Pig kidney Na^+,K^+-ATPase

Primary structure and spatial organization

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cDNAs complementary to pig kidney mRNAs coding for α- and β-subunits of Na^+,K^+-ATPase were cloned and sequenced. Selective tryptic hydrolysis of the α-subunit within the membrane-bound enzyme and tryptic hydrolysis of the immobilized isolated β-subunit were also performed. The mature α- and β-subunits contain 1016 and 302 amino acid residues, respectively. Structural data on the peptides from extramembrane regions of the α-subunit and on glycopeptides of the β-subunit underlie a model for the transmembrane arrangement of Na^+,K^+-ATPase polypeptide chains.

$(Na^+ + K^+)$-ATPase *α-Subunit* *β-Subunit* *cDNA nucleotide sequence* *Primary structure* *Glycopeptide* *Transmembrane arrangement*

1. INTRODUCTION

Na^+,K^+-activated adenosine triphosphatase (EC 3.6.1.6) localized in plasma membranes of animal cells pumps Na^+ out and K^+ in against their electrochemical potential gradients using ATP as an energy source. In such a way it maintains Na^+,K^+ homeostasis fundamental for numerous cellular processes (review [2,3]). The enzyme molecule consists of equimolar amounts of the catalytic α-subunit and the glycosylated β-subunit of unknown function (for the enzyme from pig kidney outer medulla subunit molecular masses are ~112 kDa (α) and ~44 kDa (β)). The carbohydrate moiety (~9 kDa) is linked by *N*-glycosidic bonds [4].

To elucidate the molecular basis of the Na^+,K^+-ATPase function we studied different levels of structural organization of the enzyme molecule [5–12]. The paper presents the main points of our research into the enzyme primary structure: cDNA nucleotide sequences corresponding to the mRNA coding regions and the deduced amino acid sequences of α- and β-subunits [13–15]; independent determination of amino acid sequences of extramembrane hydrophilic fragments of the α-subunit polypeptide chain [16] as well as sequence analysis of tryptic peptides of the β-subunit (including three glycosylated ones); a model for the spatial arrangement of the enzyme molecule.

Preliminary results were reported at the International Symposium on Ion Transport through Membranes (Nagoya, Japan, November 4–7, 1985) [1]

2. MATERIALS AND METHODS

Na^+,K^+-ATPase from pig kidney outer medulla was isolated according to a modified Jørgensen procedure [4]. 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) at 37°C for 10 min [16]; the water-soluble peptides were separated by reverse-phase HPLC and sequenced. The membrane portion of the hydrolysate was treated with neuraminidase and then with trypsin again; the

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hydrophilic peptides were isolated as in the above experiment.

The subunits of Na^+,K^+-ATPase were separated after enzyme dissociation [4] by HPLC on an Ultro Pac TSK-G 3000 SWG column (LKB) in 5% SDS. The isolated β-subunit was immobilized on thiol glass CPG/thiol (Pierce) [17], the detergent washed out and the conjugate treated with 10% trypsin for 16 h. In the course of hydrolysis cysteine-free peptides were removed from the support. Cysteine-containing peptides were solubilized with β-mercaptoethanol and carboxymethylated. Individual peptides were isolated from these fractions by reverse-phase HPLC.

RNA was isolated from outer medulla of pig kidney by selective precipitation with LiCl in the presence of urea and the vanadyl ribonucleoside complex [18]. The mRNA fraction was obtained after two cycles of chromatography on oligo(dT)-cellulose [19]. Use was made of either $oligo(dT)_{12-18}$ or 'random' primers (mean-statistic DNA hydrolysate) to initiate synthesis of the first cDNA strand [20]. The second strand was synthesized by DNA polymerase I and RNase H [21]. Double-stranded DNA was inserted in plasmid pBR322 cleaved with *PstI* by the dG:dC tailing technique [22] and cloned into *E. coli* MH1. The clone libraries were screened with the following specific oligonucleotide probes: for the α-subunit – 5′-TT(T,C)TG(A,G)AAGGC(A,G)TC-(T,C)TT-3′, 5′-CC(T,C)TC(A,G,T,C)GG(A,G)-AA(T,C)TG(T,C)TC-3′, 5′-TT(T,C)TC(T,C)TG-(A,G)TT(A,G,T,C)GC(T,C)GT-3′ and for the β-subunit – 5′-GCCGCCCAGGCCGAAGTAC-CCATGGTGCCAC-3′, 5′-(A,G)TTCCAGAT-(A,G)AA(T,C)TT-3′ corresponding to peptides Lys-Asp-Ala-Phe-Gln-Asn (528–533), Glu-Gln-Phe-Pro-Glu-Gly (556–561), Glu-Ala-Asn-Gln-Glu-Asn (427–433) (α-subunit) and Val-Gly-Thr-Met-Glu-Tyr-Phe-Gly-Leu-Gly-Gly (223–233), Lys-Phe-Ile-Trp-Asn (13–17) (β-subunit), respectively [13–15,23,24].

