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Polypeptide Molecular Weights of the (Na^+,K^+)-ATPase from Porcine Kidney Medulla†

J. William Freytag and Jacqueline A. Reynolds*

ABSTRACT: The molecular weights of the polypeptide chains from (Na^+,K^+)-ATPase of porcine kidney medulla have been determined by analytical sedimentation equilibrium. The α-subunit molecular weight is 93 900, and the β subunit is a glycoprotein with a polypeptide molecular weight of 32 300 (41 400 including protein and carbohydrate). Amino acid and carbohydrate compositions are presented together with related properties (i.e., partial specific volumes, extinction coefficients, and hydrophobic/hydrophilic amino acid content).

Plasma membranes of animal cells contain an ion pump, (Na^+,K^+)-ATPase, that maintains the intracellular space high in potassium and low in sodium. This membrane-bound (Na^+,K^+)-ATPase transduces the energy from ATP hydrolysis to the countertransport of Na^+ and K^+ against a concentration gradient. The pump protein has been purified in membrane-bound form by a number of investigators [e.g., see Jorgensen (1974), Uesugi et al. (1971), Dixon & Hokin (1974), and Hokin et al. (1973)] and as a detergent-solubilized protein–lipid complex by Hastings & Reynolds (1979) and Esmann et al. (1979). In all purified preparations thus far investigated, two polypeptide chains are observed under denaturing conditions in sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The catalytic subunit (α) has an *apparent* molecular weight between 85 000 and 106 000 while the β polypeptide has *apparent* molecular weights varying between 36 000 and 65 000 and contains covalently bound carbohydrate.

Despite extensive kinetic studies of (Na^+,K^+)-ATPase from a wide variety of species and organs together with structural investigations of conformational changes during the ATP hydrolysis cycle, we are lacking the most fundamental information required for a mechanistic model. The absolute number of polypeptide chains in the minimal functioning unit is still a subject of controversy, and this information cannot be obtained in the absence of rigorous molecular weight determinations of the catalytic and glycoprotein subunits. Absolute molecular weights and polypeptide chain stoichiometry have been determined by sedimentation equilibrium for the (Na^+,K^+)-ATPase from dogfish shark rectal gland (Hastings & Reynolds, 1979; Esmann et al., 1979). Although both groups obtain the same molecular weights for the individual polypeptides (α = 106 000 and β = 36 000–40 000), different chain stoichiometries were found for the minimal functioning unit, $\alpha_2\beta_4$ and $\alpha_2\beta_2$, respectively. These data are not available for the mammalian enzyme, but estimates of polypeptide stoichiometry and the minimal molecular weight of a functioning unit have been published by using indirect methods [e.g., cross-linking (Craig & Kyte, 1980) and active site binding (Jorgensen, 1980)]. *Apparent* polypeptide molecular weights have been reported for the mammalian enzyme by

† From the Whitehead Medical Research Institute and the Department of Physiology, Duke University Medical Center, Durham, North Carolina 27710. *Received April 13, 1981.* This study was supported by Research Grant HL 22570 from the National Institutes of Health. J.W.F. was a recipient of a Muscular Dystrophy Association Postdoctoral Fellowship.

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using the empirical procedures of sodium dodecyl sulfate–polyacrylamide gel electrophoresis and gel filtration in guanidinium chloride.

In this study, we have purified the α and β polypeptides from the (Na^+,K^+)-ATPase of porcine kidney medulla and determined their respective molecular weights by the rigorous technique of analytical sedimentation equilibrium. This information allows a calculation of possible minimal molecular weights of the functioning unit, and the results are compared with estimates from indirect procedures.

Experimental Procedures

Materials. Fresh porcine kidneys were obtained from a local abattoir immediately after animal death and either used at once or stored at −70 °C. Membrane-bound (Na^+,K^+)-ATPase was isolated from outer medulla microsomes by the method of Jorgensen (1974), i.e., titration of the membrane fraction with sodium dodecyl sulfate and isolation of the extracted membrane by means of discontinuous density gradient centrifugation. The purified membrane fraction is recovered at a density corresponding to 1.117 g/cm^3.

All chemicals were standard reagent grade with the exception of a specially pure grade of sodium dodecyl sulfate produced by BDH Chemical Corp. and marketed by Gallard-Schlesinger.

