Biochimica et Biophysica Acta, 641 (1981) 55—70

BBA 79121

STUDIES ON (Na^+ + K^+)-ACTIVATED ATPase

XLVII. CHEMICAL COMPOSITION, MOLECULAR WEIGHT AND MOLAR RATIO OF THE SUBUNITS OF THE ENZYME FROM RABBIT KIDNEY OUTER MEDULLA

W.H.M. PETERS, J.J.H.H.M. DE PONT, A. KOPPERS and S.L. BONTING

Dept. of Biochemistry, University of Nijmegen, 6500 HB Nijmegen (The Netherlands)

(Received July 23rd, 1980)

Key words: (Na^+ + K^+)-ATPase subunit; Amino acid composition; Phospholipid composition; Carbohydrate content; Molecular weight determination; Molar absorption coefficient; (Rabbit kidney)

Summary

(1) The subunits of a purified preparation of (Na^+ + K^+)-ATPase from rabbit kidney outer medulla have been completely separated by gel filtration in sodium dodecyl sulfate. During the gel filtration procedure 3—4% of the phospholipids present in the purified enzyme preparation remain bound to the separated subunits even after repeated gel filtration.

(2) The composition of the bound phospholipids does not greatly differ from that in the original enzyme preparation, which indicates that there is no specific binding of phospholipids to the subunits. The isolated β-subunit contains twice as much phospholipid per mg protein as the α-subunit.

(3) The α-subunit is more hydrophobic (43%) in its amino acid composition than the β-subunit (38%). The α-subunit contains more alanine and histidine and much less tyrosine and lysine than the β-subunit; the other amino acids show little difference. The amino acid composition of each subunit closely resembles that of the corresponding subunit of the enzyme from dog and lamb kidney, duck salt gland, shark rectal gland and electric eel electroplax.

(4) Both subunits are glycoproteins, although the β-subunit contains 5-times more carbohydrate per g protein than the α-subunit. Both subunits contain glucosamine, galactose, mannose, sialic acid and also glucose, which appears to be covalently bound. Galactosamine, in small amounts, is only detected in the β-subunit.

Abbreviation: SDS, sodium dodecyl sulfate.

(5) The molecular weights of the subunits have been determined by sedimentation equilibrium analysis in the absence of detergent. The molecular weight of the α-subunit is 131 000 (excluding phospholipids), and of its protein part 120 600. The molecular weight of the β-subunit is 61 800 (excluding phospholipids), and of its protein part 42 800.

(6) In the native enzyme complex, the α/β protein mass ratio is 3.04, which gives an α/β molar ratio of 1 : 1.

(7) The molar absorption coefficients at 280 nm for the α- and β-subunits are determined as 143 000 and 78 000 $l \cdot mol^{-1} \cdot cm^{-1}$, respectively. In the native enzyme complex the α/β absorbance ratio at 280 nm is 2.02, which gives an α/β molar ratio of 1 : 1.

(8) Comparison of the amino acid composition of the subunits and of the native enzyme indicates that the most probable α/β molar ratio is 1 : 1.

(9) Since these three independent methods all give the same result, we conclude that the α/β molar ratio is 1 : 1. In combination with enzyme molecular weight data this means that the enzyme must be composed of two α- and two β-subunits ($\alpha_2\beta_2$), giving a protein molecular weight of 326 800.

Introduction

Although there is general agreement on the presence of two different subunits in the $(Na^+ + K^+)$-ATPase complex from various sources [1,2], there are conflicting reports about the molecular weights of the two subunits and about the subunit composition of the enzyme molecule. Compositions of $\alpha_2\beta_1$ [3—5], $\alpha_2\beta_2$ [2,6—8], $\alpha_2\beta_3$ [9], $\alpha_2\beta_4$ [10,11] and $\alpha_8\beta_x$ [12] have been proposed for $(Na^+ + K^+)$-ATPase from various sources. The confusion concerning the molecular weights appears to be due to the unreliability of molecular weight determinations of glycoproteins by SDS-polyacrylamide gel electrophoresis and gel filtration. In addition, the Coomassie brilliant blue staining method for determining the relative amounts of the subunits leads to inaccurate results, since different proteins show different degrees of staining.

In this paper we report the separation and isolation of the two subunits of a purified $(Na^+ + K^+)$-ATPase preparation from rabbit kidney outer medulla. We find that both subunits contain considerable amounts of carbohydrate and minor amounts of lipids, which have been quantitatively determined. We also report the amino acid composition of each subunit. Furthermore, we describe the molecular weight determination of the isolated subunits by means of sedimentation equilibrium analysis. Three independent approaches have been used to calculate from these data the subunit composition of the enzyme complex.

Methods

Enzyme preparation. A highly purified, membrane-bound preparation of $(Na^+ + K^+)$-ATPase is obtained from rabbit kidney outer medulla by using the method of Jørgensen [13]. The specific activity ranges from 1.4 to 2.0 mmol ATP hydrolyzed/mg protein per h. On SDS-polyacrylamide gel electro-

phoresis there are only two protein bands. The low molecular weight band stains for carbohydrate with the periodic acid-Schiff staining method.

Solubilization of the enzyme. The enzyme (4—8 mg protein) is solubilized in 1.5 ml buffer solution, which contains: 25 mM Tris-HCl (pH 7.4), 1 mM EDTA, 6% SDS (w/v), 3% β-mercaptoethanol (v/v). The mixture is left at room temperature for 3 h under gentle shaking, and is then centrifuged at 180 000 × *g* for 30 min. The clear supernatant is used for gel filtration. The small remaining pellet contains no detectable protein.

Gel filtration procedure. The supernatant, containing 4—8 mg protein, is loaded on a Sephadex G-200 superfine (Pharmacia) gel filtration column (2.6 × 95 cm, Pharmacia), which is eluted with a buffer solution containing: 25 mM Tris-HCl (pH 7.4), 1 mM EDTA, 0.1% SDS. The flow rate is 3 ml/h and 2-ml fractions are collected. The flow direction in the column is from bottom to top, which prevents clogging of the column and gives better separation of the subunits. The elution process is followed by monitoring the 280 nm absorption of the samples. All subunit preparations used for phospholipid analysis or molecular weight determination by sedimentation equilibrium analysis were subjected to at least a second gel filtration procedure in order to remove non-protein bound lipids.

