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SOLUBLE AND ACTIVE RENAL Na,K-ATPase WITH MAXIMUM PROTEIN MOLECULAR MASS 170,000 ± 9,000 DALTONS; FORMATION OF LARGER UNITS BY SECONDARY AGGREGATION

Jaakko R. Brotherus Department of Medical Chemistry, University of Helsinki, Finland

Jesper V. Møller Department of Medical Biochemistry, Aarhus University, 8000 Aarhus C, Denmark

Peter L. Jørgensen Institute of Physiology, Aarhus University 8000 Aarhus C, Denmark

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SUMMARY: Purified membrane-bound Na,K-ATPase from pig kidney was solubilized with nonionic detergent, dodecyloctaethylenglycol monoether ($C_{12}E_8$) as 70-90% active protein units with $S_{20,w}$ 7.4 ± 0.2 and maximum molecular mass 170,000 ± 9,000 daltons indicating that the soluble complex predominantly consisted of protomeric αβ-units. Inactivation of Na,K-ATPase by excess $C_{12}E_8$ was not related to the aggregation state of the protein. On storage both the soluble Na,K-ATPase and soluble Ca-ATPase (115,000 daltons) from sarcoplasmic reticulum underwent secondary aggregation which may account for previous reports of higher molecular weights.

INTRODUCTION: Pure Na,K-ATPase from outer renal medulla (1) or from shark rectal glands (2,3,4) contain two proteins, the α-subunit with molecular weight close to 100,000 and the β-subunit, a sialoglycoprotein with protein molecular weight close to 40,000. It is uncertain whether the minimum active protein unit of Na,K-ATPase contains one α-subunit (6,7) in αβ or $\alpha\beta_2$ structures, or if it contains two α-subunits in $\alpha_2\beta_2$ (5,8) or $\alpha_2\beta_4$ (3) oligomeric structures. The subunit structure has been studied after solubilization, but Na,K-ATPase is often inactivated by detergents in concentrations near the cmc (9). Soluble complexes with molecular weights 140,000 (10) and 190,000 (11) were prepared, but they had no Na,K-ATPase activity. In contrast, Na,K-ATPase from the rectal glands of the dogfish remained active after solubili-

ABBREVIATIONS: $C_{12}E_8$ = octaethyleneglycol-mono-n-dodecyl ether; SDS = sodium dodecyl sulphate; DTT = dithiothreitol, TES = tris-hydroxymethyl-2-aminoethane sulfonic acid; Na,K-ATPase (EC 3.6.1.3) = membrane adenosine 5 triphosphat phosphohydrolase requiring Na^+, K^+, and Mg^{2+} for full activity; K-phosphatase (EC 3.6.1.7) = membrane acylphosphate phosphohydrolase requiring K^+ and Mg^{2+} for full activity.

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zation with Lubrol WX (3) or $C_{12}E_8$ (4). Sedimentation equilibrium analysis gave molecular weights 379,000 and 265,000 suggesting that oligomeric structures, $\alpha_2\beta_4$ or $\alpha_2\beta_2$, are reguired for expression of the Na,K-ATPase activity.

The purpose of the present work has been to identify the minimum protein unit of the soluble and active Na,K-ATPase from the outer medulla of mammalian kidney and to examine if the detergent inactivation is related to subunit dissociation of the soluble Na,K-ATPase. The enzyme was purified in membrane-bound form and solubilized at increasing ratios of $C_{12}E_8$/protein to identify the range where the enzyme remains active and the range where the Na,K-ATPase and K-phosphatase activities are lost. Sedimentation velocity analysis was performed immediately after solubilization to determine the aggregation state of the soluble protein. Molecular masses were estimated from the sedimentation coefficients and determinations of the Stokes radius and the buoyant density factor (12) on Na,K-ATPase isolated by gel filtration. The data show that the renal Na,K-ATPase can be solubilized with $C_{12}E_8$ as catalytically competent protein units with molecular mass 170,000 $\pm$ 9,000 daltons or lower and that loss of Na,K-ATPase activity at higher $C_{12}E_8$ concentrations is unrelated to subunit dissociation. Secondary aggregation of the soluble complex interferes with attempts at obtaining rigorous molecular weights by sedimentation equilibrium analysis.

