimates because our observations show that the Stokes radius was overestimated due to secondary aggregation.

Sedimentation equilibrium studies. In equilibrium centrifugation, $M_p(1 - \phi\rho)$ is determined directly from the distribution of the complex [26,30], but in a series of centrifugations we were unable to obtain sedimentation equilibrium for the fully active enzyme at $C_{12}E_8$/protein ratio of 2.9, probably because time-dependent self-aggregation of the particles proceeded during the prolonged centrifugations. Similarly, sedimentation equilibrium was not obtained for the fully active $(Na^+ + K^+)$-ATPase emerging from the chromatography column (Fig. 7). This enzyme had sedimentation coefficients in the range 10–12 S, with evidence for heterogeneity of the sedimenting boundary. In equilibrium centrifugations we obtained curvilinear plots of ln$C$ versus r^2 as an indication of heterogeneity. Using only the slope of the portion of the curve close to the cell bottom, apparent molecular masses of 273000–386000 daltons were obtained (Table II).

As shown in Fig. 11, only slight deviations from linearity were observed in plots of ln$C$ versus r^2 for the partially inactive enzyme at $C_{12}E_8$/protein ratio of 5.9. Calculation from the slopes gave M_r 168000 ± 5000 for this enzyme preparation (Table II). These values should also be considered as maximum estimates because heavier components concentrate in the bottom region of the cell.

Discussion

This work led to methods for rapid preparation of enzymatically stable soluble $(Na^+ + K^+)$-ATPase from the purified membrane-bound enzyme of mammalian renal medulla. As assayed in $C_{12}E_8$ solution, the maximal specific activities of soluble $(Na^+ + K^+)$-ATPase are equal to those of the purified membrane-bound enzyme. After mixing with sonicated phospholipid the soluble protein inserts into vesicles by the freeze-thaw sonication procedure to catalyze active Na^+, K^+ transport at high rates. The fully active $(Na^+ + K^+)$-ATPase in $C_{12}E_8$ thus reconstitutes directly

without addition of denaturing detergents, such as cholate.

The hydrodynamic studies confirm and extend our previous conclusion [9] that the active soluble $(Na^+ + K^+)$-ATPase, as examined within a few hours after solubilization, consists of $\alpha\beta$-units with admixture of a small fraction, 10–20%, of aggregated enzyme units. The high activity of the soluble $\alpha\beta$-units shows that a permanent association to $(\alpha\beta)_2$-dimers is not necessary for the $(Na^+ + K^+)$-ATPase activity. Each $\alpha\beta$-unit may possess sites for binding of ATP [32] and phosphorylation [33] and the $\alpha\beta$-unit forms the minimum asymmetric unit cell in the vanadate-induced two-dimensional crystal of the membrane-bound $(Na^+ + K^+)$-ATPase. The $\alpha\beta$-unit is, therefore, the minimum functional protein unit of $(Na^+ + K^+)$-ATPase, both in $C_{12}E_8$ solution and in the membrane-bound state.

The soluble $(Na^+ + K^+)$-ATPase binds nucleotides with the same capacity as the membrane-bound $(Na^+ + K^+)$-ATPase and analysis of the effects of K^+ on the binding suggests that cooperative interactions between binding sites are relieved on solubilization [35]. With one site for ATP binding and phosphorylation per $\alpha\beta$-unit [32,33], the data on our soluble preparation thus support the notion that a single $\alpha\beta$-unit performs the entire $(Na^+ + K^+)$-ATPase cycle. However, one cannot immediately exclude the possibility that temporary interactions between soluble $\alpha\beta$-units occur at some stage of the complex reaction mechanism. This requires studies of the enzymatic properties of the soluble $(Na^+ + K^+)$-ATPase in line with those demonstrating the properties of monomeric Ca^{2+}-ATPase from sarcoplasmic reticulum. One molecule of ATP or fluorescein isothiocyanate is bound per polypeptide chain and the soluble monomeric enzyme is capable of undergoing the complex conformational transitions involved in the Ca^{2+}-ATPase cycle [36]. The soluble Ca^{2+}-ATPase in $C_{12}E_8$ sediments at 5 S and the boundary has phosphatase activity as assayed in the analytical ultracentrifuge [37].