Methods. The α and β polypeptides from purified, membrane-bound (Na^+,K^+)-ATPase were separated by gel filtration chromatography in either sodium dodecyl sulfate or guanidinium chloride. Protein (3.4 mg) was solubilized in 1.0 mL of 0.52 M sodium dodecyl sulfate, 0.6 M β-mercaptoethanol, and 0.025 M *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid (Tes) (pH 7.5). The solution was chromatographed on a Sepharose 6B column (1.6 × 90 cm) equilibrated in 0.0173 M sodium dodecyl sulfate, 0.025 M Tes (pH 7.5), and 0.001 M ethylenediaminetetraacetic acid (EDTA). The α-polypeptide fraction was pooled, concentrated, and rechromatographed on a second Sepharose 6B column (0.9 × 55 cm) using an elution buffer containing 0.00346 M sodium dodecyl sulfate, 0.025 M Tes (pH 7.5), and 0.001 M EDTA. Bound detergent was measured by using ^{35}S-labeled sodium dodecyl sulfate (Makino et al., 1973). The α polypeptide was also transferred into 8 M guanidinium chloride, 0.025 M dithiothreitol, and 0.5 M Tris-HCl (pH 8.5) by exhaustive dialysis at 75 °C. Cysteine residues were alkylated with iodoacetic acid, and the sample was dialyzed against 8 M guanidinium chloride and 0.1 M sodium phosphate (pH 7.0).

The nonalkylated β polypeptide which was eluted from the gel filtration column in the presence of sodium dodecyl sulfate aggregated irreversibly when transferred to 8 M guanidinium chloride even in the presence of reducing reagent. Therefore, the purified, membrane-bound (Na^+,K^+)-ATPase was dissolved directly in 8 M guanidinium chloride, 0.5 M Tris-HCl (pH 7.5), and 0.02 M dithiothreitol. The polypeptides were alkylated with iodoacetamide and chromatographed on a Sepharose CL-4B column (1.6 × 80 cm) in 8 M guanidinium chloride and 0.5 M Tris-HCl (pH 8.5). The catalytic chain eluted in the void volume, and the β chain was well included as a symmetrical peak with a K_d of 0.6. Fractions were pooled and concentrated.

Solvent densities were measured in an Anton Paar densimeter at 20 °C. Amino acid compositions were obtained from 24-, 48-, and 72-h hydrolysates on a Beckman 119 amino acid analyzer. Tryptophan was determined spectrophotometrically by using the method of Edelhoch (1967). Amino sugars were assayed on the analyzer following 6-h hydrolysis in 4 N HCl, 100 °C. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was carried out according to Weber & Osborn (1969) with minor modifications.

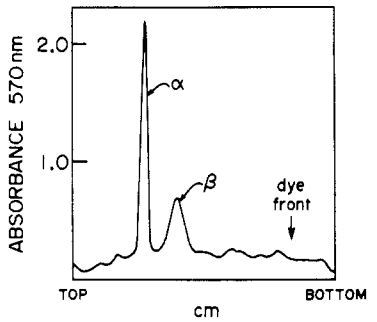

FIGURE 1: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of membrane-bound (Na^+,K^+)-ATPase.

Sedimentation equilibrium measurements were performed on a Beckman Model E analytical ultracentrifuge equipped with a photoelectric scanner. Sample size in all experiments was 100 μL, and initial optical densities were between 0.06 and 0.17 absorbance units. Equilibrium was judged to have been attained when the distribution of protein in the centrifugal field did not change over 8-h intervals.

Partial specific volumes for protein and carbohydrate were calculated according to Cohn & Edsall (1943) and Gibbons (1966). The partial specific volume of sodium dodecyl sulfate was determined in this laboratory and has been published elsewhere (Steele et al., 1978). Estimated corrections to the buoyant density factor in concentrated guanidinium chloride solutions were made according to Lee & Timasheff (1979).

Results and Discussion

Membrane-bound (Na^+,K^+)-ATPase purified as described under Experimental Procedures contained greater than 95% α and β polypeptides by the criterion of sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Figure 1). Specific activities between 13 and 18 IU were routinely obtained. Comparison of these activities with those reported by Jorgensen for mammalian kidney enzyme suggests substantial inactivation (maximum observed hydrolytic activity by Jorgensen = 36–40 IU); however, other laboratories report specific activities lower than (Craig & Kyte, 1980) or the same as (Lane et al., 1979) those observed by us. Hastings & Reynolds (1979) and Esmann et al. (1979) found a variable amount of inactivated enzyme in preparations from dogfish shark rectal gland that could be separated from highly active protein (28–36 IU) by either ion-exchange or gel filtration chromatography of the detergent–lipid–enzyme complex.

The α and β polypeptides were purified as described under Experimental Procedures in sodium dodecyl sulfate and 8 M guanidinium chloride. Both appeared as single symmetrical bands on sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The amino acid compositions, carbohydrate analyses, and associated calculated properties of the two chains are given in Table I. It is interesting that the content of hydrophobic amino acid residues is somewhat higher in the β polypeptide than in the catalytic subunit.

