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Topology of Na^+,K^+-ATPase

Identification of the extra- and intracellular hydrophilic loops of the catalytic subunit by specific antibodies

Yu.A. Ovchinnikov, N.M. Luneva, E.A. Arystarkhova, N.M. Gevondyan, N.M. Arzamazova, A.T. Kozhich, V.A. Nesmeyanov and N.N. Modyanov

Shemyakin Institute of Bioorganic Chemistry, USSR Academy of Sciences, 117871 Moscow, USSR

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To study the topology of Na^+,K^+-ATPase monoclonal antibodies (MAbs) specific for membrane-bound enzyme were produced. Using immunofluorescence staining of viable cells or smears of a pig kidney embryonic (PKE) cell line, two groups of MAbs were selected, namely those binding to extra- or intracellular portions of the α-subunit. The extracellular location of peptide loop 804–841 linking the Vth and VIth intramembrane hydrophobic segments was proved using MAb VG_2. Another MAb, IIC_9, interacting with PKE cells only after membrane perforation (4% formaldehyde and 0.1% Tween-20), was shown to bind to the hydrophilic loop 868–945. The antigenic determinants recognized by MAb IIC_9 and VG_2 are located in peptides 887–904 and 810–825, respectively. The C-terminus of the α-subunit molecule was positioned on the outer side of the cytoplasmic membrane utilizing affinity-purified antibodies to the synthetic peptide corresponding to fragment 999–1008.

$(Na^+ + K^+)$-ATPase; Monoclonal antibody; Hydrophilic loop; Transmembrane arrangement

1. INTRODUCTION

The Na^+,K^+-ATPase molecule consists of equimolar amounts of two polypeptides: the catalytic α-subunit and β-subunit with still unknown functions. Determination of the primary structures of Na^+,K^+-ATPases from different sources [1–9] initiated studies on the membrane topography of the enzyme molecule which should be crucial for the understanding of the molecular mechanism of the enzyme action.

Limited proteolysis of the membrane-bound enzyme [10] and theoretical calculation of the hydrophobicity profile of its subunits [3,11] allowed us to propose a model for the arrangement of the pig kidney Na^+,K^+-ATPase protomer in the membrane. To confirm and fine-tune this model we obtained monoclonal antibodies (MAbs) to Na^+,K^+-ATPase and isolated peptides carrying antigenic determinants. In addition, antibodies to the synthetic peptide covering the 999–1008 region of the α-subunit were produced in order to confirm the extracellular location of the C-terminus of the molecule.

Correspondence address: N.N. Modyanov, Shemyakin Institute of Bioorganic Chemistry, USSR Academy of Sciences, ul. Miklukho-Maklaya 16/10, 117871 GSP Moscow V-437, USSR

2. MATERIALS AND METHODS

Na^+,K^+-ATPase was isolated from pig kidney (outer medulla) according to the modified procedure of Jørgensen [12]. The homogeneous subunits were isolated as described in [13]. Trypsin hydrolysis of the membrane-bound enzyme was performed as in [10]. Insoluble membrane-bound tryptic peptides were immobilized on thiol-glass (CPG/Thiol, Pierce, USA) [14] and then cleaved with cyanogen bromide in 70% formic acid. Individual peptides were purified by HPLC and sequenced.

Hybridomas producing MAbs to Na^+,K^+-ATPase and its subunits were obtained according to De Fazekas and Scheidegger [15] using the myeloma cell line PAI. Balb/c mice were im-

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munized with the purified membrane-bound enzyme according to the procedure proposed in [16]. Hybridomas were cloned by the method of limited dilutions [17]. Preparative amounts of MAbs were obtained from the ascitic fluid of mice and purified by affinity chromatography on protein A-Sepharose 4B [18]. The isotype and subclass of MAbs were determined by double immunodiffusion [19]. Affinity constants of MAbs were calculated from ELISA data [20]. ELISA was also used for screening of anti-ATPase MAb-producing hybridoma clones and search for epitope-bearing peptides: to perform the assay the membrane-bound Na^+,K^+-ATPase or its subunits (1 μ/ml) or water-soluble tryptic peptides (5–10 μg/ml) were introduced into 96-well microtiter plates. The samples dissolved in formic acid were introduced in 50 μl aliquots and air-dried. The peptides purified by HPLC before ELISA were dissolved in PBS after evaporation of acetonitrile.