Nucleotide sequences of cDNA inserts from the positively hybridized clones were determined according to Maxam and Gilbert [25] and Sanger et al. [26]. Hydrophobicity profiles of the amino acid sequence were calculated by two methods [27–29], the segment length being equal to 21 (α) or 13 (β) residues.

3. RESULTS AND DISCUSSION

To determine the primary structure of Na^+,K^+-ATPase we analysed the nucleotide sequences corresponding to mRNAs of both subunits. Structural information for synthesis of specific oligonucleotide probes was procured from amino acid sequence of peptide fragments. The selective tryptic digestion of the native membrane-bound Na^+,K^+-ATPase was performed under the conditions for extensive hydrolysis of exposed regions of the α-subunit at complete retention of glycoprotein integrity [16]. As a result amino acid sequences covering almost all the exposed regions of the catalytic subunit polypeptide chain were established.

Immobilization of the isolated β-subunit according to [17] allowed us to remove the detergent from the conjugate and to perform the exhaustive trypsinolysis of a rather hydrophobic glycosylated protein. Most of the generated fragments, among them all three glycopeptides, were isolated and sequenced.

The experiments on hybridization of $poly(A^+)$ RNA with specific oligonucleotide probes showed that mRNAs coding for the α- and β-subunits of Na^+,K^+-ATPase contain ~4000 bp (26–27 S) and ~3000 bp (22–23 S), respectively. Thus, the translated part of the β-subunit mRNA is about twice as short as the 5′- and 3′-untranslated regions.

Clone libraries created by means of random (2×10^5 clones) and oligo(dT) (10^5 clones) primers were screened with specific oligonucleotide probes (see section 2); as a result ~50 (α) and 15 (β) positively hybridized clones were identified. We determined nucleotide sequences of overlapping cDNA fragments with the following coordinates: 1403–1852 [12]; 1146–2465; 1296–2880; 723–1946; 220–1186; −60–1260; −240–602; 1910–3216 (α-subunit) (fig.1) and −120–1103; 327–879; 565–1002; −80–448 (β-subunit) (fig.2). They cover at least twice translated mRNA regions.

The complete cDNA nucleotide sequences and the deduced amino acid sequences of the Na^+,K^+-ATPase subunits are shown in figs 1 and 2. N-terminal sequence analysis of the mature α-subunit [4] disclosed five additional residues in the primary translation product cleaved off during

