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Analysis of free sulfhydryl groups and disulfide bonds in Na^+,K^+-ATPase

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The content of free SH groups and disulfide bonds in the purified pig kidney Na^+,K^+-ATPase was determined by ammetric titration with silver nitrate. In the native enzyme, most of the free SH groups are masked due to their location in the polypeptide chain regions poorly accessible to SH reagents. Denaturation with 5% SDS and 8 M urea makes these regions accessible thus revealing 22 free SH groups/mol of the protein. After complete blocking of free SH groups with silver ions, 8 SH groups/mol of the protein are being released upon sulfitolysis which indicates the presence of four disulfide bonds in the enzyme. At least one disulfide bridge is located in the α-subunit whereas the β-subunit contains three disulfide bonds.

Na^+,K^+-ATPase, membrane-bound; SH-group, free; Disulfide bond; Ammetric titration

1. INTRODUCTION

Na^+,K^+-ATPase is an obligatory component of plasma membranes of eukaryotic cells and performs the translocation of sodium and potassium ions against their electrochemical potentials. The enzyme consists of equimolar amounts of the α-subunit (110 kDa) and β-subunit (35 kDa, protein moiety, and 10 kDa, carbohydrate portion) [1,2]. The enzyme was shown to contain 11–14 cysteine residues, 8 or 9 of them belong to the α-subunit and 1 or 2 to the β-subunit. Modification of cysteine residues (2–6) with alkylating reagents results in inhibition of the enzymatic activity [3–7]. According to previous data [8] stability of the β-subunit in the native membrane to proteases is mainly determined by existence of disulfide bonds. One disulfide bond is located between cysteine residues in positions 158 and 174, whose cleavage inactivates the enzyme [9]. According to [3,10–13] several (2–7) disulfide bonds can exist, but it was not proved convincingly. Analysis of the primary structure revealed 30 cysteine residues, 23 of them belonging to the α-subunit and 7 to the β-subunit [1,2].

The analysis of accessible free SH groups (readily accessible and masked) and determination of the number of disulfide bonds in Na^+,K^+-ATPase is the aim of the present study.

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2. MATERIALS AND METHODS

Na^+,K^+-ATPase from pig kidney outer medulla was isolated according to the modified Iorgensen procedure [14]. The enzyme specific activity was 1500 mkmol P_i/h·mg of the protein at 37°C.

Sulfhydryl groups were detected by ammetric titration [15] with 0.5×10^{-3} M silver nitrate in 20 ml of 0.1 M ammonium-nitrate buffer (pH 7.6). To identify free SH groups the enzyme (2–4 mg) was preincubated for 5 or 30 min at 37°C in 0.1 M ammonium-bicarbonate buffer, pH 7.5, containing 2 mM EDTA, with or without denaturation reagent (8 M urea, or 5% SDS, or 6 M guanidine chloride). Disulfide bonds in Na^+,K^+-ATPase were determined after complete mercaptidation of all free SH groups in saturated solution of sodium sulfite added to the sample, that followed by detection of released SH groups per mol of the protein.

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Incubation of the enzyme with 10 mM *N*-ethylmaleimide (NEM) was carried out for 16 h in the presence of 20% glycerol, 2 mM EDTA and 20 mM imidazole-HCl buffer (pH 7.5, at 4°C). The protein concentration in the incubation medium was between 0.2 and 0.4 mg/ml.

The α-subunit in the native membrane-bound enzyme was selectively and exhaustively digested with 1% trypsin in 0.1 M NH_4HCO_3 (pH 7.5) and 2 mM EDTA at 37°C for 10 min [8].

After high speed centrifugation of the hydrolysate, the membrane portion was treated with 5% trypsin for 6 h in buffer as in the above experiment.