The specificity of MAbs was determined by immunoblotting after SDS-PAGE of Na^+,K^+-ATPase on a 4–15% polyacrylamide gradient gel [21].

For immunofluorescence analysis, the pig embryonic cell line (PKE) donated by the All-Union Institute of Experimental Veterinary was used. Viable cells were stained by the 'sandwich' technique [17] at 4°C in the presence of 20 mM sodium azide. To perforate plasma membranes, PKE cells were fixed before staining for 10 min with 4% formaldehyde and then incubated in 0.1% Tween-20 or Triton X-100 for 15 min. MAb IIC_9 (5 mg/ml) was immobilized on CNBr-activated Sepharose 4B according to the manufacturers's instructions. The supernatant from the tryptic hydrolysate containing about 20 mg of the peptides was applied to a 2 ml-immunosorbent column. The bound peptides were eluted with 1 M acetic acid and further purified by HPLC [14].

The decapeptide corresponding to the α-subunit 999–1008 was synthesized following the solid phase technique [22]. The peptide obtained (p999, 1 mg) was conjugated with keyhole limpet hemocyanin [23]. Rabbits were immunized by a 2-fold subcutaneous injection of the conjugate (500 μg) with a 2-week interval. Anti-peptide antibodies (α-p999) were purified by affinity chromatography on Sepharose 4B with the immobilized synthetic peptide. Immunofluorescence staining of viable PKE cells with anti-peptide antibodies (250 μg/ml) was carried out according to the above described procedure. The stained cells were analyzed on an EPICS-5 flow cytofluorimeter (Coultronics, France). The proteolytic treatment of PKE cells was performed under conditions providing cell viability (0.3% trypsin, 45 min, 37°C).

3. RESULTS AND DISCUSSION

The two-dimensional scheme for the transmembrane arrangement of Na^+,K^+-ATPase has been proposed in our laboratory on the basis of the hydrophobicity profile of the enzyme subunits and data obtained from limited proteolysis of the native membrane-bound complex [3,11]. In order to verify and fine-tune this model we employed immunochemical methods. Topology of the enzyme α-subunit was studied by means of MAbs to Na^+,K^+-ATPase [14].

The fusion of myeloma cells with splenocytes of mice immunized with the membrane-bound Na^+,K^+-ATPase resulted in the large number of hybridoma clones. The properties of several MAbs are summarized in table 1. Notably only one of these antibodies, IIF_2, interacted with the SDS-denatured α-subunit.

The topological study involved not only identification of the exposed regions of the molecule but also determination of their orientation relative to the plasma membrane. Therefore, at the initial stage, we tried to find out to which fragments of the polypeptide chains (intracellular or extracellular) the obtained MAbs were bound. To do this the immunofluorecence staining of PKE cells was performed. MAbs capable of staining the viable cells were specific to extracellular ATPase fragments. In contrast, MAbs that stained cells only after perforation of the cell membrane were specific for cytoplasmic domains [17,24]. Results of immunofluorecence staining of PKE cells are listed in table 1. Of 11 MAb studies, 7 were shown to bind to extracellular fragments of the enzyme.

In order to localize the antigenic determinants in the polypeptide chains of the membrane-bound Na^+,K^+-ATPase, the enzyme was subjected to limited proteolysis (1% trypsin, 10 min). The hydrophilic portion of the catalytic α-subunit was

Table 1

Characteristics of monoclonal antibodies to the α-subunit of Na^+,K^+-ATPase

Clone	Isotype and subclass of MAbs	Affinity constant (10^8 M^{-1})	Immunofluorescence staining of PKE cells	
			Viable cells	Cells with perforated membrane
IIA_2	IgG2b	1.2	–	+
IIA_5	IgG2b	2	–	+
IIF_6	IgG2b	5.5	+	+
IIC_9	IgG1	0.8	–	+
IIF_2	IgG2b	2	–	–
IE_1	IgG2b	5	+	+
IVE_3	IgG1	4.8	+	+
VG_2	IgG2b	4.6	+	+
VG_4	IgG2b	1.4	+	+
IID_6	IgG2b	20	+	+
IIE_{12}	IgG2b	10	+	+

virtually, completely cleaved off [10,11]. The water-soluble part of this hydrolysate included peptides derived from the exposed regions of the molecule and bearing antigenic determinants for at least some of the obtained MAbs. According to ELISA MAb IIC_9 interacted the most actively with hydrophilic peptides.

