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Detailed structural analysis of exposed domains of membrane-bound Na^+,K^+-ATPase

A model of transmembrane arrangement

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Exposed regions of the α- and β-subunits of membrane-bound Na^+,K^+-ATPase were in turn hydrolyzed with trypsin. Resistance of the β-subunit to proteolysis was shown to be due mainly to the presence of disulfide bridge(s) in the molecule. A model for the spatial organisation of the enzyme in the membrane was proposed on the basis of detailed structural analysis of extramembrane regions of both subunits.

Membrane-bound enzyme; Na^+,K^+-ATPase; Tryptic digestion; Exposed domain; Transmembrane arrangement

1. INTRODUCTION

Determination of the complete primary structures of Na^+,K^+-transporting adenosine triphosphatases from diverse sources [1-11] initiated a new stage in studying the molecular organisation of the enzyme - a direct positioning of certain structural elements of subunit polypeptide chains. The urgent task here is reliable identification of exposed and intramembrane domains, i.e. design of a two-dimensional scheme for the transmembrane protein folding. Such schemes for the polypeptide molecules in the lipid bilayer differing in the number of transmembrane segments in the C-terminal part of the α-subunit have been previously proposed from calculation of the hydrophobicity profiles [1-11].

A methodological approach used to obtain experimental data for modelling of the enzyme molecule in the membrane is based on limited proteolysis of the membrane-bound Na^+,K^+-ATPase. This implies that only the exposed protein regions will be attacked proteolytically and that the intramembrane moiety is safely protected by the lipid environment.

Most of the information concerning the exposed portions of the catalytic subunit had been obtained previously from structural analysis of the extramembrane peptides formed on selective tryptic hydrolysis of the α-subunit entering the membrane-bound enzyme [1,3,12]. The membrane moiety of the hydrolysate, being a unique composition of membrane segments of the α-subunit and intact glycoprotein, became an object for studying the exposed domains of the latter. The original preparation and the samples preliminarily treated with neuroaminidase or β-mercaptoethanol were subjected to tryptic hydrolysis. Data on structures of water-soluble peptides of all three hydrolysates were used to determine the exposed regions of the β-subunit. The influence of terminal sialic acids and disulfide bridges on the β-subunit's resistance to proteolysis was evaluated. Structural analysis of products of proteolytic cleavage of both subunits underlies the modelling of folding of the enzyme molecule [13].

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2. MATERIALS AND METHODS

Purification of Na^+,K^+-ATPase from pig kidney outer medulla, limited hydrolysis of the α-subunit entering the membrane-bound enzyme and peptide analysis were carried out as in [12,13]. For selective isolation of cysteine- and tryptophan-containing peptides, tryptophan residues were modified with 2-nitrophenylsulphenyl chloride and reduced to 2-SH Trp [14]. The fraction of the desired peptides was obtained by covalent chromatography on thiol-Sepharose [14].

The membrane-bound part of the initial hydrolysate was treated with neuroaminidase (0.5 U/mg protein) in 25 mM imidazole buffer, containing 3 mM EDTA (pH 6.0) at 24°C for 4 h, or with 300 mM β-mercaptoethanol in 0.1 M NH_4HCO_3 in the presence of 3 mM EDTA (pH 7.3) at 37°C for 20 min, and centrifuged ($140\,000 \times g$, 2.5 h). Pellets of both samples were homogenised in 0.1 M NH_4HCO_3 (pH 7.3) (1 mg/ml) and treated at 37°C with 50 μg trypsin for 3 h and with 25 μg of the enzyme for 20 min, respectively. The hydrolysates were centrifuged ($140\,000 \times g$, 2.5 h) and water-soluble peptides of the supernatants were separated by HPLC and sequenced as described in [15].

3. RESULTS AND DISCUSSION

The purified membrane-bound Na^+,K^+-ATPase is a suitable system for topological investigations, since it is isolated as a homogeneous functionally active enzyme within the plasma membrane [16].