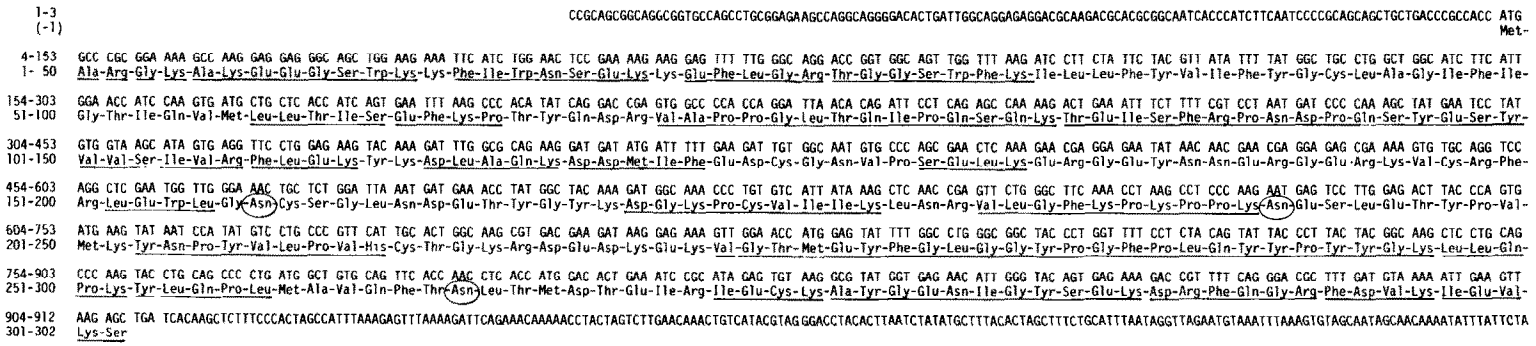

Fig.1. Nucleotide sequence of cDNA coding chain and amino acid sequence of the Na^+,K^+-ATPase α-subunit. Sequences of isolated hydrophilic peptides are underlined.