Protein determination. Protein determinations are performed, according to the method of Lowry et al. [14] after trichloroacetic acid precipitation as described by Jørgensen [13]. Bovine serum albumin is used as standard. Absolute protein determinations are achieved by adding the weights of the separate amino acid residues after amino acid analysis.

Amino acid analysis. The protein samples are hydrolyzed in 5.7 M HCl at 105°C for 4, 6, 24, 48 and 72 h in sealed evacuated glass tubes in darkness, using norleucine as internal standard. The hydrolysate is analysed on a modified JEOL type JLC-6AH amino acid analyzer. Tryptophan is determined by using the spectrophotometric method of Edelhoch [15].

Sugar analysis. All sugar analyses, except those of amino sugars, are carried out after hydrolysis in 2 M HCl for 2.5 h at 100°C in sealed evacuated glass tubes. Neutral sugars are assayed by gas-liquid chromatography according to the method of Langeveld et al. [16]. Glucose is also determined enzymatically by using the hexokinase/glucose-6-phosphate dehydrogenase method (Boehringer, Mannheim, F.R.G.). Sialic acid is measured using the fluorometric assay of Hammond and Papermaster [17]. Glucosamine and galactosamine are determined on the amino acid analyzer. Sucrose is determined enzymatically by using the β-fructosidase-hexokinase/glucose-6-phosphate dehydrogenase method (Boehringer, Mannheim, F.R.G.).

Phospholipid analysis. Total lipid extraction is carried out with $CHCl_3$/CH_3OH (2 : 1, v/v), and the extract is washed with 0.1 M KCl [18]. Phospholipid analysis of the lipid extract is carried out by two-dimensional thin-layer chromatography on silica gel, containing 4% alkaline magnesium silicate [19]. The phosphate content of the lipid spot is measured using a modified Fiske-Subbarow method [19].

Removal of SDS from the subunit preparations. Dowex AG 1 × 2 (200—400 mesh) is converted to the acetate form according to the method of Weber and Kuter [20], and is stored in 0.05 M Tris/acetate buffer (pH 7.4). Before

use the resin is equilibrated in 0.05 M phosphate buffer (pH 7.4). This resin preparation has been used in two different ways.

Method 1: 0.5 ml α-subunit solution (280 nm absorbance 0.4—0.8 in a 1-cm cuvet) is passed through a 0.5 × 4 cm column of resin. The column is washed with 0.05 M phosphate buffer (pH 7.4). Monitoring the 280 nm absorbance of the eluate shows when the protein is leaving the column. This method cannot be used for SDS removal from the β-subunit, because this subunit adheres to the resin.

Method 2: 2 ml subunit solution (280 nm absorbance 0.4—1.2) are dialysed against 200 ml 0.05 M phosphate buffer (pH 7.4), in which 30—40 ml resin slurry are gently stirred for 48 h at 4°C. This method is suitable for both α- and β-subunit solutions.

Both methods give nearly complete removal of SDS, as checked with [^{35}S]-SDS (38.9 mCi/mg, New England Nuclear). After treatment with method 1, there remains at most 0.02% and with method 2 at most 0.25% of the amount of SDS originally present.

Molecular weight determinations. The molecular weights of the subunits are determined by sedimentation equilibrium analysis in 0.05 M phosphate buffer (pH 7.4) in the absence of detergent and reducing agent.

After removal of SDS, each subunit solution is dialyzed for at least 24 h against 0.05 M phosphate buffer (pH 7.4), which is also used as reference solution. Each solution is centrifuged for 30 min at 200 000 × g in a Beckman airfuge to remove possible protein aggregates. The resulting subunit solutions (280 nm absorbance 0.3—0.6) are then centrifuged at 8000—12 000 rev./min for 48 h at 20°C in a Beckman model E ultracentrifuge, equipped with monochromator, ultraviolet detection unit and An-H-Ti type rotor.

Molecular weights are calculated by means of the following equation:

$$M = \frac{2RT \ln A}{\omega^2(1 - \bar{v}\rho)\, r^2}$$

where A is the 280 nm absorbance measured at a certain point in the cell, r is the distance from this point to the rotor axis, ρ is the density of the solvent (1.006 g/cm^3), $\bar{v}$ is the partial specific volume of the subunit, and ω is the angular velocity of the rotor.

The partial specific volumes of the α- and β-subunits are calculated from their known chemical composition by the equation:

$$\bar{v} = \frac{\Sigma \bar{v}_r w_r}{\Sigma w_r}$$

where $\bar{v}_r$ is the partial specific volume of a given component and w_r its percentage of weight [21,22]. The partial specific volumes of amino acids, carbohydrates and phospholipids are taken from the data of Cohn and Edsall [21], Gibbons [22] and Tanford et al. [23], respectively.

Sedimentation coefficients are determined with a standard double sector centerpiece in the An-H-Ti type rotor at a rotor speed of 68 000 rev./min by means of ultraviolet detection.

Miscellaneous. SDS-polyacrylamide gel electrophoresis is carried out accord-

ing to the method of Laemmli [24]. Periodic acid-Schiff staining for glycoproteins is carried out according to the method of Segrest and Jackson [25], except that the incubation times for periodic acid and sodium metabisulfite are both reduced to 20 min.

Statistical significance of differences between averages is calculated with the Student's *t*-test.

Results

Subunit separation

After solubilization of the purified ($Na^+ + K^+$)-ATPase in 6% SDS (w/v) the solution is not entirely clear. The insoluble material, which is not protein, can be removed by centrifugation. The clear supernatant, which does not display any ATPase activity, is subjected to gel filtration in 0.1% SDS on a Sephadex G-200 superfine column. The elution profile shows two protein peaks, which are completely separated from each other (Fig. 1). The first protein peak represents the α-subunit and the second one the β-subunit, as judged by SDS-polyacrylamide gel electrophoresis. Each peak gives only one band after Coomassie brilliant blue staining (not shown). At an elution volume of about 450 ml a minor third peak is visible, which consists entirely of SDS and probably results from light scattering by SDS micelles.