Calculation of the hydrophobicity profile of the α-subunit allowed the prediction of eleven probable transmembrane clusters [1,3]: 89–114, 123–142, 284–306, 313–341, 530–554, 569–597, 780–803, 842–867, 909–930, 946–971, 973–994. Previously, we established conditions for trypsinolysis of the membrane-bound enzyme (0.1 N NH_4HCO_3, pH 7.3; 1% trypsin, 10 min), providing exhaustive hydrolysis of the exposed regions of the polypeptide chain of the catalytic subunit [3,12]. Among the water-soluble peptides isolated from the hydrolysate we identified three fragments, viz. 529–544, 545–589, 590–598, and thus the moderately hydrophobic stretches 540–544 and 569–597 could not be considered as embedded in the lipid bilayer.

This paper presents the results of detailed screening of the tryptic hydrolysate of the native membrane-bound Na^+,K^+-ATPase, and the isolation and identification of additional fragments of the exposed parts of the α-subunit (fig.1). A highly informative experiment on the directed isolation of cysteine- and tryptophan-containing peptides from the hydrolysate was performed by the recently described modification procedure. This includes conversion of tryptophan residues into SH-tryptophan and selective covalent chromatography on thiol-Sepharose [14]. Of great importance here is the identification of peptides 913–931 and 973–998 in the water-soluble hydrolysate fraction. This means that the rather hydrophobic sequences 909–930 and 973–994 are in fact, exposed outside the membrane.

Fig.1 outlines results of the structural analysis of the extramembrane peptides of the α-subunit of Na^+,K^+-ATPase. The sequences of isolated peptides (in total about 600 amino acid residues) cover the following regions of the polypeptide chain: 1–87, 150–257, 348–766, 826–841, 876–931, and 973–1016, thus providing experimental evidence of the exposure of these regions in the α-subunit. Other regions of the polypeptide chain can be considered as a sum of membrane-bound fragments. Of eleven probable transmembrane segments previously suggested only seven belong to this portion of the molecule. According to the hydrophobicity profile their coordinates are: 89–114, 123–142, 284–306, 313–341, 780–803, 842–867, 946–971. More precise determination of the boundaries of intra- and extramembrane regions is impossible from the experiment because of the methodical limits, particularly trypsin specificity. In addition, proteolysis may induce a slight disturbance in the spatial disposition of the protein fragments originally located in the polar membrane zone.

The above data make it possible to propose the following scheme for the α-subunit folding in the plasma membrane. (Henceforth, the coordinates of hydrophobic sequences are assumed to be the boundaries of the transmembrane segments.) The cytoplasmic location of hydrophilic regions 1–87, 143–283 and 342–779 situated ahead of rod I and between II–III and IV–V membrane rods seems obvious, since they include the known markers of the intracellular regions of the α-subunit (com-