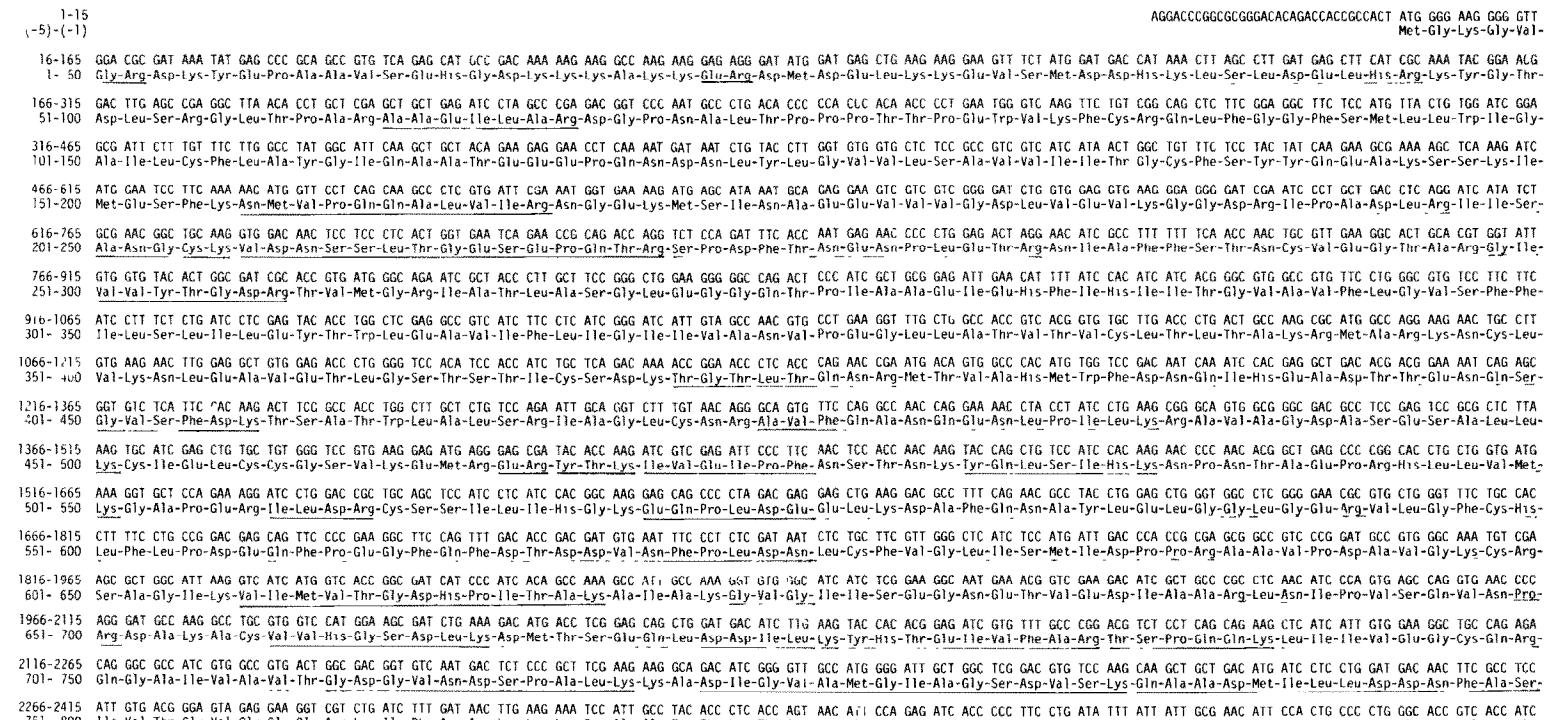

Fig.2. Nucleotide sequence of cDNA coding chain and amino acid sequence of the Na^+,K^+-ATPase β-subunit. Amino acid sequences whose complete or partial structures were determined by analysis of corresponding peptides are underlined.

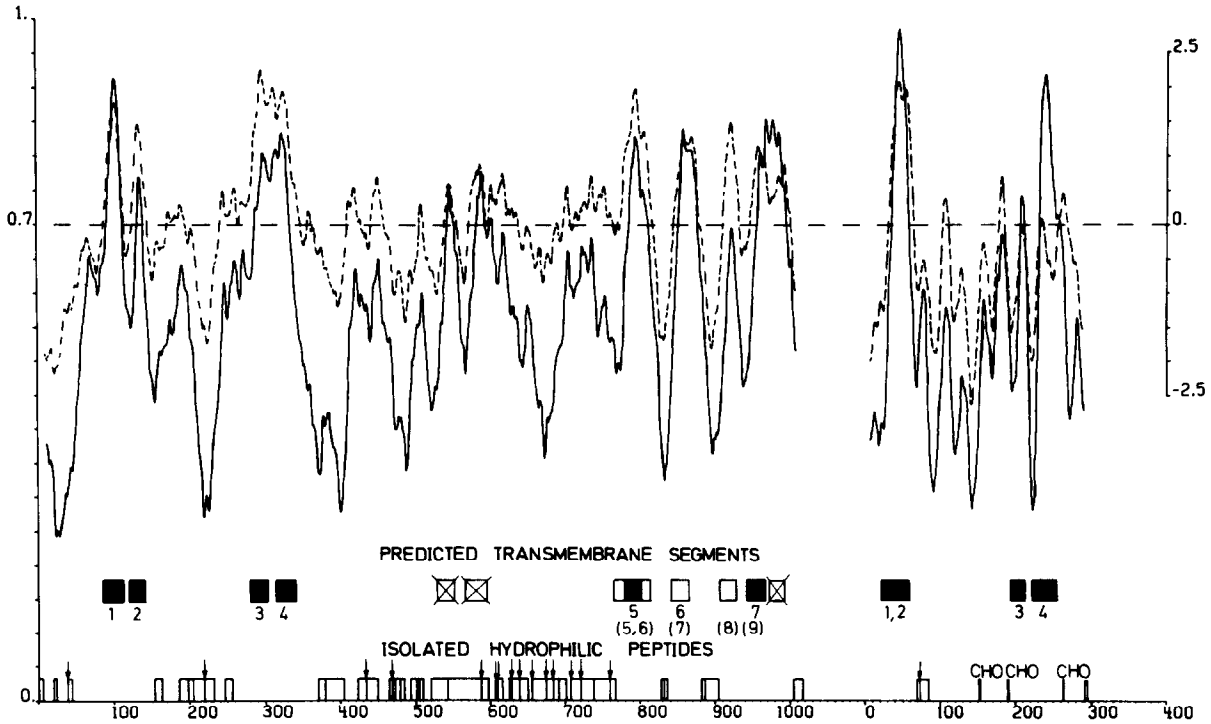

Fig.3. Hydrophobicity profile of Na^+,K^+-ATPase α- and β-subunits. Broken line [29]; solid line [27,28]. *N*-glycosylation sites are marked with CHO. Points of unspecific trypsin splits are indicated by arrows.

processing. The initiation ATG codon foregoes the sequence coding for the N-terminus of the β-subunit. Structures of tryptic peptides Arg-Pro-Gly-Gly-Trp-Val-Glu-Lys-Glu-Thr-Tyr-Tyr (α, 1005–1016) and Ile-Glu-Val-Lys-Ser (β, 298–302) completely coincide with the sequences preceding termination codons. Thus 1016 amino acid residues compose the α-subunit polypeptide chain (M_r 112235) and the β-subunit is made up of 302 amino acid residues (M_r of the protein moiety 34958).

