Evidence for a diprotomeric structure of Na,K-ATPase

Accurate determination of protein concentration and quantitative end-group analysis

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Received 20 November 1985

Three methods were used to assess protein concentration in membrane-bound Na,K-ATPase preparations: standard Lowry assay, Kjeldahl nitrogen determination and amino acid analysis. While the first two methods showed excellent agreement, the third one always gave a lower value which varied drastically depending on the condition of sample treatment before amino acid analysis. This result reinforces the Lowry method in assessing the true concentration of Na,K-ATPase protein and suggests 250 kDa to be a true estimate of the molecular mass of the smallest ligand-binding unit of the enzyme. The cyanate method reveals two NH_2-terminal residues of the β-subunit (NH_2-Ala) and one such residue of the α-subunit (NH_2-Gly) per ligand-binding unit. From the data on equimolarity of the α- and β-subunits in Na,K-ATPase this suggests that the enzyme molecule is composed of two $\alpha\beta$-protomers, one possessing a modified (presumably an N-blocked) α-subunit.

$(Na^+ + K^+)$-ATPase *Enzyme structure* *Subunit modification* *Protein assay* *End-group analysis*

1. INTRODUCTION

One of the main questions currently debated in Na,K-ATPase studies is whether the enzyme molecule consists of 1 or 2 $\alpha\beta$-protomers, the answer being of great importance for concepts on the enzyme mechanism. Earlier studies on the maximal capacity of the purest and most active preparations to bind specific ligands revealed 3.5–4.0 nmol ligand-binding units per mg Lowry protein [1,2] suggesting the molecular mass of the unit to be $\geq$250 kDa which is roughly twice as great as that of the $\alpha\beta$-protomer [3,4]. These data were, however, disregarded later in reports where the Na,K-ATPase protein concentration was assessed by amino acid analysis and an essentially lower value than that found in the Lowry assay was obtained [5–7]. This observation led to the conclusion that each single $\alpha\beta$-protomer serves as a ligand-binding (and probably enzyme) unit [8]. However, the large inconsistency of the authors' estimates of the error made by the Lowry assay, 12% [9] and 35% [6] to as much as 89% [7] was very puzzling.

To clear up this point, 3 methods (standard Lowry assay, Kjeldahl nitrogen determination and amino acid analysis) are used here to assess the protein concentration in a pure membrane-bound Na,K-ATPase preparation, together with the quantitative determination of NH_2-terminal residues of the enzyme subunits in the preparation. The results support the view that the functional Na,K-ATPase unit consists of 2 $\alpha\beta$-protomers.

2. EXPERIMENTAL

The membrane-bound Na,K-ATPase from pig kidney outer medulla prepared according to Jørgensen [10] by the method in [11] had a specific activity of 25–30 μmol P_i/mg protein per min, was

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/86/$3.50

90–95% pure and had an approximate 3:1 α/β subunit mass ratio judging from the analysis of gradient polyacrylamide gel stained after electrophoresis in the presence of SDS and urea. Before the analytical experiments, the preparation was transferred into deionized water and freed of soluble contaminants.

Lipids were extracted by method II of Slayback et al. [12] into an ethyl acetate/acetone mixture, and both the protein pellet and the upper organic phase were collected and washed.

Organic phosphate was determined as P_i [11] after sample ashing in perchloric acid [13].

Lowry assays were performed according to the original procedure [14] with bovine serum albumin as a standard, whose concentration was assessed using the published $A^{1\%}_{1cm}$ value (6.68 at 279 nm [15]). Although our own determination, based on the nitrogen content in the albumin (16.4%, deduced from [16]) and total nitrogen assay, gave a lower value (6.42 ± 0.03, corrected for the light-scattering effect [17]) the 6.68 value was used for a direct comparison of our results with those of other authors. Where indicated a modified version of the Lowry method [18] was used.

Total nitrogen was assessed by the Bohley procedure [19], a microversion of the Kjeldahl method, with an accuracy within 1–2% in experiments with substances of known nitrogen content. The Na,K-ATPase protein nitrogen was evaluated by subtraction of nitrogen pertaining to lipids, polysaccharides and ammonia admixtures. The first was assessed by multiplying the organic phosphate content in the enzyme preparation by the value of the molar nitrogen/phosphate ratio in the lipid extract; the ratio was found to be constant (1.20 ± 0.02) independently of the preparation. The nitrogen of protein-bound polysaccharides was estimated from the data on the amino sugar content in pig kidney Na,K-ATPase subunits [3,20]; this amounted to less than 1.5% of the protein nitrogen. Inorganic ammonia was assessed as total nitrogen omitting sample ashing and did not exceed 1% of the total nitrogen of the sample. The nitrogen content in the protein moiety (16.6%) was calculated from data on the amino acid composition of the membrane-bound Na,K-ATPase (see below) and from that on the amide nitrogen content (5.60 ± 0.05% of protein nitrogen, or 37% of the sum of Asx and Glx). The amide nitrogen was determined as the ammonia liberated upon sample treatment at 100°C with 2 N HCl [21].