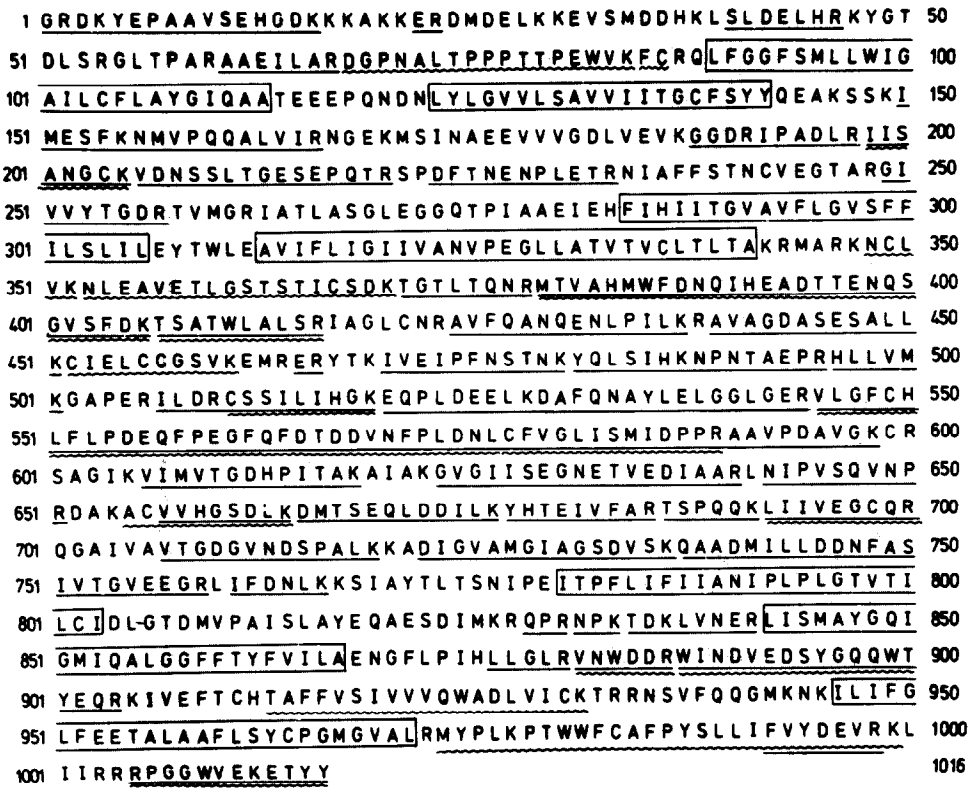

Fig.1. Amino acid sequence of the α-subunit. Peptides resulting from limited tryptic hydrolysis of membrane-bound Na^+,K^+-ATPase (1%, 10 min) are underlined: (——) before and (~~~) after modification of tryptophan residues. Suggested transmembrane segments are framed.

ponents of the catalytic site Asp 369 [17], Lys 501 [18,19], Asp 710, 714 [20,21], Lys 719 [22]) and the points of primary proteolytic cleavage characteristic of E_1 and E_2 enzyme forms (bonds 30–31, 266–267, 438–439 [23]).

Orientation of hydrophilic loops 868–945 and 804–841 relative to the plasma membrane was shown by means of monoclonal antibodies IIC_9 and VG_2 whose antigenic determinants are located in peptides 887–904 and 810–825, respectively [24]. In addition to the exposed C-terminus and hydrophilic loop 804–841, the extramembrane region of the molecule involves short junctions I–II and III–IV of membrane segments (115–122 and 307–312). The latter contain no trypsin-sensitive bonds and it is still unclear whether they are really exposed.

The first experimental data on the spatial disposition of certain fragments of the β-subunit polypeptide chain resulted from the isolation and deciphering of the glycopeptide structure containing asparagine residues 157, 192 and 264 [15]. They imply that the corresponding sites of the protein molecule are exposed on the outer surface of the plasma membrane.

The hydrophobicity analysis shows that the polypeptide chain of the β-subunit can involve up to four (23–67, 197–214, 223–260) intramembrane rods [3,25,26].

The first stage of tryptic hydrolysis of the membrane-bound enzyme [12] yielded a preparation containing intact glycoprotein serving as a suitable model for studying the spatial arrangement of the β-subunit. The main problem here is the strong resistance of this subunit as with the native enzyme to the action of proteases: trypsin, chymotrypsin, *Staphylococcus aureus* protease (unpublished).

Probably, the stability of the β-subunit polypeptide chain is rooted in the protein structure. The

shielding effect produced by the carbohydrate chains evenly distributed on the long C-terminal part can be a factor affecting the protein resistance. Therefore, removal of terminal sialic acids with neuroaminidase could increase the rate of proteolytic cleavage. Neuroaminidase treatment of the native enzyme does not inhibit its ATPase activity and, evidently, does not disturb the integrity of its spatial structure [27]. Peptides of the tryptic hydrolysate of the desialiated protein were isolated and analysed as in [15]. Fig.2 depicts the identified peptides. Noteworthy are fragments 75–84, 233–236 and 253–157 formed due to splitting the trypsin-unspecific bonds. In the hydrolysate we found the peptides entering the composition of fragments 197–214 and 223–260. However, the conclusions concerning the exposure of these regions were of preliminary character only, since complete trypsinolysis of the β-subunit took rather a long time (3 h).