MATERIALS AND METHODS: Na,K-ATPase was purified in membrane-bound form from pig kidney outer medulla by selective extraction of plasma membranes with SDS in presence of ATP, followed by isopycnic zonal centrifugation (1). The preparation had specific activities of 40-48 µmol P_i/min·mg protein for Na,K-ATPase and 5.9-6.9 for K-phosphatase at 37° C. The membrane-bound Na,K-ATPase had sedimentation coefficients of 400-500 S (1) and all Na,K-ATPase activity was sedimented by centrifugation for 10 min at 100,000 rpm in the Beckman Airfuge. This centrifugation was used as an operational criterion for separation of soluble Na,K-ATPase from the non-solubilized membrane residue. For solubilization the pellet was resuspended to 0.2 mg protein/ml in 120 µl 10 mM TES, 1 mM EDTA, 1 mM DTT, pH 7.5 containing 150 mM KCl or 130 mM NaCl, 20 mM KCl and the specified concentrations of $C_{12}E_8$. After incubation for 30 min at 20°C the non-solubilized residue was removed by centrifugation for 10 min at 100,000 rpm.

For Na,K-ATPase assay, 40 µl of the supernatant with NaCl 130 mM, KCl 20 mM were mixed with 4 µl 100 mM ATP, 100 mM $MgCl_2$. After 20-60 sec at 20°C the reaction was stopped with 1 ml icecold 0.5 n HCl containing 30 mg ascorbic acid, 5 mg ammoniumheptamolybdate and 10 mg SDS and the tubes were transferred to ice. For colour development 1.5 ml containing 30 mg sodium-meta-arsenite,

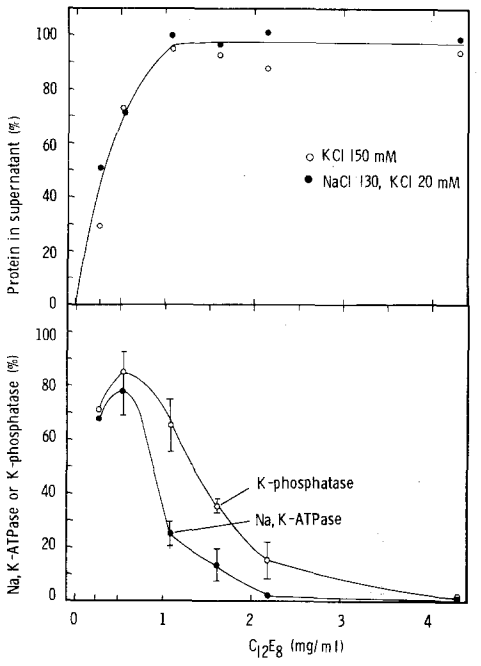

Figure 1. Soluble protein, Na,K-ATPase and K-phosphatase activities after solubilization of pure membrane-bound Na,K-ATPase with increasing concentrations of $C_{12}E_8$ in presence of 150 mM KCl (o) or 130 mM NaCl, 20 mM KCl (o). Enzyme assay as described under Methods. Activities are expressed in per cent of the activity of untreated membrane-bound Na,K-ATPase that was assayed under identical conditions.

30 mg sodium-citrate, and 30 µl acetic acid were added, the tubes were heated for 10 min at 37°C and absorbance was read at 850 nm. For K-phosphatase assay, 40 µl of the supernatant with KCl 150 mM were mixed with 4 µl 200 mM $MgCl_2$, 100 mM p-nitrophenylphosphate. After 20-120 sec at 20°C, 2 ml 0.2 M NaOH, 0.05 M Na_2EDTA were added and absorbance was read at 410 nm (22).

Sedimentation velocities were measured in a Beckman model E centrifuge equipped with photoelectric scanner. Sedimentation coefficients were calculated from linear plots of radial distance of protein boundary versus time from 10 to 70 min after reaching 44,770 rpm.

Gel filtration was done on 1.5 x 90 cm columns of Sepharose 6B eluted downwards at 5 ml/h at 15°C with 150 mM KCl, 1 mM DTT, 1 mM EDTA, 10 mM TES pH 7.5, 150 mg/ml ^{14}C-$C_{12}E_8$ ($4 \cdot 10^4$cpm/mg from CEA, Saclay, France). Samples of 4 mg Na,K-ATPase protein were solubilized by 10 mg ^{14}C-$C_{12}E_8$ ($4 \cdot 10^4$ cpm/mg) in 1 ml of the elution buffer containing 20% glycerol. Fraction volumes were determined by weighing. Peak positions were estimated as in Ref. 12 using Blue Dextran and NaCl analyzed by flame photometry as markers for the void volume and total volume. The columns were calibrated with marker proteins with known Stokes radii (13).

Protein was determined by the Lowry procedure after precipitation with trichloraceticacid as standardized with membrane-bound Na,K-ATPase in which the protein concentration had been determined by quantitative aminoacid analysis. Phospholipid and cholesterol were assayed as before (1). Hexoses were determined by the anthrone reaction (14), hexosamines by the Elson-Morgan reaction (15) and sialic acid by the thiobarbituric acid method (16).