Immunoaffinity chromatography was chosen as the key technique for isolation of the immunogenic peptides. To do this MAb IIC_9 immobilized on Sepharose 4B was loaded with the freshly prepared water-soluble part of the tryptic hydrolysate. Immunosorbent-bound fragments were eluted with 1 M acetic acid. Peptide fractions binding MAb IIC_9 in ELISA were lyophilized, salted out and purified further by reverse-phase HPLC (Nucleosil C_{18}). The N-terminal sequence analysis of the fragments allowed their assignment to the particular regions of the α-subunit polypeptide chain. Of the three peptides obtained (881–886, 887–904, and 881–904), only two (887–904 and 881–904) bound MAb IIC_9 during solid-phase ELISA. It is quite possible that the largest fragment was hydrolyzed during the elution from immunosorbent. Since MAb IIC_9 stained PKE cells only after perforation of the plasma membrane, when antibody penetrated the cell, the 868–945 hydrophilic loop connecting the VIth and VIIth intramembrane segments of the α-subunit was located in cytoplasm (fig.1).

The ELISA study of the membrane-bound fraction of the tryptic hydrolysate containing inramembrane fragments of the α-subunit and intact β-subunit [10] revealed MAb VG_2 to be the most active. Since this MAb interacted with viable cells, its epitope was exposed outside the cell. To localize the VG_2 determinant, the membrane-bound fragments from the tryptic hydrolysate were im-