In searching for other approaches to overcome the resistance of the β-subunit to proteolysis, the original preparation was treated with β-mercaptoethanol for reduction of one or two suggested internal disulfide bonds [28,29]. Our results show that it is the preliminary treatment that facilitates fast exhaustive tryptic hydrolysis of the exposed domains of the β-subunit. Thus, glycoprotein stability is possibly determined by disulfide bridges. Fig.2 presents the results of structural screening of the hydrolysate. Particular attention was paid to identification of the fragments that could be ascribed to the membrane sector due to their high hydrophobicity index. However, the experimental results demonstrate that peptides 22–26, 71–84, 182–202, 223–247 and 248–272 belong to the exposed protein areas (peptide 834–841 of the α-subunit that marked the N-terminal boundary of the VI membrane rod was also found in the hydrolysate).

Thus, limited proteolysis unraveled only one hydrophobic region (34–61) in the membrane-bound β-subunit. This fragment may span the lipid bilayer once, since to traverse it twice a secondary structure other than the α-helix would be admitted, which is unlikely to happen. Thus, the C-terminal part of the protein molecule (62–302) forms the single extracellular domain, and the N-terminal hydrophilic region (1–33) is, obviously, exposed to the cytoplasm.

The above structural analysis of the exposed subunit regions by stepwise limited tryptic hydrolysis of the membrane-bound enzyme became the experimental basis for a detailed model of the transmembrane organisation of the enzyme $\alpha\beta$-protomer (fig.3) [13]. It allowed determination of the composition of the Na^+,K^+-ATPase membrane sector that is actually involved in cation transfer across the hydrophobic barrier of the lipid bilayer. The amino acid composition of the membrane portion is characterised by an extremely low content of charged residues. Of eight transmembrane segments 20–28 residues in length, only the 4th and 7th rods of the α-subunit include three charged residues of glutamic acid (327, 953, 954) located in the hydrophobic portion of the lipid bilayer. This unique situation implies carboxyl groups participate directly in the transport. Thus the primary targets for directed mutagenesis are evident, that would elucidate the functional role of certain amino acid residues in the vector transfer of cations.

Naturally, this model proposed mainly on the basis of only one methodological approach cannot

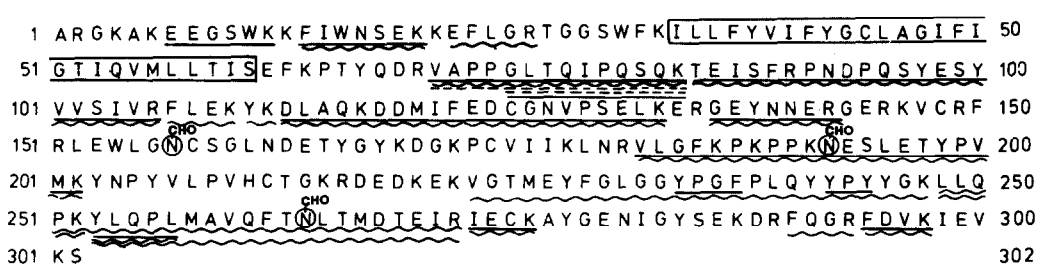

Fig.2. Amino acid sequence of the β-subunit. Peptides obtained from tryptic hydrolysis: (– – –) membrane-bound β-subunit (5%, 1 h), (——) desialiated membrane-bound β-subunit (2.5%, 3 h), (——) reduced membrane-bound β-subunit (5%, 20 min).