RESULTS: It is seen from Fig. 1 that the efficiency of solubilization with $C_{12}E_8$ was the same in media containing 150 mM KCl as in media containing 130 mM NaCl and 20 mM KCl. The K-phosphatase or Na,K-ATPase activities of the solubilized preparations could be measured directly by additions of a small volume of $MgCl_2$ plus p-nitrophenylphosphate or of $MgCl_2$ plus ATP, resulting in less than 10% dilution of the samples. After solubilization at a $C_{12}E_8$/protein ratio of 2.7 where about 70% of the protein was solubilized both the Na,K-ATPase and the K-phosphatase activities of the soluble protein remained within 70-90% of the specific activity of the untreated membrane-bound Na,K-ATPase. The soluble Na,K-ATPase was rather stable at 20°C declining at rates between -0.01 h^{-1} and -0.05 h^{-1}. Inactivation rates were much faster at 37°C, from -0.01 min^{-1} to -0.04 min^{-1}. Protein solubilization was complete at $C_{12}E_8$/protein ratios of 5-6, but only 30% of Na,K-ATPase and 60-70% of K-phosphatase activities were retained. At this ratio the activity could be restored by removal of $C_{12}E_8$ with BioBeads-SM 2 (not shown). At $C_{12}E_8$/protein ratios of 9-10 and higher both enzyme activities were irreversibly lost.

Both active and inactive Na,K-ATPase sedimented as a single boundary consisting of a major and a minor overlapping component. The major component comprised about 85% of the total protein absorption. As shown by data in Table I the average $S_{20,w}$ decreased from 7.4 S for active and partially active Na,K-ATPase to 6.3 S at a high $C_{12}E_8$/protein ratio. The sedimentation was not affected by the rise of protein concentration from 0.2 to 0.8 mg/ml (Table I). Since the protein profile was also unaltered by addition of excess $C_{12}E_8$, there is no evidence for a change in the aggregational state caused by the inactivation of the enzyme in excess detergent. The reason for the relatively small decrease of $S_{20,w}$ under these conditions is uncertain. It could be due to a change in the shape of the solubilized complex following inactivation, resulting in an increased frictional coefficient with the solvent.

The molecular mass of the soluble protein particles can be estimated from the sedimentation coefficient if the Stokes radius and the effective partial

Table I

Sedimentation coefficients of soluble Na,K-ATPase

Protein mg/ml	$C_{12}E_8$ mg/ml	KCl mM	NaCl mM	Sedimentation coefficient	n
0.2	0.54	150		7.4 ± 0.2	4
0.2	0.54	20	130	7.1	1
0.2	1.08	150		7.4 ± 0.2	4
0.2	1.08	20	130	7.3	1
0.2	4.3	150		6.8	1
0.2	8.6	150		6.3 ± 0.2	4
0.8	2.2	150		7.1	2
0.8	4.3	150		7.0	2

Solubilization as in Fig. 1 at varying concentrations of $C_{12}E_8$, KCl and NaCl. Sedimentation coefficients were determined as in Methods. The values were reduced to standard conditions using the calculated partial specific volume of solubilized Na,K-ATPase (Table II) together with density and viscosity data from standard handbooks.

specific volume of the hydrodynamic particle is known (12). The data obtained by gel chromatography on Sepharose 6B columns are shown in Table II. The sedimentation coefficient of 7.4 ± 0.2 S, the R_S of 72 ± 4 Å, and the buoyant density factor of 0.355 gave estimated molecular masses of 170,000 ± 9,000 (S.E.)daltons for the protein moiety of the active Na,K-ATPase. This represents an overestimate rather than an underestimate of the molecular mass of the enzyme protein because the observations discussed below suggest that the R_S values are overestimated due to secondary aggregation. It has also been found that gel filtration overestimates the Stokes radii of other detergent solubilized membrane proteins as compared to that of water soluble protein standards (13).

Rigorous determinations of the molecular weight of the protein part of the hydrodynamic particle requires sedimentation equilibrium analysis. To reduce background absorbance, attempts were made to separate excess detergent and lipid from the soluble Na,K-ATPase by gel exclusion on Sepharose 6B columns equilibrated with $C_{12}E_8$. We found that the partition coefficient was dependent on the detergent concentration. In columns equilibrated with a high detergent concentration, 1 mg/ml $C_{12}E_8$, the protein was inactivated and eluted as well defined peaks with K_d= 0.39-0.40 corresponding to R_S=67-69Å as estim-

Table II

Chemical compostion, buoyant density factor and Stokes radius of soluble Na,K-ATPase isolated by gel filtration on Sepharose 6B