Amino acid analyses of membrane-bound preparations were done in triplicate on a D-500 Durrum analyzer following hydrolysis of the sample for 24, 48 and 72 h at 110°C in the presence of 6 N HCl with or without 50% (v/v) formic acid (acid hydrolysis) or for 0.5, 1, 2 and 4 h at 155°C in the presence of 5 N KOH (alkaline hydrolysis [22]), with L-norleucine being added as an internal standard. Before acid hydrolysis the samples were either dried in hydrolysis ampoules with or without preliminary precipitation with 5% trichloroacetic acid, or mixed with acid without drying following ultrasonic treatment. The protein concentration was calculated from the sum of amino acids found upon acid hydrolysis, corrected for Trp content (1.35 ± 0.07 mol%, alkaline hydrolysis data), half-cystine content (2.17 mol% [20]) and Ser contained in phospholipids (~20% of total Ser).

NH_2-terminal residues were assayed by the cyanate method of Stark [23,24], 5 nmol of polypeptide being used per analysis instead of 0.5 μmol, with or without preliminary lipid extraction. Carbamylation was carried out in the presence of either 6 M guanidinium chloride (delipidated preparation and albumin) or 5% SDS, 8 M urea and 50 mM dithiothreitol (membrane-bound preparation; in this case sodium cyanate replaced potassium cyanate). Blanks were prepared omitting cyanate and urea. After carbamylation samples were transferred into deionized water, sonicated, assayed for nitrogen content, and the weighed aliquots were taken for cyclization. Carbamylated L-[U-^{14}C]phenylalanine was introduced at this stage (~10^6 cpm per aliquot) to control occasional losses during the steps preceding addition of the norleucine standard, the latter being introduced before the acid hydrolysis of hydantoins into the parent amino acids.

3. RESULTS

Table 1 presents the results of some experiments on determination of protein concentration in different Na,K-ATPase preparations by the Lowry and nitrogen assays. In a number of experiments with 10 different preparations these 2 methods appeared to agree within 3%, the standard error in

Table 1

Determination of protein concentration by nitrogen assay and by the method of Lowry et al. in Na,K-ATPase preparations

Preparation no.	Organic phosphate content (μmol · mg protein^{-1})	Protein concentration by nitrogen assay (mg · ml^{-1})	Protein concentration by Lowry assay (mg · ml^{-1})	
			Standard procedure [14]	Modified procedure [18]
Membrane-bound preparations				
612	0.89	2.53 ± 0.03	2.57 ± 0.02	–
622	0.77	0.86 ± 0.01	0.87 ± 0.01	–
731	1.06	2.08 ± 0.01	2.12 ± 0.01	2.08 ± 0.05
Delipidated preparations				
611/622	<0.028	1.17 ± 0.02	0.96 ± 0.01	1.18 ± 0.01
631/632	<0.039	1.03 ± 0.03	0.71 ± 0.02	0.98 ± 0.02

Mean values of 4–8 independent determinations (± SE) are presented

each case being ± 2%. It can be seen that both methods give the same result irrespective of the phospholipid content in the enzyme preparation. The original Lowry procedure fails to recover all the protein in the delipidated preparations consisting of hardly dispersed protein aggregates; the difficulty can be overcome in a modified version of the method [18] where prolonged treatment of the sample with SDS in a strongly alkaline medium precedes the assay.

Amino acid analysis gives a lower estimate of the protein concentration than the Lowry and nitrogen assays do. The maximal yield of amino acids depends drastically on the particular preparation procedure of the sample for hydrolysis and on the hydrolysis conditions, the highest yield being achieved when the most favourable conditions for protein solubilization are ensured (table 2). The underestimation of the Na,K-ATPase protein concentration by amino acid analysis can be explained, at least partly, by poor solubility of the sample rendering a part of the protein material unsusceptible to hydrolysis. This observation could give a rationale for the severe disagreement in the authors' estimates of the difference between amino acid analysis and Lowry data (see section 1).