Phospholipid content of the subunits

Organic phosphate has been determined in the elution fractions in order to determine when the phospholipids are leaving the column. Fig. 1 shows that the major part of the phospholipids is separated from the proteins and emerges as a broad peak after the two subunit peaks. Two minor phosphate peaks elute together with the two protein peaks. The phosphate/protein ratio remains constant after a second gel filtration of each subunit (after concentration to 1.5 ml on a Minicon-B concentrator) as illustrated by Fig. 2.

In order to determine whether this phosphate content represents bound phospholipids, the eluate of each subunit fraction is concentrated to about 0.1 ml and is extracted with $CHCl_3/CH_3OH$ (2 : 1, v/v). In both cases at least 90% of the phosphate is extractable by $CHCl_3/CH_3OH$. Two-dimensional thin-layer chromatography of the extract yields the phospholipid composition, which is shown in Table I together with that of the native ($Na^+ + K^+$)-ATPase preparation. There is considerable similarity between these phospholipid compositions, even though the remaining phospholipids in the α- and β-subunits represent only 2 and 5%, respectively, of the total phospholipid content of the native enzyme preparation. Only the phosphatidylinositol content of the isolated subunits seems to be significantly lower than that of the native enzyme preparation.

Amino acid composition of the subunits

The amino acid compositions of the two subunits and the intact enzyme are shown in Table II. The main differences between the two subunits concern the four amino acids: alanine, histidine, tyrosine and lysine, the first two being much higher and the latter two much lower in the α-subunit than in

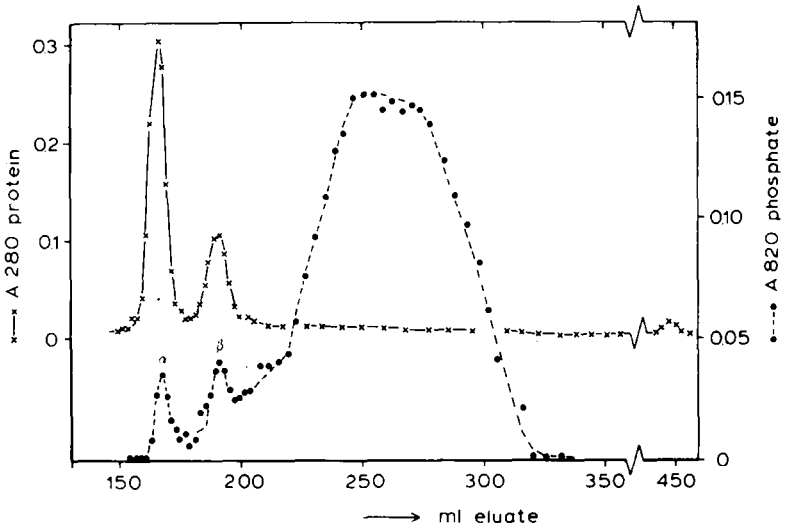

Fig. 1. Protein (X———X) and phospholipid (•- - - - -•) patterns of the solubilized ($Na^+ + K^+$)-ATPase after gel filtration on Sephadex G-200 superfine.

the β-subunit. The percentage of nonpolar (hydrophobic) amino acids in the α-subunit (43.2%) is higher than that for the β-subunit (37.7%), as is consistently found for the enzyme isolated from different species and organs (Table III).

Carbohydrate composition of the subunits

Both subunits contain carbohydrates and are thus glycoproteins. The results

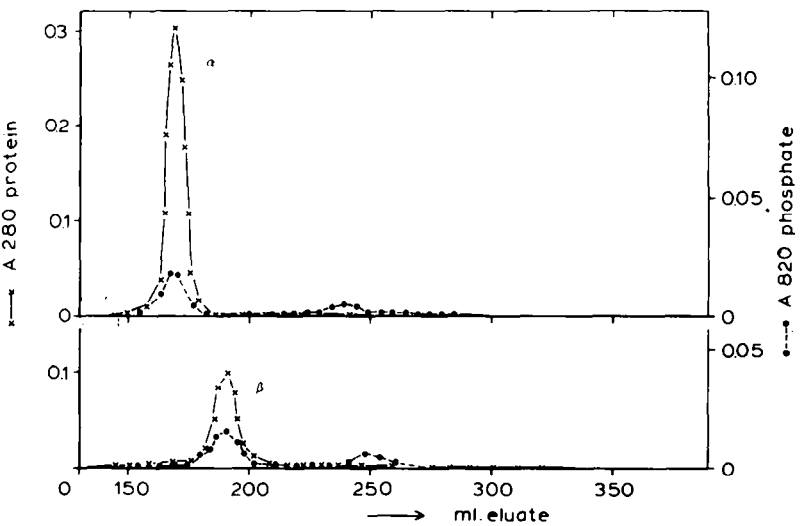

Fig. 2. Protein (X———X) and phospholipid (•- - - - -•) patterns after a second Sephadex G-200 superfine gel filtration procedure of α- and β-subunit preparations.

TABLE I

PHOSPHOLIPID COMPOSITION OF ISOLATED α- AND β-SUBUNITS AND INTACT ENZYME

Values are given with standard error of the mean; n is the number of preparations analyzed.

Phospholipid	Phospholipid composition (% of total)		
	α-subunit (n = 3)	β-subunit (n = 3)	total enzyme * (n = 15)
Sphingomyelin	23 ± 6	17 ± 4	18 ± 0.6
Phosphatidylcholine	31 ± 5	37 ± 2	36 ± 0.7
Phosphatidylserine	11 ± 2	11 ± 2.5	13 ± 0.9
Phosphatidylinositol	1 ± 1	1 ± 1	6 ± 0.3
Phosphatidylethanolamine	33 ± 7	33 ± 4.5	28 ± 1.0
μg P/mg protein	0.74	1.66	33.1

* Data derived from de Pont et al. [26].

of the carbohydrate determinations, listed in Table IV, show good reproducibility, except for the gas chromatographic determination of glucose, which has also been observed by Perrone et al. [28].