3. RESULTS AND DISCUSSION

Titration of free SH groups in native membrane-bound Na^+,K^+-ATPase from pig kidney (see table 1) revealed 1–4 SH groups per mol of protein, i.e. 2.5±0.75 on average. This small number of detectable SH groups proves that the majority of thiol groups are in the poorly accessible sites of the polypeptide chains. A short (5 min) preincubation of the enzyme with different denaturation reagents (5% SDS; 8 M urea; 6 M guanidine chloride or 8 M urea plus 1% SDS) induced a structural change in the polypeptide chains thus increasing the accessibility of free SH groups. Under these conditions, an average 4.9 ± 0.24 SH groups/mol of the protein were identified. Prolonged incubation (30 min) of the protein under the same conditions favoured the identification of an even larger number of SH groups, on average 12.6 ± 0.5 SH groups.

Information obtained by mercaptidation of free readily accessible SH groups coincides well with that on NEM alkylation [3,5,10,13]. However, when adding the sodium sulfite (0.5 ml of the saturated solution, 4 h incubation) to the sample, besides 8 M urea and 1% SDS, 28–29 SH groups/mol of protein were detected, half of which were titrated in the absence of the reducing agent. This number of identified SH groups corresponds to that obtained from protein structural analysis. Thus Na^+,K^+-ATPase has 15 free SH groups and 14 groups were revealed upon sulfitolysis. So the enzyme is expected to contain either seven disulfide covalent bonds or a combination of disulfide bonds and masked free SH groups. To determine the total number of free SH groups, along with the masked ones, we elaborated the method for their detection and complete blocking.

Table 2 presents the analysis results of all free SH groups identified in Na^+,K^+-ATPase preparations from various isolation experiments. The content of easily reacting SH groups (5–15, i.e. 9.46 ± 0.46 on an average) appeared to be the most changeable.

Table 1

Free SH groups of Na^+,K^+-ATPase from different isolation experiments detected by ammetric titration

Addition to buffer[a]	Time pre-incubation	SH groups mol/mol of protein	
		Mean values[b]	Fluctuation
–	5	2.5 ± 0.75	1–4
8 M urea	5	5.5 ± 0.25	5–6
	30	13.8 ± 0.72	12–14
5% SDS	5	4.6 ± 0.4	4–5
	30	13.0 ± 0.97	11–15
6 M guanidine chloride	5	4.5 ± 0.4	4–5
	30	12.5 ± 0.72	11–14
8 M urea, 1% SDS	5	6.1 ± 1.2	5–7
	30	13.0 ± 0.8	11–15

[a] Buffer for preincubation of the protein: 0.1 M NH_4HCO_3 (pH 7.5), containing 2 mM EDTA

[b] Values are mean ± SD of five separate determinations

The number of masked SH groups varied from 9 to 15, i.e. 11.95 ± 0.56. The ratio of free easily reacting SH groups to masked groups was 1.1 : 1.45 (1.22 ± 0.07) in 50% of all the experiments, whereas 0.33 : 0.66 (0.49 ± 0.06) in the others. Apparently various conformations of Na^+,K^+-ATPase can be estimated from this ratio. It is noteworthy that the number of all free SH groups in all the enzyme preparations appeared to be very stable and varied from 20.0 to 22.5 SH groups per mol of protein, i.e. 21.44 ± 0.22 on average.

The complete mercaptidation of all free SH groups enabled the analysis of disulfide bridges by their reduction with sodium sulfite. The number of SH groups identified by sulfitolysis in various enzyme preparations after mercaptidation varied from 8.0 to 11.0 and was 8.6 ± 0.29 SH groups per mol of protein on average, 4 disulfide bonds being detected in 75% of experiments and 5 disulfide bonds in 25% of tests. The overall quantity of revealed cysteine residues in the enzyme varied from 28.7 to 31.0 (30.0 ± 0.38). The true number of disulfide bonds in mercaptidation preparations upon sulfitolysis (table 2) coincided completely

Table 2

SH groups of Na^+,K^+-ATPase from different isolation experiments detected by ammetric titration