To determine the concentration of Na,K-ATPase polypeptides in the enzyme preparation, their NH_2-terminal residues (NH_2-Gly and NH_2-Ala for the α- and the β-subunit, respectively

Table 2

Protein mass recovered at amino acid analysis (percent of that revealed by nitrogen assay)

Sample treatment	Yield (%)
(a) Standard acid hydrolysis of the sample	
– precipitated with trichloroacetic acid and dried before addition of 6 N HCl	62 ± 2
– dialyzed against water and dried before addition of 6 N HCl	79 ± 2
– dialyzed against water, sonicated and mixed with concentrated HCl to 6 N concentration, without drying	87 ± 1
(b) Acid hydrolysis in the presence of 50% formic acid	89 ± 1
By maximal yield of each amino acid under all examined conditions, including alkaline hydrolysis	91 ± 1

[20]) were assayed. The cyanate method of Stark [23,24] was chosen for this purpose as it ensures a full recovery of the residues under examination and allows one to carry out an α-amino group modification in the presence of urea, a strong denaturing agent compatible with SDS, without the interference of cyanate produced on urea decomposition. Preliminary experiments on quantitation of NH_2-Asp in bovine serum albumin (M_r 66296 [16]) revealed 100 ± 5% of the residue (not shown). Table 3 summarizes the results of 5 independent experiments with 3 different Na,K-ATPase preparations where, on the average, 3.8 ± 0.5 nmol NH_2-Gly and 8.2 ± 1.4 nmol NH_2-Ala were found in 1 mg of protein assessed by nitrogen content. Upon correction for preparation purity this corresponds to 250 ± 26 kDa protein mass per NH_2-Gly and 116 ± 16 kDa per NH_2-Ala. From the results presented in table 3 it is clear that the unequal amount of these residues is due neither to incomplete denaturation of the samples, nor to partial proteolysis of the α-subunit nor to experimental error. In a special experiment on analysis of an acidic chloroform/methanol extract less than 1 nmol of any NH_2-terminal residue was found per mg of protein taken for the extraction (not shown) which apparently precludes a putative γ-subunit [25] as being responsible for the increased amount of NH_2-Ala.

Table 3

Quantitative NH_2-terminal analysis of Na,K-ATPase

Amino acids found	Yield of amino acids (nmol·mg protein^{-1}) minus blank values				
	Experiment no.				
	1	2	3	4	5
Asx	2.3	2.4	−0.1	0.5	2.0
Thr	0.5	0.4	0.4	0.3	0.4
Ser	0.7	0.1	0.1	0.5	1.5
Glx	2.5	1.8	0.6	1.9	1.5
Pro	−0.1	0.3	–	0.6	–
Gly	3.5	3.1	3.8	4.2	4.2
Ala	7.1	7.9	7.1	8.4	10.4
Val	0.6	1.1	0.4	0.9	1.1
Ile	0.3	0.6	0.2	0.2	0.2
Leu	1.1	1.4	0.6	0.7	0.7
Tyr	0.2	0.3	0.1	–	–
Phe	0.5	0.6	0.1	0.3	0.1
Protein mass[a] (kDa) per					
NH_2-Gly	257	290	250	226	226
NH_2-Ala	127	114	134	113	91

[a] The protein purity was ~90% (expts 1 and 2) and ~95% (expts 3–5). It was estimated just before use and taken into account in the calculations

The preparation used in expts 1 and 2 was stored in 25 mM Tris-HCl (pH 7.5)/1 mM EDTA solution at 0°C for a year. The preparation used in expts 3 and 4 was freshly prepared. Both the preparations were delipidated before carbamylation in the presence of 6 M guanidinium chloride. The preparation used in expt 5 was freshly isolated in the presence of 0.1 mM phenylmethylsulfonyl fluoride; this protease inhibitor (0.5 mM) was also present at carbamylation of the membrane-bound enzyme carried out in the presence of 5% SDS, 8 M urea and 50 mM dithiothreitol

4. CONCLUSION

It follows from the above that being standardized with bovine serum albumin, the Lowry method reproducibly gives a true estimate of the protein concentration in Na,K-ATPase preparations. This result reinforces the earlier data on the content of ligand-binding units in the enzyme, ~4 nmol units per mg of Lowry protein (see section 1), and leads to the conclusion that the mass of the binding unit (the enzyme molecule) is ~250 kDa.

It follows also that there are 2 free α-amino groups of the β-subunit and one such group of the α-subunit per Na,K-ATPase molecule and this is sufficient to conclude that 2 β-subunits are involved. Bearing in mind the data on the equimolarity of the α- and β-polypeptides in the enzyme molecule [4,26–28] one can conclude further that 2 α-subunits are present as well, half of them modified in such a way that they cannot be detected at NH_2-terminal analysis; presumably they bear a blocked α-amino group.

The general conclusion from this work is that Na,K-ATPase is an $\alpha_2\beta_2$-diprotomer. Each diprotomer probably contains one modified and one unmodified α-subunit and this might be of significance for folding and/or functioning of the enzyme.

ACKNOWLEDGEMENTS

I am much indebted to Professor A.S. Spirin for constant interest in this work and to Mr A.I. Vassin for amino acid analyses. Professor R.L. Post's comments on the results as well as his suggestion to use the term 'diprotomer' instead of 'dimer' are gratefully acknowledged. The continuous help and encouragement of my wife Helena Chetverina were of invaluable significance throughout this work.

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