The variability in the glucose content would suggest that it derives from an extraneous source. Three possibilities of such an extraneous source have been investigated:

(1) Sucrose used in preparation and storage of the ($Na^+ + K^+$)-ATPase preparation. This possibility, also suggested by Perrone et al. [28], has been tested

TABLE II

AMINO ACID COMPOSITION OF α- AND β-SUBUNITS AND INTACT ENZYME

Averages for analyses on two preparations after 4, 6, 24, 48 and 72 h hydrolysis. The values for serine are extrapolated to zero time of hydrolysis, those for valine and isoleucine to infinite time of hydrolysis. The average S.E. in these values is 0.13 mol/100 mol amino acids. Values are expressed as mol per 100 mol amino acids.

Amino acid	α-subunit	β-subunit	Intact enzyme
Aspartic acid	10.5	9.9	9.95
Threonine	6.0	4.1	5.6
Serine	7.2	6.6	6.95
Glutamic acid	10.4	11.8	10.7
Proline	4.6	5.8	4.6
Glycine	8.0	9.9	8.65
Alanine	8.2	4.3	7.2
Cysteine	0.9	0.7	0.8
Valine	6.7	5.7	6.2
Methionine	2.3	2.1	2.6
Isoleucine	6.5	5.0	5.8
Leucine	9.3	7.1	9.35
Tyrosine	2.3	4.8	2.7
Phenylalanine	4.0	5.3	4.5
Tryptophan	1.6	2.4	not determined
Histidine	1.9	0.5	1.6
Lysine	5.6	9.9	6.7
Arginine	4.7	4.7	4.8

TABLE III

PERCENTAGE OF NON-POLAR (HYDROPHOBIC) AMINO ACIDS IN THE α- AND β-SUBUNITS OF ($Na^+ + K^+$)-ATPase FROM DIFFERENT SOURCES

Values calculated from our results and from data given in the cited references, taking proline, alanine, valine, methionine, isoleucine, leucine, phenylalanine and tryptophan as non-polar amino acids. —, no data available for tryptophan.

Source	Rabbit kidney	Dog kidney	Lamb kidney	Duck salt gland	Shark rectal gland	Electric eel electroplax	Mean ± S.D.
α-subunit	43.2	44.5	44.4	—	45.0	44.7	44.4 ± 0.7
β-subunit	37.7	41.7	38.2	43.0	42.2	42.2	40.8 ± 2.3
Reference	this paper	11	27	3	28	28	

by means of enzymatic sucrose determination on several purified subunit preparations. No sucrose is detected, although good recovery is obtained for added sucrose.

(2) Glycolipids present in the plasma membrane. Lipid extracts of native sucrose-free ($Na^+ + K^+$)-ATPase have been hydrolyzed and analyzed for glucose. Variable, minor amounts of glucose (less than 0.4 g/100 g protein) are found in these lipid extracts, but these can only account for a minor part of the amounts of glucose found in the subunits.

(3) Sephadex, an anhydroglucose polymer, is used in the gel filtration column. In the eluate of the Sephadex column, glucose can be detected after hydrolysis. However, the amounts of glucose found can again only account for a minor part of the glucose in the subunit preparations.

We must thus conclude that the glucose found is largely really present in the subunits and is probably covalently bound.

Subunit molecular weights

After SDS removal by method 1, the α-subunit gives a sedimentation coeffi-

TABLE IV

CARBOHYDRATE COMPOSITION OF α- AND β-SUBUNITS

Averages for multiple determinations on four preparations are given with standard error of the mean. n.d., not detectable.

Carbohydrate	α-subunit		β-subunit	
	mol/100 mol amino acid	g/100 g protein	mol/100 mol amino acid	g/100 g protein
Mannose	0.3 ± 0.1	0.5 ± 0.1	2.5 ± 0.1	3.9 ± 0.2
Galactose	0.9 ± 0.1	1.5 ± 0.2	5.5 ± 0.4	8.6 ± 0.6
Glucose	0.9 ± 0.2	1.4 ± 0.4	2.0 ± 0.5	3.2 ± 0.8
Glucosamine	2.0 ± 0.2	4.0 ± 0.4	10.1 ± 0.5	19.5 ± 1.0
Galactosamine	n.d.	n.d.	0.3 ± 0.1	0.6 ± 0.2
Sialic acid	0.35 ± 0.05	1.1 ± 0.1	3.2 ± 0.2	8.6 ± 0.4
Total	4.4 ± 0.3	8.5 ± 0.6	23.6 ± 0.8	44.4 ± 1.5

cient of 7.6 S, while after method 2 a sedimentation coefficient of 4.6 S is found. This suggests that in the former case the subunit exists in a dimeric form. After standing at room temperature aggregation takes place in the subunit solution obtained with method 2, as indicated by a deviation from linearity in the plot of ln A vs. r^2 in the high concentration range.

After SDS removal by either method, the β-subunit has a sedimentation coefficient of 6.3 S, which is reduced to 4.7 upon addition of 2 mM dithioerythritol, indicating transformation of a dimeric to a monomeric form. Further aggregation of the 6.3 S form takes place after standing at room temperature for several days, a process which can be delayed by dilution.

Partial specific volumes of both subunits are calculated as described under Methods and the results are shown in Table V. The results of the sedimentation analysis studies are presented in Table VI. The molecular weight values for the α- and β-subunits, using only the linear part of the ln A vs. r^2 plot with correction for non-covalently bound phospholipids and taking into account that the β-subunit occurs in dimeric form, are 131 000 and 61 800, respectively. The molecular weights of the protein part are 120 600 and 42 800.