Characteristics SH groups[a]	SH groups mol/mol of the protein	
	Mean values[b]	Fluctuation
Readily accessible	9.46 ± 0.76	5.0–13.0
Masked	11.95 ± 0.56	9.0–15.0
Total free	21.44 ± 0.22	20.0–22.0
Released upon sulfitolysis	8.68 ± 0.29	7.9–11.0
Total values	30.0 ± 0.33	29.8–32.3

[a] Buffer for preincubation of the protein: 0.1 M NH_4HCO_3 (pH 7.5), containing 2 mM EDTA, 8 M urea, 5% SDS
[b] Values are mean ± SD of twelve separate determinations

with that calculated as the difference between the number of cysteine residues in the protein and the number of free SH groups per mol.

Upon enzyme incubation with NEM only 13–14 SH groups were alkylated and 9.3 masked SH groups remained unblocked. Additional NEM treatment of the hydrolysate obtained from a 10 min trypsinolysis (T_{10}) led to alkylation of all the masked SH groups thus revealing the real number of disulfide groups in the native protein.

The determination of 4 disulfide bonds in the enzyme posed the question of their distribution between the Na^+,K^+-ATPase subunits. The characteristic feature of the enzyme's structural organization is the high sensitivity of the catalytic α-subunit to trypsinolysis. It was shown earlier [8] that limited proteolysis of the native membrane-bound enzyme affected only the hydrophilic region of the α-subunit, the β-subunit being intact. Thus after high-speed centrifugation, the hydrolysate was separated into two fractions: the supernatant (sT_{10}), containing hydrophilic fragments of the α-subunit, and the membrane sample (mT_{10}): a combination of transmembrane segments of the α-subunit and the intact glycoprotein. This model served as the basis to clarify the distribution of disulfide bridges and free SH groups in the native Na^+,K^+-ATPase. Table 3 presents the qualitative distribution of free SH groups and disulfide bonds in the hydrolysate fractions. Three disulfide bridges were located in the membrane fraction (mT_{10}), containing the β-subunit, one or two – in the exposed regions of the α-subunit (sT_{10}). Following the exhaustive trypsinolysis of membrane fraction mT_{10}, all three disulfide bridges were found in the supernatant of hydrophilic fragments of the β-subunit (sT_{360}). Thus the six cysteine residues located in the C-terminal region of the β-subunit participate in the formation of disulfide bridges. Analysis of the tryptic hydrolysate showed that the total number of SH groups and disulfide bonds in the subunits is in agreement with the results obtained for the enzyme preparations before trypsinolysis and enabled the location of the disulfide bridges in the subunits. It is now clear that three disulfide bonds reside in the β-subunit and that one bond is found in the α-subunit (25% of experiments showed two disulfide bonds in the α-subunit). While this manuscript was prepared for publication, a paper [16] appeared which confirms the presence of three disulfide bridges in the β-subunit.

The localisation of disulfide bonds in the primary structure of subunits will be reported in our next paper.

Table 3

Distribution of free SH groups and disulfide bonds in preparations of tryptic hydrolysates of native membrane-bound Na^+,K^+-ATPase

Sample[a]	SH groups mol/mol of the protein		
	Free	Released upon sulfitolysis	Total
[b] T_{10}	22 ± 0.1	8 ± 0.1	30 ± 0.1
sT_{10}	12.6 ± 0.8	3.4 ± 0.9	16.0 ± 2.1
mT_{10}	8.26 ± 1.9	6.12 ± 0.2	14.4 ± 1.0
Sum	20.86 ± 1.8	9.52 ± 1.2	30.4 ± 0.5
[c] T_{10}	21.27	8.3	29.57
mT_{10}	8.47	5.55	14.02
sT_{360}	5.0	6.28	11.28
mT_{360}	2.0	0	2.0

[a] Sample was preincubated in 0.1 M NH_4HCO_3 buffer (pH 7.5), containing 2 mM EDTA, 8 M urea, 5% SDS
[b] Values are mean ± SD of five separate determinations
[c] Values of one preparative experiment

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