Ultracentrifugation within the first few hours after solubilization shows that that the major component (80–90%) of the soluble and active Na, K-ATPase has $s_{20,w}$ 7.2–7.4 S and a maximum molecular weight of 170 000. The molecular weight estimates for the partially or completely inactive protein at higher detergent concentration are 141 000–159 000 by sedimentation velocity and 168 000 by sedimentation equilibrium analysis. Since the sedimentation coefficients for the active $(Na^+ + K^+)$-ATPase ($s_{20,w} = 7.4$ S) and inactive enzyme ($s_{20,w} = 7.3$ S) were almost identical, the conclusion that the soluble $(Na^+ + K^+)$-ATPase has maximum molecular weight 170 000 does not depend on determination of Stokes radii, which is complicated by aggregation of particles during chromatography. The data provide an upper limit for the molecular weight of the active, soluble $(Na^+ + K^+)$-ATPase of 170 000, but rigorous estimates of the molecular weight were not obtained because secondary aggregation occurred during the prolonged centrifugations. This aggregation also explains that the determinations based on sedimentation velocity overestimates the true molecular weight of the $\alpha\beta$-unit, since they depend on the determination of the Stokes radii by column chromatography.

Inactivation of $(Na^+ + K^+)$-ATPase and potassium phosphatase activity in the range of $C_{12}E_8$/protein ratios between 3 and 6 is not due to subunit dissociation, but our data show that the loss of enzymatic activity can be explained by delipidation. The phospholipid/protein weight ratio in fractions at the leading edge of the protein peak after chromatography in $C_{12}E_8$ is related to the enzyme activities in the same way as previously observed in relipidation experiments [31]. The removal of phospholipid thus limits the range of $C_{12}E_8$ concentrations where renal $(Na^+ + K^+)$-ATPase can remain active. This is in contrast to Ca^{2+}-ATPase, where $C_{12}E_8$ and other detergents can replace phospholipids without loss of activity [40].

Time-dependent secondary aggregation during column chromatography is demonstrated by the change in $s_{20,w}$ from 7.2–7.4 S in the freshly soluble preparation to 10–12 S for the fully active dimeric or trimeric $(Na^+ + K^+)$-ATPase in the fractions from the chromatography column. Similar observations were made with soluble Ca^{2+}-ATPase from sarcoplasmic reticulum with $s_{20,w} = 5.0$ S and M_r 115 000 in $C_{12}E_8$ [9]. The active Ca^{2+}-ATPase was aggregated to dimers and trimers after passage over the Sepharose 6B col-

umns [9].

Previous hydrodynamic studies of enzymatically active soluble preparations of $(Na^+ + K^+)$-ATPase from shark rectal gland gave molecular weights of 380 000 [3] and 257 000–276 000 [5] and oligomeric $\alpha_2\beta_4$ or $\alpha_2\beta_2$ structures were proposed. Recently, incubation with 8 mM glutaraldehyde for 40 min at room temperature followed by SDS-polyacrylamide gel electrophoresis was used as a technique for estimating the distribution among protomer and oligomer species in these preparations [38]. The pattern of covalently cross-linked products in the gel suggested that $(\alpha\beta)_2$-units comprised only 24 and 28% of the total protein in the soluble preparations from shark rectal gland. With soluble renal $(Na^+ + K^+)$-ATPase prepared with the method developed by us [9] cross-linking with glutaraldehyde showed that the $\alpha\beta$-units comprised 54 and 70% of total protein at $C_{12}E_8$/protein ratios of 3 and 50, respectively [38]. The fraction of total protein which is detected as covalently cross-linked $\alpha\beta$-heterodimers by gel electrophoresis in SDS after cross-linking with glutaraldehyde is necessarily a minimum estimate of the true content of $\alpha\beta$-units in the soluble preparation. During the prolonged incubation at high concentrations of glutaraldehyde (0.7%) the bifunctional reagent will also form bridges between $\alpha\beta$-units at rates depending on the frequency of collisions in the solution. When the differences between the techniques are considered, the observation of 50–70% $\alpha\beta$-units by glutaraldehyde cross-linking therefore compares well with our hydrodynamic studies showing that $\alpha\beta$-units form more than 80% of protein particles in both the active and the inactive soluble preparation. At variance with our results Craig's [38] cross-linking experiments suggested that association between $\alpha\beta$-units was required for $(Na^+ + K^+)$-ATPase activity of the soluble enzyme. More recently, it was found that this turnover-dependent cross-linking of α-subunits of soluble $(Na^+ + K^+)$-ATPase in $C_{12}E_8$ could be due to an effect of ionic strength [39]. Using $(Na^+ + K^+)$-ATPase from outer renal medulla as well as the procedures for purification and solubilization with $C_{12}E_8$ developed by us [9,10], Craig [39] now confirms our conclusion in previous [9] and in the present work that soluble $(Na^+ + K^+)$-ATPase can be prepared in which the monomer $\alpha\beta$-unit forms more than 85% of the mass of soluble particles.