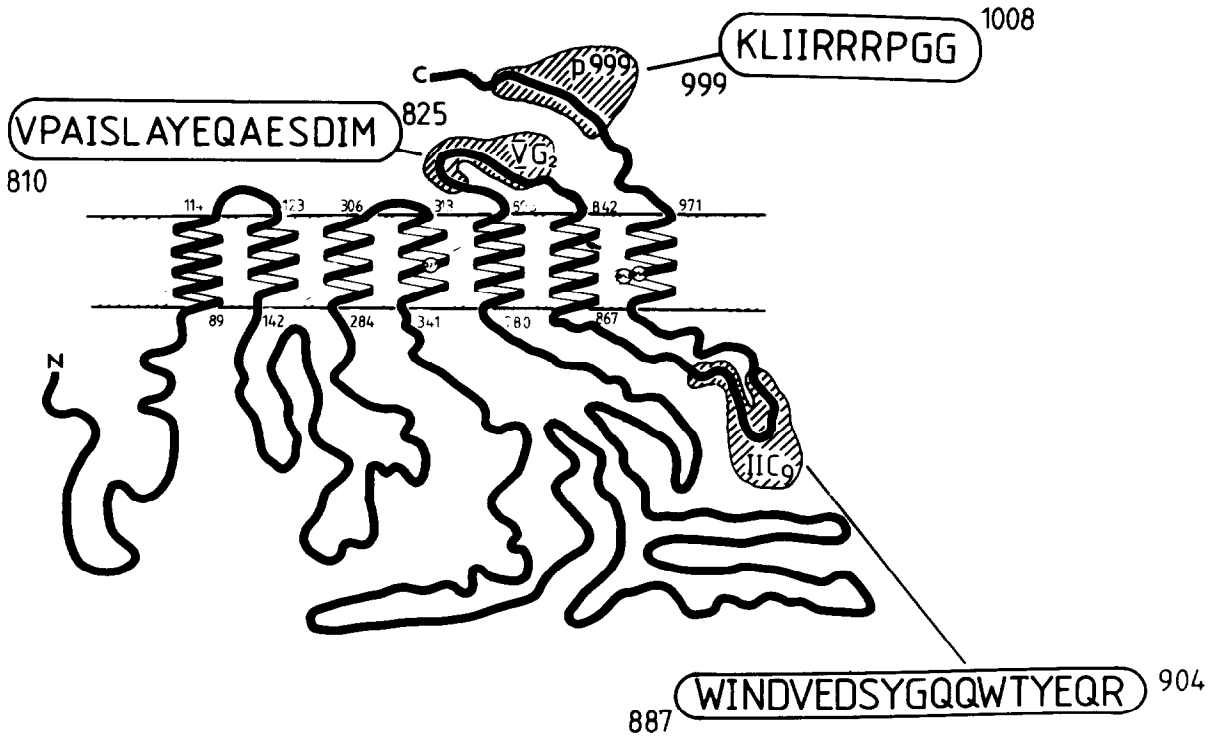

Fig.1. Model for spatial organization of the α-subunit of Na^+,K^+-ATPase. Regions of the polypeptide chain corresponding to antigenic determinants recognized by MAb IIC_9, VG_2 and α-p999 antibodies are marked. Coordinates of transmembrane segments are given as in [11].

mobilized on thiol-glass support under conditions identical to those described for covalent binding of the individual β-subunit [13]. After delipidation, the immobilized peptides were treated with cyanogen bromide and the supernatant containing cysteine-free peptides was analyzed. According to ELISA the peptide carrying epitope for MAb VG_2 was released into the supernatant. Fractionation of the peptides from this supernatant by reverse-phase HPLC followed by ELISA of the obtained fractions yielded the homogeneous peptide that interacted with MAb VG_2. The N-terminal sequence analysis revealed that this peptide could be assigned to the 810–825 region of the polypeptide chain of the α-subunit. Fig.1 shows that the extracellular orientation of this region fits the above model well.

The position of the C-terminus of the α-subunit aroused violent discussions [3,4,6]. Attempts to isolate the C-terminal peptide from the tryptic hydrolysate using MAbs were unsuccessful. Therefore the decapeptide (p999) corresponding to the 999–1008 region of the α-subunit was synthesized. The choice was made on the basis of the secondary structure prediction [25] since the β-turn of the polypeptide chain should be located here. The small peptide size implies only one epitope. Therefore it was reasonable to produce polyclonal rather than monoclonal antibodies. Antiserum was raised in rabbit by immunization with p999 conjugated to hemocyanin. Anti-peptide antibodies were purified by affinity chromatography on the immobilized p999.

The antibodies obtained (α-p999) interacted in ELISA with the membrane-bound enzyme. Upon Western blotting of Na^+,K^+-ATPase with α-p999 antibodies one band corresponding in mobility to the α-subunit was found. These data confirmed the specificity of the antibodies and justify the application of α-p999 to topological studies. Interaction of α-p999 with intact PKE cells was demonstrated by flow cytofluorimetry. Staining of cells by α-p999 antibodies and FITC-labeled anti-mouse IgG resulted in a considerable increase of fluorescence intensity (3–4 times) as compared to staining with normal rabbit immunoglobulin. Fig.2 shows the increase of fluorescence intensity of virtually all cells which proves the presence of the corresponding epitope on the surface of PKE cells. Trypsin treatment of PKE cells considerably decreased the efficacy of cell staining (fig.2) though few cells were nevertheless stained evidently due to incomplete proteolysis.

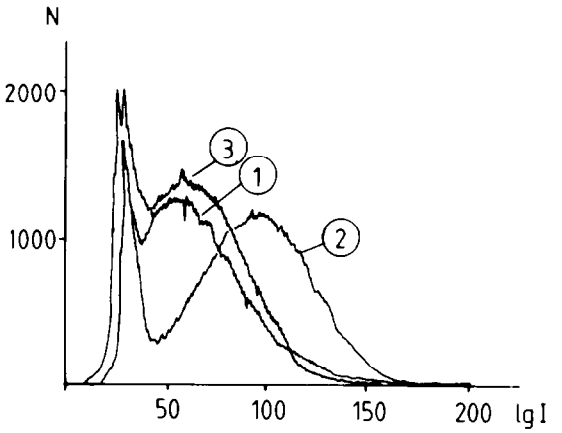

Fig.2. Cytofluorimetric analysis of intact (1,2) and trypsin-treated (3) PKE cells using normal rabbit IgG (1) or α-p999 antibodies (2,3). *N*, the number of the cells analyzed; *I*, fluorescence intensity.

So, we established the spatial orientation of the VIth intramembrane segment of the α-polypeptide and the extracellular location of its C-terminus and thus confirmed our previous model of a transmembrane arrangement for the Na^+,K^+-ATPase catalytic subunit. Immunochemical investigations of the β-subunit arrangement are now in progress.

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