Protein weight ratio of the subunits in the enzyme complex

This is the first of three independent methods used to determine the molar ratio of the subunits in the enzyme complex. The protein mass ratio of the α- and β-subunits can be determined in a direct manner, since the protein peaks of the α- and β-subunits are fully separated by means of the Sephadex G-200 superfine gel filtration procedure (Fig. 1). All fractions containing the α-subunit are collected and mixed. In a known aliquot of this mixture, after addition of norleucine as internal standard, the protein content is determined by means of amino acid analysis. The same procedure is followed for the β-subunit. The protein weight ratio of the subunits (α/β), determined for five enzyme preparations, is 3.04 (S.E. 0.06).

The protein weight ratio can also be calculated from the protein molecular weights of the α- and β-subunits of 120 600 and 42 600, respectively, obtained from sedimentation equilibrium analysis, for any given α/β molar ratio (Table VII). For example, for an α/β molar ratio of 1 : 2 the protein weight ratio is $120\,600/2 \times 42\,800 = 1.41$. Comparison of the calculated protein weight

TABLE V

PARTIAL SPECIFIC VOLUMES OF THE SUBUNITS

	α-subunit		β-subunit	
	Content (% of total, w/w)	Partial specific volume (cm^3/g)	Content (% of total, w/w)	Partial specific volume (cm^3/g)
Protein	90.7	0.7429	67.4	0.7406
Carbohydrate	7.7	0.6345	29.9	0.6314
Phospholipid	1.6	0.9742	2.7	0.9702
Complete subunit		0.7382		0.7141

TABLE VI

SEDIMENTATION COEFFICIENTS AND MOLECULAR WEIGHTS OF THE SUBUNITS

Averages with standard error of the mean are given for the indicated number of determinations. S.E. for $n = 2$ is approximated according to Davies and Pearson [29] as 0.63 X range.

	α-subunit		β-subunit	
	Monomer	Dimer	Monomer	Dimer
Sedimentation coefficients	4.6 ± 0.4 ($n = 2$)	7.6 ± 0.6 ($n = 4$)	4.7 ($n = 1$)	6.3 ± 0.4 ($n = 2$)
Molecular weights derived from sedimentation equilibrium analysis				
Molecular weight (total)	133 000 ± 4500			127 000 ± 1500 *
Molecular weight (protein + carbohydrate)	131 000 ± 4500		61 800 ± 1000 *	
Molecular weight (protein only)	120 600 ± 4600 $n = 7$		42 800 ± 1000 $n = 5$	

* Half the value of the total molecular weight of the dimer less phospholipid weight.

ratios with the experimental value shows that there is agreement for an α/β molar ratio of 1 : 1, while all other ratios give statistically significant differences (Table VII).

Absorbance ratio of the subunits in the enzyme complex

The ratio of the absorbance at 280 nm for the two subunits can be determined after their separation in the following way.

All 280 nm absorbing fractions belonging to the α-peak are collected and mixed and the 280 nm absorbance is determined. The same is done with the fractions belonging to the β-peak. The α/β absorbance ratio, determined for four preparations of each subunit, is 2.02 (S.E. 0.05).

The absorbance ratio can also be calculated from the molar absorption coefficients at 280 nm of the α- and β-subunit for a given molar α/β ratio. By

TABLE VII

PROTEIN WEIGHT RATIO OF α- AND β-SUBUNIT

Weight ratios calculated: calculated from the subunit protein molecular weights shown in Table II for the assumed subunit ratio ($n = 7$ for α-subunit, $n = 5$ for β-subunit). Weight ratio determined: determined as described in text, average with standard error of the mean is given for five determinations in five different preparations.

Subunit ratio assumed	Weight ratio calculated	Weight ratio determined
$\alpha_1\beta_2$	1.41 ± 0.06 *	
$\alpha_1\beta_{1.5}$	1.88 ± 0.08 *	
$\alpha_1\beta_1$	2.82 ± 0.12	3.04 ± 0.06
$\alpha_{1.5}\beta_1$	4.23 ± 0.19 *	
$\alpha_2\beta_1$	5.64 ± 0.25 *	

* Significantly different from the determined weight ratio ($P < 0.001$).

comparing the calculated with the determined absorbance ratio the correct molar α/β ratio can be found. The molar absorption coefficients are determined by means of the Lambert-Beer law from the 280 nm absorbance of a subunit solution, divided by the protein molar concentration. The protein molar concentration is determined by amino acid analysis, taking into account the known protein molecular weight of the subunit.

The molar absorption coefficients at 280 nm for the α- and β-subunits are 143 000 (S.E. 6000, $n = 4$) and 78 000 (S.E. 5000, $n = 4$) $l \cdot mol^{-1} \cdot cm^{-1}$, respectively (Table VIII).

For a molar α/β ratio of 1 : 2 the calculated absorbance ratio is $143\,000/2 \times 78\,000 = 0.92$. Comparison of the calculated absorbance ratios with the experimental value shows that there is agreement for an α/β molar ratio of 1 : 1, while all other ratios give statistically significant differences (Table VIII).

Amino acid composition of subunits and native enzyme

Amino acid analysis of the α- and β-subunits (Table II) shows that the molar contents of four amino acids are markedly different in the two subunits, viz., for alanine, histidine, tyrosine and lysine. From the known protein molecular weights of the subunits and the molar content of one of these amino acids the theoretically expected content of this amino acid in the native enzyme for a given α/β molar ratio can be calculated. Comparison of this value with the experimental value affords a third independent method for determining the α/β molar ratio in the native enzyme.