The variability in the state of aggregation of the different $(Na^+ + K^+)$-ATPase preparations may have at least two reasons. One possibility is that the strength of subunit interactions depends on the animal source of the enzyme, being stronger for the shark than for the pig kidney $(Na^+ + K^+)$-ATPase. This is ruled out because the range of particle sizes of active renal $(Na^+ + K^+)$-ATPase is identical to those reported for the shark enzyme after column chromatography in detergent (Table II). Another possibility is that the $(Na^+ + K^+)$-ATPase preparations from shark rectal gland [3,5] were purified in soluble form. Our observations suggest that the prolonged period of contact with the polyether-derived detergents during purification lead to secondary aggregation of the protein to dimers or trimers regardless of the initial state of aggregation. In our approach the pure $(Na^+ + K^+)$-ATPase was obtained in membrane-bound form and the measurement of sedimentation velocity could be started within 0.5 h after the addition of the detergent. Artefactual aggregation due to associations between detergent molecules in the soluble enzyme preparations is a serious complication since these interactions may be indistinguishable from specific protein-protein interactions. Formation of aggregates of micelles of non-ionic polyoxyethylene detergents depends on the size of the polar headgroups relative to the hydrophobic tail. Pure $C_{12}E_8$ micelles does not form such aggregates [41], but this conclusion may not be valid for the behaviour of mixed protein-lipid-$C_{12}E_8$ complexes in conditions where oxidizing degradation products may be formed [7,8].

It is important that the results are self-consistent in the sense that the molecular mass of the soluble particle can be accounted for in terms of an integral number of constituent polypeptide chains. The maximum molecular weights for the active $(Na^+ + K^+)$-ATPase of 170 000 or 141 000–159 000 for the inactive $(Na^+ + K^+)$-ATPase at the high detergent concentrations are too low to allow for the presence of two α-chains and two or more β-chains in the same particle, as observed earlier for $(Na^+ + K^+)$-ATPase from shark rectal gland [3,5]. This agrees with the data published earlier for inactive $(Na^+ + K^+)$-ATPase

in Triton X-100 [42] or in digitonin [43]. Recent molecular weight estimates for the α-chain and β-chain fall in the range 93000–106000 and 32000–38000 [3–5], respectively. The maximum molecular mass of 170000 does not allow for more than one α-chain and one or two β-chains in the same particle, even for the lowest choice for the molecular weight of the constituent chains. Therefore, we conclude that the active soluble $(Na^+ + K^+)$-ATPase in the present case consists predominantly of particles with only one α-chain. With maximum molecular weight 170000 for the active enzyme the particles could accomodate one or two β-subunits, but the latter alternative is improbable because at the higher detergent concentration there would not be space for two β-chains in the particle. Therefore, the higher effective molecular weights at the low detergent concentrations are more likely to reflect a tendency for self-association in these conditions. The protein molecular weight of the fully active $(Na^+ + K^+)$-ATPase at the low $C_{12}E_8$/protein ratio could, for instance, be consistent with the presence of 10–20% of $\alpha_2\beta_2$-complexes among the population of soluble $\alpha\beta$-units. This would explain the observed broadness of the sedimentation boundary, although no clear second boundary could be seen in these experiments.

It should be emphasized that the observation of the $\alpha\beta$-unit as the minimum active protein unit of soluble $(Na^+ + K^+)$-ATPase is not directly valid for the membrane-bound Na^+, K^+ pump. It is not a priori given that the active unit of $(Na^+ + K^+)$-ATPase is identical to the protein complex required for active transport, and transport across phase boundaries cannot be demonstrated in detergent solution. It is, therefore, important to relate data on the molecular size and functional properties of the soluble $(Na^+ + K^+)$-ATPase to the characteristics of the membrane-bound Na^+, K^+ pump. The demonstration that the soluble $\alpha\beta$-units reconstitute into vesicles in conditions that result in one active Na^+, K^+ pump per phospholipid vesicle may provide the basis for such experiments.

Acknowledgements

The work was supported by the Danish Medical Research Council (P.L.J.) and the Finnish Academy and Finnish Scientific Society (J.R.B.). J.R.B. was the recipient of an EMBO short-term fellowship. Vesa Räsänen of Medix Laboratories, Helsinki, Finland provided valuable help with the analytical ultracentrifuge.