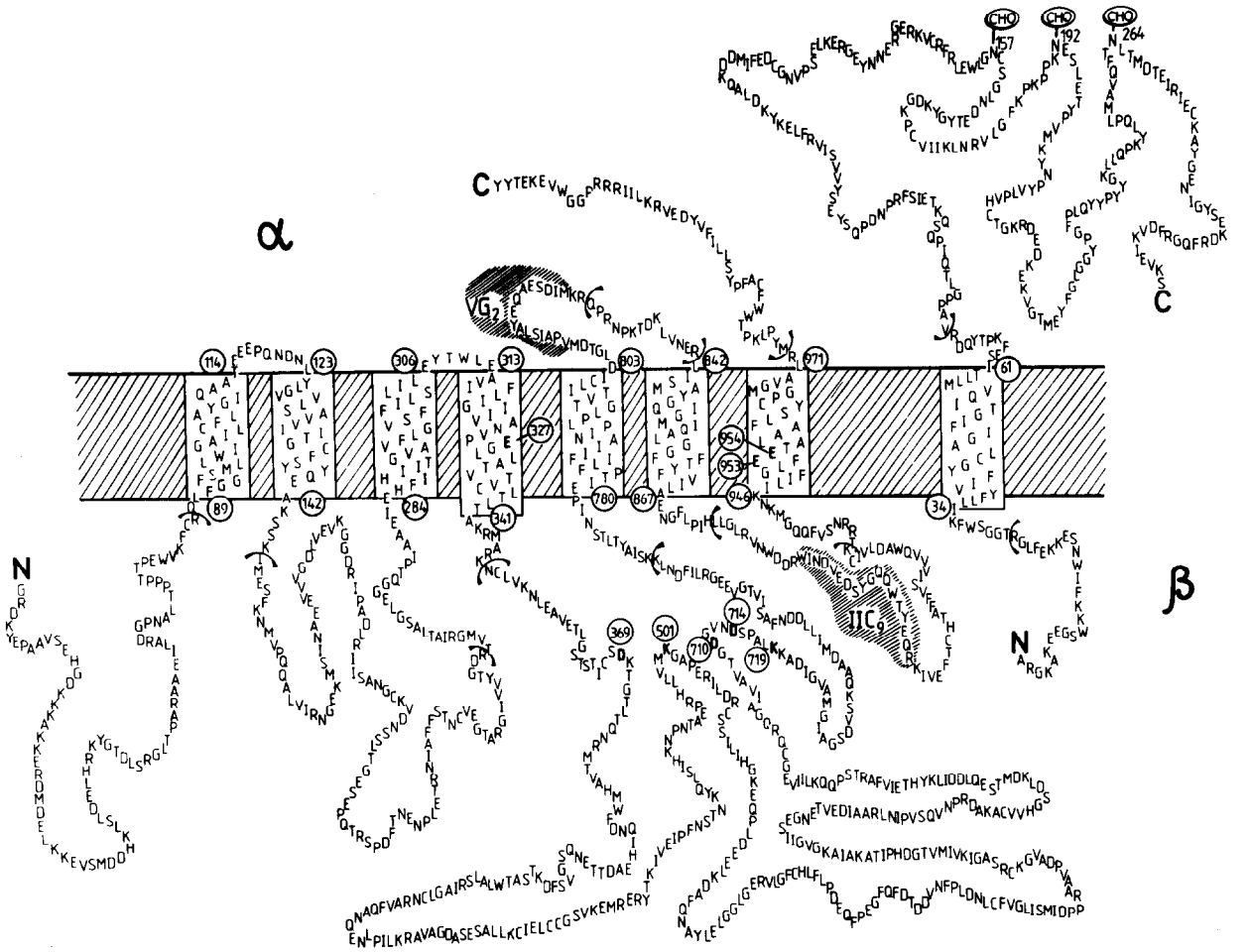

Fig.3. Scheme for the spatial organisation of the $\alpha\beta$-protomer of Na^+,K^+-ATPase. Hatched regions of α-subunit polypeptide chain correspond to epitopes of monoclonal antibodies IIC_9 and VG_2. The areas of dispostion of isolated extramembrane peptides are bracketed (details in text).

be considered as the final one. It undoubtedly requires further refinement by various methods (for example, covalent labelling of the hydrophobic portion, cross-linking experiments, immunochemical techniques, etc.). With this aim in view, we used monoclonal antibodies in additional analysis of the exposed domains of the enzyme molecule, in particular, to obtain independent data on the spatial localization of the C- and N-terminal regions of the α- and β-subunits, respectively.

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