Two known sites of the α-subunit involved in formation of the ATP-hydrolyzing catalytic site are located in the polypeptide chain as follows. A functionally important aspartic acid residue phosphorylated upon the acyl phosphate intermediate formation is localized in position 369. The Cys-Ser-Asp-Lys sequence surrounding this residue completely agrees with that reported in [30]. The 368–375 region is assumed to be a common structural element for the catalytic site of all known transport ATPases (including F_0F_1-ATPases) [31]. Another region (496–506) of the α-subunit, apparently a part of the enzyme catalytic site, contains lysine residue (501) which can be modified with an irreversible inhibitor of the enzyme – fluorescein isothiocyanate [32,33].

The cytoplasmic region comprising the major part of the protein molecule includes the catalytic site [2,3,34], where the N-terminus is localized [34,35]. The hydrophobicity profile of the α-polypeptide chain revealed eleven regions, which could serve, in principle, as intramembrane segments; their hydrophobicity exceeds level 0.7 [27] and is positive [29] (fig.3). However, comparing the above data and those on amino acid sequences of the hydrophilic peptides of the α-subunit [16], we positioned segments 89–114, 123–142, 284–306 and 313–341, but not 530–553 and 569–597, inside the membrane. The two latter rather hydrophobic stretches cannot be transmembrane, as the lipid bilayer would include sequences Arg^{544}–Val^{545} and Arg^{589}–Ala^{590} hydrolyzed by trypsin [16].