	δ(mg/g protein	$\bar{\nu}$(cm^3/g)	$\delta_i(1-\bar{\nu}_i\rho)^a_{20.w}$
Carbohydrate	151 ± 6 (5)	0.63	0.056
Phospholipid	176 ± 25 (3)	0.98	0.0038
Cholesterol	95 ± 4 (4)	0.95	0.0049
$C_{12}E_8$	783 ± 86 (5)	0.973	0.023
Protein		0.734	0.267
$(1-\phi\rho)_{20.w}$	0.355		
K_d	0.37 ± 0.02 (4)		
R_s	72 ± 4 Å (4)		

a: $\rho_{20.w}$ = 0.9982 g/cm^3

Gel filtration and determination of chemical composition of the soluble complex was done as described in Methods. The buoyant density factor was calculated from $1-\phi'\rho = (1-\bar{\nu}_p\rho) + \Sigma\delta_i\ (1-\bar{\nu}_i\rho)$ where ρ is solvent density, $\bar{\nu}_p$ and $\bar{\nu}_i$ are partial specific volumes of protein and the chemical components, and δ_i is the weight ratio of the component to protein in the particle.

ated from calibration with standard proteins. At a lower concentration, 0.15 mg/ml $C_{12}E_8$, corresponding to about 2 micelles per protein unit, active Na,K-ATPase was eluted in broad peaks with K_d = 0.34-0.39 equal to R_s 72 ± 4 Å. This enzyme had sedimentation coefficients in the range 10-11 S and molecular weights in the range 270,000-390,000 as determined by sedimentation equilibrium analysis. These results show that fully active, soluble oligomers are formed by secondary aggregation during column chromatography.

Secondary aggregation was also observed with sarcoplasmic reticulum Ca-ATPase. The monomeric polypeptide chain with molecular weight 115,000 (19) remains catalytically active after solubilization of Ca-ATPase in $C_{12}E_8$ (20). However, during column chromatography under the conditions described in Table II, the protein underwent secondary aggregation to a dimeric or trimeric state with molecular masses in the range 206,000-283,000 daltons.

Sedimentation equilibrium analysis without separation of excess lipid and detergent from the soluble Na,K-ATPase can be carried out if special precautions are taken to correct for background absorption by lipid and detergent. At a $C_{12}E_8$/protein ratio of 5-6 this approach gave molecular weights of

160,000-177,000 for the partially active Na,K-ATPase. This value is in good agreement with the molecular weights estimated from the sedimentation coefficient. For the fully active Na,K-ATPase at a $C_{12}E_8$/protein ratio of 2.7 we have not succeeded in obtaining sedimentation equilibrium for homogeneous particles. This may be attributed to the tendency for secondary aggregation of the soluble particles. Similar problems in obtaining molecular weights by sedimentation equilibrium analysis have been encountered after solubilization of Ca-ATPase in $C_{12}E_8$ (20).

DISCUSSION: It can be safely deduced from the sedimentation velocity data together with the R_s values and the effective partial specific volume that the active and soluble Na,K-ATPase has a maximum protein molecular mass of 170,000 $\pm$ 9,000 daltons. Polyacrylamide gel electrophoresis in SDS showed that the soluble Na,K-ATPase contained α-subunits and β-subunits in the same proportion as in the membrane-bound enzyme preparation i.e. in a molar ratio close to one (5,6). The maximum molecular mass of 170,000 cannot contain more than one α-subunit with molecular weight 100,000. The most likely protomer structure is therefore $\alpha\beta$, although $\alpha\beta_2$ cannot be excluded. The assignment of this maximum mass to the protein component of soluble Na,K-ATPase is in agreement with the observation of binding capacities for ATP and ouabain in the range 5.4-6 nmol/mg protein corresponding to molecular weights of 167,000-185,000 for the binding complex (7, see also 6).

Previously, solubilization in a low molecular weight form has led to loss of Na,K-ATPase activity (10,11). The Na,K-ATPase activity has only been retained in soluble preparations with the much higher molecular weights 379,000 (9) or 265,000 (4). Both determinations were obtained by sedimentation equilibrium analysis after chromatography of the soluble Na,K-ATPase. We also observe molecular weights in this range after gel chromatography of the soluble renal Na,K-ATPase, but our data show that the higher molecular weights can be explained by secondary aggregation of the micelles containing the solubilized protein. Until more suitable solubilization methods become available,

it appears that demonstration of the protomeric protein unit of Na,K-ATPase in catalytically competent form requires that sedimentation analysis is performed immediately after solubilization of the membrane-bound Na,K-ATPase.

While the Na,K-ATPase activity is readily inhibited by $C_{12}E_8$ in concentrations exceeding those required for solubilization, the Ca-ATPase remains active presumably because $C_{12}E_8$ may replace lipid attached to the Ca-ATPase without loss of activity (21). Maintenance of the Na,K-ATPase activity thus seems to pose more stringent requirements to the interaction with hydrophobic substances than the mere presence of flexible hydrocarbon chains (Cf. 22,23). This may account for the loss of Na,K-ATPase activity at high $C_{12}E_8$ concentrations by a process which is unrelated to the aggregational state of the protein.

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