From the protein molecular weights of the α-subunit (120 600) and the β-subunit (42 800) the number of amino acid residues are calculated to be 1090 and 374, respectively. From the alanine contents of 8.2 mol% in the α-subunit and 4.3 mol% in the β-subunit a content of

$$\frac{(10.90 \times 8.2) + 2(3.74 \times 4.3)}{10.90 + 2 \times 3.74} = 6.6 \text{ mol\%}$$

TABLE VIII

MOLAR ABSORPTION COEFFICIENTS OF THE α- AND β-SUBUNITS AND THEIR ABSORBANCE RATIO AT 280 nm

Molar absorption coefficients at 280 nm (determined): α-subunit, $\epsilon_{280} = 143\,000 \pm 6000\ l \cdot mol^{-1} \cdot cm^{-1}$ ($n = 4$); β-subunit, $\epsilon_{280} = 78\,000 \pm 5000\ l \cdot mol^{-1} \cdot cm^{-1}$ ($n = 4$). Averages with standard error of the mean for n different preparations are given. Determined 280 nm absorbance ratio: determined as described in the text.

Subunit ratio assumed	280 nm absorbance ratio	
	Calculated	Determined
$\alpha_1\beta_2$	0.92 ± 0.07 *	
$\alpha_1\beta_{1.5}$	1.23 ± 0.09 *	
$\alpha_1\beta_1$	1.85 ± 0.14	2.02 ± 0.06 ($n = 8$)
$\alpha_{1.5}\beta_1$	2.77 ± 0.21 *	
$\alpha_2\beta_1$	3.69 ± 0.27 *	

* Significantly different from the determined absorbance ratio ($P < 0.01$).

TABLE IX

COMPARISON OF CALCULATED AND DETERMINED CONTENTS OF FOUR AMINO ACIDS IN THE NATIVE ENZYME

The determined amino acid contents are taken from Table II. The protein molecular weights of the α- and β-subunits (120 600 and 42 800) are taken from Table VI. Δ is the absolute difference between the calculated and the determined content of an amino acid for a particular molar α/β ratio. Values expressed as mol per 100 mol amino acids.

	Alanine		Tyrosine		Histidine		Lysine		
α-subunit	8.2		2.3		1.9		5.6		
β-subunit	4.3		4.8		0.5		9.9		
Enzyme, determined	7.2		2.7		1.6		6.7		
Enzyme, calculated		Δ		Δ		Δ		Δ	Δ_{av}
$\alpha_1\beta_2$	6.6	0.6	3.3	0.6	1.35	0.25	7.35	0.65	0.53 ± 0.08 *
$\alpha_1\beta_{1.5}$	6.85	0.35	3.15	0.45	1.4	0.2	7.05	0.35	0.34 ± 0.05 *
$\alpha_1\beta_1$	7.2	0.0	2.95	0.25	1.55	0.05	6.7	0.0	0.08 ± 0.06
$\alpha_{1.5}\beta_1$	7.45	0.25	2.75	0.05	1.65	0.05	6.4	0.3	0.16 ± 0.065 *
$\alpha_2\beta_1$	7.6	0.4	2.6	0.1	1.7	0.1	6.2	0.5	0.28 ± 0.10 *

* Significantly different from zero ($P < 0.05$).

in the native enzyme at an α/β molar ratio of 1 : 2 is calculated. In Table IX the results of such calculations for all four amino acids at five different α/β molar ratios are presented. The absolute differences between the calculated and determined contents are listed and averaged for each α/β molar ratio. The minimal average difference is obtained for an α/β molar ratio of 1 : 1, and this is the only one which is not significantly different from zero. Hence, the α/β molar ratio of 1 : 1 is again the most probable ratio.

Discussion

Chemical composition of the subunits

Separation of the α- and β-subunits of ($Na^+ + K^+$)-ATPase has been reported for enzyme preparations from dog [11], lamb [27] and rabbit [6] kidney, duck salt gland [3], shark rectal gland [10,28] and electric eel electroplax [4,28]. In all these cases the enzyme has been solubilized in SDS, but different methods have been used for the subunit separation.

Our Sephadex gel filtration method gives complete separation of the two subunits. It also yields a nearly complete separation of protein and phospholipid, more than 95% of the phospholipids being eluted after elution of the α- and β-subunits. There remains, however, a small amount of phospholipid bound to each subunit, even after repeated gel filtration. This has previously been reported for some other membrane proteins [30], but not for ($Na^+ + K^+$)-ATPase. This small amount of bound phospholipids should be taken into account when determining the molecular weights of the subunits by sedimentation equilibrium analysis in the absence of detergent [31].

Since the composition of the phospholipids bound to the subunits does not differ appreciably from that of the intact enzyme, there appears to be no

specific interaction between these phospholipids and the subunits, at least after denaturation of the intact enzyme. This suggests that phospholipid binding occurs through hydrophobic interaction of the fatty acid chains with hydrophobic regions in the protein. Polar interactions with phospholipid head groups would lead to different affinities of the various phospholipids, depending on the nature of the phospholipid head group.

There is great similarity in the amino acid composition of each subunit for $(Na^+ + K^+)$-ATPase isolated by us and by others from various species and tissues [11,27,28,32]. Consequently, this is also true for the percentage of hydrophobic amino acids in each of the two subunits. The average percentages, 44% for the α-subunit and 41% for the β-subunit, are rather low compared to those found for other membrane-bound enzymes (49—60%), even keeping in mind that cysteine has been counted as a non-polar amino acid in the latter cases [33]. $(Ca^{2+} + Mg^{2+})$-ATPase from sarcoplasmic reticulum [33] is the only plasma membrane protein with about the same percentage of non-polar amino acids (47%, including cysteine) as present in the α-subunit of $(Na^+ + K^+)$-ATPase, which underlines the similarity between these two transport ATPases. The larger variability in the percentage of non-polar amino acids in the β-subunit from different sources suggests that the α-subunit has been more conservative in evolution, which is understandable in view of the catalytic function of the α-subunit.

The glycoprotein nature of the β-subunit has been widely recognized, but this is not the case for the α-subunit. Only recently, has it been reported that the α-subunit of $(Na^+ + K^+)$-ATPase of electric organ [34] and probably of brine shrimp nauplii [2] is also a glycoprotein. Sialic acid may also be present in small amounts in the α-subunit of dog kidney $(Na^+ + K^+)$-ATPase, as can be concluded from the results of Giotta [7], who labeled the sialic acid residues present in the subunits with tritium. We now clearly demonstrate that the α-subunit of rabbit kidney is a glycoprotein, despite the fact that it does not stain with the periodic acid-Schiff staining method after SDS-polyacrylamide gel electrophoresis. By means of SDS-polyacrylamide gel electrophoresis we have repeatedly and carefully checked that the α-subunit preparation is not contaminated by the β-subunit. Another interesting point is that both subunits appear to contain glucose, which is quite unusual for glycoproteins [35,36].