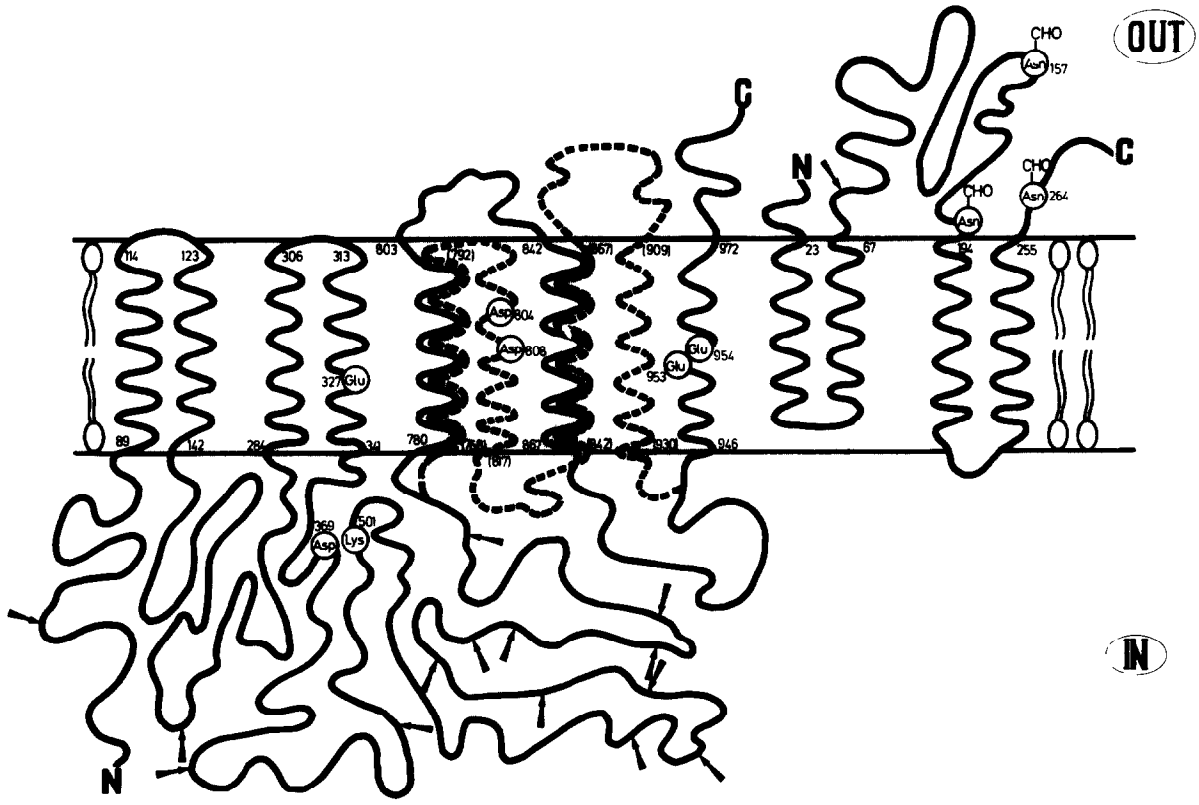

Fig.4. Model for spatial organization of the enzyme molecule. Alternative pathway of the α-subunit chain is shown by the broken line. *N*-glycosylation sites are marked with CHO. Arrows indicate points of unspecific trypsin splits.

It is hard to predict the arrangement of the remaining more hydrophobic portion of the polypeptide chain. The extracellular domain of the α-subunit should be large enough since it forms the main part of the binding site of the cardiac glycosides [2,3] (it is supposed to be 20–30% of the cytoplasmic portion according to chemical modification with impermeable reagents [7,11,34,36,37]). This domain is substantially larger at the odd rather than at the even number of transmembrane segments.

Therefore we assume that of five probable transmembrane fragments only three (780–803, 842–867, and 946–972) are localized inside the membrane, the remaining two, shorter and less hydrophobic (909–930, 979–994), being exposed outside. However, there is another way for the polypeptide chain organization. The first region can be essentially extended (768–817) preserving the high hydrophobicity level. In this case its length is sufficient to traverse the lipid bilayer twice. Just this pathway was proposed for the analogous part of Ca^{2+}-ATPase [38]. If so, segment (909–930) should be considered as transmembrane.

The polypeptide chain of the α-subunit traverses the lipid bilayer seven or nine times and its C-terminus is exposed on the outer membrane side. Even according to this model the ratio of outer and inner hydrophilic regions is smaller than was predicted from chemical labelling [7,11,34,36,37]. So the α-subunit may be of more complex organization than in fig.4. For example, one cannot exclude the presence of additional hydrophilic stretches in the lipid bilayer, since the three residues of glutamic acid (327, 953, 954) positioned in the membrane are, probably, insufficient to form a cation-conducting pathway. (In this respect the 9-rod model is preferential, since three more charged residues (Gly 779, Asp 804, 808) get into the membrane.)

Limited tryptic hydrolysis of the membrane-bound Na^+,K^+-ATPase unexpectedly revealed 15 trypsin-unspecific splits of bonds such as Ile–Val; Ala–Ala; Ile–Met, etc. marked by arrows in figs 3 and 4 [13,14,16]. Apparently, these parts of the sequence are situated on the surface of the protein globula in bends of the polypeptide chain.

To construct a model for the β-subunit transmembrane arrangement we used the following data as a basis. Modification of Na^+,K^+-ATPase with permeable hydrophobic reagents [39,40] demonstrates that a part of the β-subunit is buried in the lipid bilayer. Labelling of the exposed enzyme portion with fluorescamine [7–11] or Bolton-Hunter reagent [36] and immunochemical assay [5] showed that practically the whole hydrophilic part of the β-subunit is exposed on the outer surface of the membrane. Three glycopeptides (152–169, 182–202 and 253–272) were isolated from the tryptic hydrolysate of the β-subunit [15]. So all canonic sites of *N*-glycosylation [41] in the β-subunit (asparagine residues 157, 192 and 264) are really glycosylated, consequently these regions of the polypeptide chain are exposed on the extracellular side of the membrane.