References

1 Jørgensen, P.L. (1982) Biochim. Biophys. Acta 694, 27–68
2 Kyte, J. (1981) Nature 292, 201–204
3 Hastings, D.F. and Reynolds, J.A. (1979) Biochemistry 18, 817–821
4 Freytag, J.W. and Reynolds, J.A. (1981) Biochemistry 20, 7211–7214
5 Esmann, M., Christensen, C., Karsson, K.A., Hansson, G.C. and Skou, J.C. (1980) Biochim. Biophys. Acta 60, 1–12
6 Hall, C. and Rouho, A. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 4529–4533
7 Lever, M. (1977) Anal. Biochem. 83, 273–284
8 Papper, G. and Schubert, D. (1982) Hoppe Seylers Z. Physiol. Chem. 363, 904
9 Brotherus, J.R., Møller, J.V. and Jørgensen, P.L. (1981) Biochem. Biophys. Res. Commun. 100, 146–154
10 Jørgensen, P.L. (1974) Methods Enzymol. 32B, 277–290
11 Ottolenghi, P. (1975) Biochem. J. 151, 61–66
12 Jørgensen, P.L. and Petersen, J. (1982) Biochim. Biophys. Acta 705, 38–47
13 Kasahara, M. and Hinkle, P.L. (1977) J. Biol. Chem. 252, 7384–7390
14 Karlish, S.J.D. and Pick, U. (1981) J. Physiol. 312, 505–529
15 Penefsky, H.S. (1977) J. Biol. Chem. 252, 2891–2899
16 London, E. and Feigenson, G.W. (1978) Anal. Biochem. 88, 203–211
17 Le Maire, M., Rivas, E. and Møller, J.V. (1980) Anal. Biochem. 106, 12–21
18 Bligh, E.G. and Dyer, W.J. (1959) Can. J. Biochem Physiol. 37, 911–916
19 Bartlett, G.R. (1955) J. Biol. Chem. 234, 466–468
20 Hanel, H.K. and Dam, H. (1955) Acta Chem. Scand. 9, 677–682
21 Hewitt, B.R. (1958) Nature 182, 246–247
22 Gatt, R. and Berman, E.R. (1966) Anal. Biochem. 15, 167–171
23 Warren, L. (1959) J. Biol. Chem. 234, 1971–1975
24 Spiro, R.G. (1972) Methods Enzymol. 28, 3–43
25 Holloway, P.W. (1973) Anal Biochem. 53, 304–309
26 Tanford, C., Nozaki, Y., Reynolds, J.A. and Makino, S. (1974) Biochemistry 13, 2369–2376
27 Cohn, E.J. and Edsall, J.T. (1943) Proteins, Amino Acids and Peptides, pp. 371–381, Rheinhold, New York
28 Gibbons, R.A. (1966) in Glycoproteins (Gottschalk, A., ed), pp. 29–95, Elsevier, Amsterdam
29 Steele, J.C.H., Tanford, C. and Reynolds, J.A. (1978) Methods Enzymol. 48, 11–23
30 Reynolds, J.A. and Tanford, C. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 4467–4471

31 Ottolenghi, P. (1979) Eur. J. Biochem. 99, 113–131
32 Moczydlowski, E.G. and Fortes, P.A.G. (1981) J. Biol. Chem. 256, 2346–2356
33 Peters, W.H.M., Swarts, H.G.P., De Pont, J.J.H.H.M., Schurmans-Stekhoven, F.M.A.H. and Bonting, S.L. (1981) Nature 290, 3389–339
34 Hebert, H., Jørgensen, P.L., Skriver, E. and Maunsbach, A.B. (1982) Biochim. Biophys. Acta 689, 571–574
35 Jensen, J. and Ottolenghi, P. (1983) Biochim. Biophys. Acta 731, 282–289
36 Andersen, J.P., Møller, J.V. and Jørgensen, P.L. (1982) J. Biol. Chem. 257, 8300–8307
37 Martin, D.W. and Tanford, C. (1982) Ann. N.Y. Acad. Sci, in the press
38 Craig, W.S. (1982) Biochemistry 21, 2667–2674
39 Craig, W.S. (1982) Biochemistry 21, 5707–17
40 Dean, W.L. and Tanford, C. (1978) Biochemistry 17, 1683–1690
41 Tanford, C., Nozaki, Y. and Rohde, F. (1977) J. Physiol. Chem. 81, 1555–1560
42 Clarke, S. (1975) J. Biol. Chem. 250, 5459–5469
43 Winter, C.G. and Moss, A.J. (1979) in Na, K-ATPase: Structure and Kinetics (Skou, J.C. and Nørby, J.G., eds.), pp. 25–32, Academic Press, London
44 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275