Subunit molecular weights

Many values for the subunit molecular weights of $(Na^+ + K^+)$-ATPase have been reported. The most widely used method has been SDS-polyacrylamide gel electrophoresis, which has yielded values in the range of 84 000—110 000 for the α-subunit and of 38 000—60 000 for the β-subunit [2], also in our hands. A gel filtration method, with proteins of known molecular weight as standards, has yielded values between 127 000 and 139 000 for the α-subunit [11] and between 35 000 and 45 000 for the β-subunit [11,37]. Neither of these methods is very reliable for determination of absolute molecular weight values [38,39].

A more reliable and accurate method is sedimentation equilibrium analysis. Kyte [11] reports a value of 147 500 obtained with this technique for the

α-subunit of the dog kidney enzyme, while Hastings and Reynolds [10] obtain values of 106 400 for the α-subunit and 51 700 for the β-subunit of the shark rectal gland enzyme. Our values for the rabbit kidney ($Na^+ + K^+$)-ATPase subunits are 131 000 (S.E. 4500) for the α-subunit and 61 800 (S.E. 1000) for the β-subunit.

A complication arises from a tendency of the subunits to aggregate, resulting in high molecular weight values with the sedimentation equilibrium technique. Kyte [11] finds under his conditions dimerization of the α-subunit (sedimentation coefficient 8.7—10.4 S) and the value of 147 500 mentioned above is half the value actually determined by him. In our case we have used the value determined for the α-subunit preparations from which detergent is removed by method 2, yielding a monomeric form (4.6 S, as compared to a 7.6 S dimeric form by method 1). We find that the β-subunit, after detergent removal by method 2, is present in a dimeric form (6.3 S, reduced to 4.7 S in the presence of dithioerythritol), hence we use half the molecular weight value actually determined.

The considerable differences between the subunit molecular weights obtained by sedimentation equilibrium analysis could be due to species differences, Kyte using dog kidney, Hastings and Reynolds shark rectal gland, and ourselves rabbit kidney as source. However, the differences may also be due to the different conditions: Kyte [11] and we work in the absence of detergent, while Hastings and Reynolds [10] make measurements on a detergent-solubilized preparation. This may explain why our value is closer to that of Kyte than to that of Hastings and Reynolds. Our protein molecular weight for the β-subunit (42 800) agrees within experimental error with the value of 43 800 (protein only) of Lane et al. [27] for lamb kidney, calculated on the assumption that there is one histidine per mol subunit.

Subunit ratio

The subunit protein weight ratio has been reported for ($Na^+ + K^+$)-ATPase from various sources [2,10]. Most of these data were obtained from quantitative scanning of the gels preparaed by SDS-polyacrylamide gel electrophoresis and stained with Coomassie brilliant blue. This method is unreliable, because the staining intensity is not the same for equal amounts of different proteins [10,40,41]. Hastings and Reynolds [10] have tried to avoid this problem by quantitative determination of the difference in staining of the α- and β-subunits. However, this procedure introduces a new problem, because the method of Lowry et al, [14] of protein determination, used for this purpose, does not give absolute values [42]. It may give a different color intensity for the same concentrations of different proteins [43], especially in the case of glycoproteins [44]. Therefore, we have used an absolute protein determination method, quantitative amino acid analysis in the presence of an internal standard.

The molar subunit ratio has been determined by means of three independent methods: protein weight ratio of the subunits, molar absorbance ratio of the subunits and calculation from the contents of four amino acids which are greatly different in the two subunits (alanine, histidine, tyrosine, lysine). By all three methods an α/β molar ratio 1 : 1 is obtained.

The same conclusion has recently been reached by Craig and Kyte [45]

who find that upon cross-linking of a detergent-treated enzyme preparation which cupric phenanthroline, an $\alpha\beta$ dimer is formed without changing the ratio of the remaining unreacted α- and β-subunits.

Subunit composition of the native enzyme

The 1 : 1 α/β molar ratio implies that the native enzyme contains an equal number of each subunit. For an $\alpha\beta$ dimer we arrive at a value of 163 400 (protein only) or 196 500 (including carbohydrates and lipids) by adding up our molecular weight values of the subunits. The latter value agrees with the particle weight of approx. 200 000, obtained by Winter and Moss [46] by sedimentation equilibrium analysis of a digitonin-solubilized dog kidney enzyme preparation, which has no overall ATPase activity but only partial activities and ouabain binding capacity and which according to these authors would represent an $\alpha\beta$ dimer.

Considerably higher molecular weight values are reported for the protein part of the smallest completely active ($Na^+ + K^+$)-ATPase particle. Sedimentation equilibrium analysis of a detergent-solubilized preparation of shark rectal gland enzyme has yielded values of 276 000 [9] and 380 000 [10], while radiation inactivation of the human erythrocyte enzyme gives a value of 330 000 ± 30 000 [47]. In view of our α/β molar ratio of 1 : 1 and our subunit protein molecular weights, this indicates that the native enzyme consists of an $\alpha_2\beta_2$ tetramer with a calculated protein molecular weight of 326 800.

The molecular weight of the whole enzyme, including carbohydrate and about 60 mol of phospholipids, would then be about 440 000. This is in good agreement with the finding that in gel filtration the ($Na^+ + K^+$)-ATPase complex appears in about the same position as apoferritin (molecular weight 466 000) [9]. The $\alpha_2\beta_2$ composition is also in agreement with results of electron microscopical studies of Haase and Koepsell [48] on freeze-fractured rat kidney enzyme preparations, which appear to show the presence of four subunits per enzyme particle.