Calculation of hydrophobicity of the β-subunit polypeptide chain revealed three probable intramembrane hydrophobic segments: 23–67, 197–214 and 223–260 (fig.3). If the central part of the first stretch (made up of 28 uncharged residues) forms a single transmembrane rod (34–61), the considerable portion of the N-terminal region of 7–8 amino groups is in the cytoplasm. Inaccessibility of the latter to modification with impermeable reagents [7,11,36] might be due to the protection by the cytoplasmic portion of the α-subunit, but alternative arrangement of the N-terminal part seems more probable. Fragment (23–67) involving 75% of the hydrophobic residues is organized in a hairpin structure, since its length is enough to cross the hydrophobic part of the lipid bilayer twice (fig.4).

The arrangement of the β-subunit polypeptide chain, in particular, of the N-terminus will be clarified on analysis of the exposed β-subunit formed by trypsin treatment of the membrane-bound enzyme. This experiment is in progress. For instance, peptide (71–84) isolated from this hydrolysate clearly indicates the position of the C-terminal boundary of the first hydrophobic stretch. The middle part of the polypeptide chain including the above peptide and two glycosylated asparagines (157 and 192) forms the long hydrophilic extracellular domain (70–194). The hydrophobic segments (197–214 and 223–260) linked by 8 charged residues (Lys-Arg-Asp-Glu-Asp-Lys-Glu-Lys) are located between glycosylated asparagines (192 and 264). The extremely hydrophobic character of fragment (223–260) demonstrates its preferentially intramembrane location. If so, one should assume the existence of another transmembrane rod formed by a rather short and less hydrophobic segment 197–214. (This stretch can be, in principle, elongated due to plunging of a part of the hydrophilic sequence 215–22 into the membrane if opposite charges are neutralized in the α-helix structure through formation of salt bridges.) The connection between transmembrane rods is, apparently, exposed on the membrane cytoplasmic side. Only this region can enclose the papain split site of the β-subunit that results in two large membrane-bound glycosylated fragments [42]. The hydrophilic character of the β-subunit C-terminal part demonstrates its extracellular orientation.

The proposed arrangement with four intramembrane segments agrees quite well with the three-dimensional model revealed by electron microscopy of two-dimensional crystals of the individual β-subunit [12]. Moreover, the cross-section of the hydrophobic area of the analogous three-dimensional model of the whole enzyme [10,11] can insert 11–13 α-helices that fit the sum of the predicted hydrophobic stretches of both subunits.

Comparison of the sequences of the catalytic subunits of Na^+,K^+-ATPase from ray electroplax [43], pig [14] and sheep [44] kidneys revealed an exceptionally high degree of homology (~90% even for diverse species). These polypeptides have rather extended regions of the sequences similar to those of other ion pumps (sarcoplasmic reticulum Ca^{2+}-ATPase [38] and *E. coli* K^+-ATPase [45]). Most of the homologous fragments of this family of ATP-driven ion pumps are localized in cytoplasmic portions that reflect similarity of catalytic mechanisms.

Analysis of the intron-exon arrangement of Na^+,K^+-ATPase genes brings to light a possible functional role of the structural units encoded by certain exons. We sequenced a part of the sole or one of a few human genes encoding the enzyme α-subunit [46]. (Intron positions in the sequenced part correspond to coordinates 2342–2343, 2566–2567, 2712–2713, 2843–2844 and 2945–2946 in fig.1.) For instance, localization of some introns coincides perfectly with the boundaries of the above homologous regions or marks the C-terminus boundaries of predicted transmembrane segments as in the case of the visual rhodopsin gene [47].

The primary structure of the β-subunit is more variable (sequences of proteins from pig kidney [15] and ray electroplax [48] differ by 38.7%). Presumably it means that the β-subunit is less substantial for the enzyme functioning than the α-subunit. Replacement points are rather evenly distributed along the polypeptide chain except for the first hydrophobic conserved segment (the sites of *N*-glycosylation and location of all the cysteine and tryptophan residues also coincide). A search for counterparts of the β-subunit among the components of ion-transporting ATPases revealed no regions of significant homology.

Determination of the primary structure of Na^+,K^+-ATPase and design of the preliminary model for its transmembrane arrangement paved the way for elucidation of the real spatial organization and molecular mechanism of functioning of this universal system for active ion transport.

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