The α and β polypeptides were homoegeneous in the ultracentrifuge as evidenced by ln OD vs. r^2 plots that were linear throughout the sample column. Calculation of molecular weights from the centrifuge data requires the knowledge of the buoyant density factor. Thus, one measures $M_p(1 - \phi'\rho)$ directly where M_p is the molecular weight of the protein and $1 - \phi'\rho$ is the buoyant density factor. The latter term is equal to $(1 - \bar{v}_p\rho) + \sum_i \delta_i(1 - \bar{v}_i\rho)$ where $\bar{v}_p$ is the partial specific

Table I: Amino Acid and Carbohydrate Compositions

	mol/10^5 g α	mol/polypeptide α	mol/10^5 g β	mol/polypeptide β
Lys	48.1	45.2	46.0	14.9
His	15.4	14.4	15.3	5.0
Arg	43.5	40.8	37.6	11.6
Asx	86.5	81.2	69.0	22.3
Thr[a]	54.1	50.8	51.8	16.7
Ser[a]	56.9	53.5	66.9	21.6
Glx	91.8	86.2	77.4	25.0
Pro	37.1	34.8	48.0	15.5
Gly	70.8	66.5	76.8	24.8
Ala	71.7	67.3	63.0	20.4
Cys[c]	26.7	25.1	20.0	6.5
Val[b]	58.7	55.2	64.8	20.9
Met[c]	18.7	17.6	21.8	7.0
Iles[b]	55.8	52.4	45.2	14.6
Leu[b]	87.9	82.5	94.4	30.5
Tyr[a]	27.9	26.2	35.5	11.5
Phe	37.1	34.8	49.9	16.1
Trp[d]	14.7	13.8	17.4	5.6
hexose[e]	0	0	72.9	23.6
hexosamine[f]	0	0	45.9	14.8
sialic acid[g]	0	0	23.4	7.6
hydrophilic	0.438		0.404	
hydrophobic	0.313		0.341	
neutral	0.248		0.255	
$A^{1cm}_{1mg/mL}$	1.20		1.44 (protein) 1.13 (protein + CHO)	
g of CHO/g of protein			0.28	
$\bar{v}$	0.737		0.740 (protein) 0.716 (protein + CHO)	

[a] Extrapolated to zero time. [b] Value at 72 h. [c] Determined as cysteic acid and methionine sulfone after performic acid oxidation. [d] Determined spectrophotometrically in 8 M guanidinium chloride. [e] Assayed by the phenol-sulfuric acid procedure of Dubois et al. (1956). [f] The 4-h hydrolysates, 4 N HCl, 100 OC, amino acid analyzer. [g] Assayed according to Warren (1969).

Table II: Molecular Weights of α and β Polypeptides[a]

	solvent[b]	$M(1-\phi'\rho)$	ρ (g/cm^3)	$\delta_{NaDodSO_4}$	δ_{CHO}	$M_{protein}$[c]	$M_{protein+CHO}$
α[d]	8.2 M GdmCl	1.18×10^4	1.189			95 400 (95 900)	
α[e]	3.45×10^3 M $NaDodSO_4$	3.55×10^4	1.0	0.89		92 400	
β[f]	8.3 M GdmCl	5.98×10^3	1.195		0.28	32 300 (32 100)	41 400 (41 100)

[a] $1-\phi'\rho$ is the buoyant density factor, $(1-\bar{v}_p\rho) + \Sigma_i\delta_i(1-\bar{v}_i\rho)$. $\bar{v}_p$ = partial specific volume of the protein; $\bar{v}_i$ = partial specific volume of bound component i, δ_i = grams of component i per gram of protein. [b] GdmCl, guanidinium chloride; $NaDodSO_4$, sodium dodecyl sulfate. [c] Values in parentheses are obtained by using estimated δ values for bound water and GdmCl according to the method of Lee & Timasheff (1979). Values not in parentheses are uncorrected for preferential solvent interactions with the exception of measurements made in $NaDodSO_4$ where $\delta_{NaDodSO_4}$ was measured directly. [d] 21 900 rpm. [e] 12 000 rpm. [f] 30 000 rpm.