Acknowledgements

The authors wish to thank Mr. M. van de Gaag for carrying out the amino acid analyses, Miss C. Duyf, Drs. J. Langeveld and G. Hoelen for assistance with the carbohydrate analyses. They are indebted to Professor Dr. H.J. Hoenders for making the analytical ultracentrifuge available and for valuable discussions concerning the molecular weight determinations of the subunits.

References

1 Jørgensen, P.L. (1975) Q. Rev. Biophys. 7, 239—274
2 Peterson, G.L., Ewing, R.D., Hootman, S.R. and Conte, F.P. (1978) J. Biol. Chem. 253, 4762—4770
3 Hopkins, B.E., Wagner, H. and Smith, T.W. (1976) J. Biol. Chem. 251, 4365—4371
4 Dixon, J.F. and Hokin, L.E. (1974) Arch. Biochem. Biophys. 163, 749—758
5 Hokin, L.E., Dahl, J.L., Deupree, J.P., Dixon, J.F., Hackney, J.F. and Perdue, J.F. (1973) J. Biol. Chem. 248, 2593—2605
6 Jørgensen, P.L. (1974) Biochim. Biophys. Acta 356, 53—67
7 Giotta, G.J. (1976) J. Biol. Chem. 251, 1247—1252

8 Lane, L.K., Copenhaver, J.H., Lindenmayer, G.E. and Schwartz, A. (1973) J. Biol. Chem. 248, 7197—7200
9 Esmann, M., Skou, J.C. and Christiansen, C. (1979) Biochim. Biophys. Acta 567, 410—420
10 Hastings, D.F. and Reynolds, J.A. (1979) Biochemistry 18, 817—821
11 Kyte, J. (1972) J. Biol. Chem. 247, 7642—7649
12 Hansen, O., Jensen, J., Nørby, J.G. and Ottolenghi, P. (1979) Nature 280, 410—412
13 Jørgensen, P.L. (1974) Biochim. Biophys. Acta 356, 36—52
14 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
15 Edelhoch, H. (1967) Biochemistry 6, 1948—1954
16 Langeveld, J.P.M., Veerkamp, J.H., Monnens, L.A.A. and van Haelst, V.J.C. (1978) Biochim. Biophys. Acta 514, 225—238
17 Hammond, K.S. and Papermaster, D.S. (1976) Anal. Biochem. 74, 292—296
18 Folch, J., Lees, M. and Sloane-Stanley, G.H. (1957) J. Biol. Chem. 266, 497—509
19 Broekhuyse, R.M. (1978) Biochim. Biophys. Acta 152, 307—315
20 Weber, K. and Kuter, D.J. (1971) J. Biol. Chem. 246, 4504—4509
21 Cohn, E.J. and Edsall, J.T. (1943) Proteins, Amino Acids and Peptides, pp. 370—375, Reinhold Publishing Corp., New York
22 Gibbons, R.A. (1966) in Glycoproteins, Their Composition, Structure and Function (Gottschalk, A., ed.), pp. 29—95, Elsevier, Amsterdam
23 Tanford, C., Nozaki, Y., Reynolds, J.A. and Makino, S. (1974) Biochemistry 13, 2369—2376
24 Laemmli, U.K. (1970) Nature 227, 680—685
25 Segrest, J.P. and Jackson, R.L. (1972) Methods Enzymol. 28, 54—63
26 De Pont, J.J.H.H.M., van Prooyen-van Eeden, A. and Bonting, S.L. (1978) Biochim. Biophys. Acta 508, 464—477
27 Lane, L.K., Potter, J.D. and Collins, J.H. (1979) Prep. Biochem. 9, 157—170
28 Perrone, J.R., Hackney, J.F., Dixon, J.F. and Hokin, L.E. (1975) J. Biol. Chem. 250, 4178—4184
29 Davies, O.L. and Pearson, E.S. (1934) J. Roy. Stat. Soc., Suppl. 1, 76—93
30 Helenius, A. and Simons, K. (1975) Biochim. Biophys. Acta 415, 29—79
31 Steele, J.H.C., Tanford, C. and Reynolds, J.A. (1978) Methods Enzymol. 48, 12
32 Hokin, L.E. (1974) Ann. N.Y. Acad. Sci. 242, 12—24
33 Guidotti, G. (1972) Annu. Rev. Biochem. 41, 731—748
34 Churchill, L., Peterson, G.L. and Hokin, L.E. (1979) Biochem. Biophys. Res. Commun. 90, 488—490
35 Spiro, R.G. (1970) Annu. Rev. Biochem. 39, 599—638
36 Marshall, R.D. (1972) Annu. Rev. Biochem. 41, 673—702
37 Lane, L.K., Gupte, S.S., Collins, J.H., Wallick, E.T., Johnson, J.D. and Schwartz, A. (1979) in (Na^+ + K^+)-ATPase, Structure and Kinetics (Skou, J.C. and Nørby, J.G., eds.), pp. 33—44, Academic Press, New York
38 Maddy, A.H. (1976) J. Theor. Biol. 62, 315—326
39 Nielsen, T.B. and Reynolds, J.A. (1978) Methods Enzymol. 48, 3—11
40 Van Kley, H. and Hale, S.M. (1977) Anal. Biochem. 81, 485—487
41 Pierce, J. and Suelter, C.H. (1977) Anal. Biochem. 81, 478—480
42 Chiappelli, F., Vasil, A. and Haggerty, D.F. (1979) Anal. Biochem. 94, 160—165
43 Westley, J. and Lambeth, J. (1960) Biochim. Biophys. Acta 40, 364—366
44 Wu, A.M., Wu, J.C. and Herp, A. (1978) Biochem. J. 175, 47—51
45 Craig, W.S. and Kyte, J. (1980) J. Biol. Chem. 255, 6262—6269
46 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, New York
47 Ellory, J.C., Green, J.R., Jarvis, S.M. and Young, J.D. (1979) J. Physiol. 295, 10P—11P
48 Haase, W. and Koepsell, H. (1979) Pflügers Arch. 381, 127—135