volume of the protein and $\bar{v}_i$ is the partial specific volume of each bound component, i. The $\bar{v}$ values for protein and carbohydrate are calculated as described under Experimental Procedures.[1] The $\bar{v}$ of sodium dodecyl sulfate and the binding as grams of detergent per gram of protein have been determined experimentally. When a concentrated solvent such as 8 M guanidinium chloride is used, the preferential interaction of water and the salt must also be included in the buoyant density factor. Hade & Tanford (1967) have shown that for most proteins this correction decreases the partial specific volume of the protein by approximately 0.01 cm^3/g. More recently, Lee & Timasheff (1979) have discussed a more accurate estimate of the binding of water and guanidinium chloride to proteins and the ensuing effect on partial specific volumes. Table II presents the molecular weights of the α and β polypeptides from porcine kidney medulla in sodium dodecyl sulfate and guanidinium chloride. The numbers in parentheses are obtained by applying the correction to the buoyant density factor by the method of Lee & Timasheff (1979). The results not in parentheses assume no correction for preferential solvent interaction. It is apparent that the two procedures give molecular weights well within the experimental error of the analytical ultracentrifuge. The molecular weight of the α chain is 93 900, and that of the *protein* portion of the β chain is 32 300. Addition of the weight of carbohydrate to the latter number gives a molecular weight of 41 400 for *protein plus carbohydrate*.

With the molecular weights of the individual polypeptide chains known from a nonempirical analytical procedure, one can now calculate the possible molecular weights of the intact (Na^+,K^+)-ATPase as a function of the α to β molar ratio. The

[1] The reliability of the calculations used herein for partial specific volumes and buoyant density factors has been demonstrated previously. Grefrath & Reynolds (1974) calculated $\bar{v}$ for a glycoprotein containing 55% carbohydrate as 0.67 g/cm^3 as compared to the experimentally determined value of 0.68 g/cm^3. Similarly, Reynolds & Tanford (1976) summarize the molecular weight determinations of two lipid binding proteins by sedimentation equilibrium in the presence of a variety of bound detergents. The results were later confirmed by sequence analysis and demonstrated an error of less than 10%.

Table III: Calculated Molecular Weight of Porcine Kidney (Na^+,K^+)-ATPase

polypeptide stoichiometry	protein mol wt	α/β wt ratio
$\alpha\beta$	126 200 ± 7 600	2.9 ± 0.4
$\alpha\beta_2$	158 500 ± 9 500	1.5 ± 0.2
$\alpha_2\beta$	220 100 ± 13 200	5.8 ± 0.8
$\alpha_2\beta_2$	252 400 ± 15 100	2.9 ± 0.4
$\alpha_2\beta_3$	284 700 ± 17 100	1.9 ± 0.2
$\alpha_2\beta_4$	317 000 ± 19 000	1.5 ± 0.2

ref	g of protein/ mol of active site	wt ratio
Jorgensen (1980)[a]	278 000	2.3
Craig & Kyte (1980)[b]	180 000–500 000	2.15

[a] Procine, canine, and lamb kidney/protein weight ratio determined as staining ratios in $NaDodSO_4$-polyacrylamide gel electrophoresis. [b] Canine kidney/protein weight ratio determined by quantitative amino acid analysis after separation by $NaDodSO_4$-polyacrylamide gel electrophoresis.

results are shown in Table III. Craig & Kyte (1980) have reported a α/β mass ratio of 2.15 ± 0.15 for (Na^+,K^+)-ATPase from canine kidney medulla by using quantitative amino acid analysis after separation of the polypeptides by polyacrylamide gel electrophoresis. Jorgensen (1980) obtained one active site per 278 000 g of protein for (Na^+,K^+)-ATPase from porcine, canine, and lamb kidney. An examination of Table III indicates that the structure $\alpha_2\beta_3$ is most consistent with these data. On the other hand, Moczydlowski & Fortes (1981) have reported one active site per 175 000 g of protein by using the (Na^+,K^+)-ATPase from electric eel. The subunit molecular weights from this species have not been measured rigorously, but if they do not differ significantly from those of dogfish shark rectal gland or porcine kidney medulla, $\alpha\beta_2$ stoichiometry would be consistent with this observation. [$\alpha\beta_2$ = 178 400 ± 10 000 for dogfish shark rectal gland enzyme and 158 500 ± 9500 for porcine kidney medulla (Na^+,K^+)-ATPase.]

The subunit molecular weights are known for only two sources of (Na^+,K^+)-ATPase, dogfish shark rectal gland and canine kidney medulla. Even in these two systems, variable estimates of polypeptide stoichiometry and minimal molecular weight of the functioning unit are obtained. In light of the recent studies of Moczydlowski & Fortes (1981), who report both higher specific activities and lower molecular weight (grams of protein per active site) than have been previously observed, we must consider the possibility that the (Na^+,K^+)-ATPase can be structurally altered (partially inactivated) by many of the commonly used preparative procedures.

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