Volume 336, number 3, 521–524 FEBS 13472 December 1993

Topology of Na,K-ATPase α subunit epitopes analyzed with oligopeptide-specific antibodies and double-labeling immunoelectron microscopy

Gang Ning[a,c], Arvid B. Maunsbach[a,c,*], Yang-Jae Lee[b,c], Jesper Vuust Møller[b,c]

[a]*Department of Cell Biology at the Institute of Anatomy,* [b]*Institute of Biophysics and* [c]*Biomembrane Research Center, University of Aarhus, DK-8000 Aarhus C, Denmark*

Received 11 November 1993

Using four oligopeptide-specific polyclonal antibodies, we mapped the α subunit of Na,K-ATPase by double-labeling immunoelectron microscopy combined with negative staining. The results show that the epitopes of the N-terminus (Gly^{1}–His^{13}), C-terminus (Ile^{1002}–Tyr^{1016}) and Leu^{815}–Gln^{828} are located on the same face of crystallized Na,K-ATPase membranes from pig kidney, whereas the epitope Asn^{889}–Gln^{903} is present on the opposite side. The present study demonstrates the cytoplasmic location of C-terminus and that Leu^{815}–Gln^{828} is exposed on the cytoplasmic and Asn^{889}–Gln^{903} on the extracellular side. The results are consistent with an eight- or ten-segment model, and support the existence of an M5/M6 loop and the presence of one transmembrane segment between Leu^{815}–Gln^{828} and Asn^{889}–Gln^{903}.

Na,K-ATPase; α-Subunit; Immunoelectron microscopy; Topology; Membrane epitope; Double labeling; Oligopeptide-specific antibody

1. INTRODUCTION

Na,K-ATPase is responsible for active sodium transport in essentially all animal cells. Its catalytic α subunit has been purified, cloned, and the primary structure has been deduced in several tissues and species [1–5]. To date there are a number of proposed models for the Na,K-ATPase α subunit based on data obtained from biochemical and biophysical studies [1,2,5–8]. These models all agree that the N-terminus is present on the cytoplasmic side of the plasma membrane, but the C-terminus has been located either on the extracellular [6,9] or on the cytoplasmic side [10,11].

In this investigation we have applied immunolabeling combined with electron microscopy to show the location of epitopes on isolated Na,K-ATPase membranes in order to recognize sidedness as well as co-location of epitopes on single pieces of membrane. We used four polyclonal antibodies raised against defined oligopeptides of the Na,K-ATPase α_1 subunit and immunolabeled the epitopes on two-dimensional crystals of the enzyme.

2. MATERIALS AND METHODS

Protein A conjugated to 5 and 10 nm colloidal gold was obtained from BioCell Research Laboratories (Cardiff, UK). Oligopeptides (supplied by Neosystem A.S., Strasbourg) were synthesized by FMOC (fluorenylmethoxycarbonyl) solid phase chemistry.

*Corresponding author. Fax: (45) (86) 19-8664.

2.1. *Oligopeptide antibodies*

The oligopeptides corresponding to the sequence of selected parts of the Na,K-ATPase α subunit [3], plus an additional N-terminal or C-terminal cysteine (see Table I), was used for coupling to keyhole limpet hemocyanin according to Green et al. [12]. Rabbits were given booster injections subcutaneously 3 times with 2-week intervals of 0.2 mg protein with Freund's incomplete adjuvant, and then injections at 4-week intervals followed by collections of blood 1 week after each injection. The development of a titer of the sera against Na,K-ATPase, which reached a maximal level after 5–8 injections, was followed by ELISA according to standard techniques [13], with purified or SDS denatured Na,K-ATPase adsorbed to ELISA wells. In competitive ELISA experiments, all antisera reacted selectively and with high affinity against the peptides used for their production. There was no significant cross-reactivity with sarcoplasmic reticulum Ca-ATPase. After degradation of Na,K-ATPase with trypsin, four distinct immunoreactive bands were observed in immunoblots with each of the three C-terminal oligopeptide antibodies (Fig. 1, bands 1–4). Apparent molecular masses of bands 1–4 range from 24–40 kDa. Consistent with a specific epitope location only two of the antisera (L28 against peptide 889–903 and L21 against 1002–1016, but not L16 against 815–828) reacted with band 5, which corresponds to the 19 kDa C-terminal fragment previously described by Karlish and collaborators [14]. The C-terminal assignment of the 19 kDa fragment (peptide 831–1016) was confirmed by N-terminal sequencing (Lee, le Maire, Denoroy and Møller, unpublished data). Bands 1–4, which react with all three C-terminal antibodies, may be assumed to single out those C-terminal polypeptides, which start before M5 and therefore will include the 815–828 epitope, as is observed. The N-terminal antibody (L25 against 1–13) did not react well in immunoblotting experiments, but was characterized by strong reactivity with undigested Na,K-ATPase, both in ELISA and electron microscopy experiments. A cytosolic location of this epitope is supported by the finding that treatment with trypsin abolishes immunoreactivity and removes cytosolic portions, including the N-terminus of the α subunit [15].

2.2. *Preparation of renal Na,K-ATPase membranes*

Na,K-ATPase was purified in membrane-bound form from the

outer medulla of pig kidney [16]. The enzyme was stored at −20°C in 250 mM sucrose, 12.5 mM imidazole, and 0.5 mM EDTA at pH 7.5. The specific ouabain-inhibitable Na,K-ATPase activity was 2300 μmol $P_i \cdot h^{-1} \cdot mg^{-1}$ protein at 37°C. Two-dimensional crystals of the enzyme were induced with vanadate [17]. The membranes were first incubated with 0.33 μg/ml phospholipase A_2 [18] and then dialyzed at 4°C overnight against 10 mM imidazole buffer, pH 7.5, containing 5 mM $MgCl_2$, 5 mM $CaCl_2$ and 1 mM NH_4VO_3.

2.3. *Immunolabeling and electron microscopy*

The Na,K-ATPase membranes (0.1 mg protein/ml) were applied to carbon films supported by 300 mesh nickel grids. After 1–2 min, excess fluid was sucked away and the grids rinsed with phosphate-buffered saline (PBS) to remove non-attached membranes from the grids. The membranes were then subjected to single labeling with each of the four antibodies, which were detected with protein A conjugated to 5 nm colloidal gold. For double-labeling, the membranes were first incubated with one of the four primary antibodies, which was detected with protein A conjugated to 5 (or 10) nm gold, followed by labeling with one of the other three antibodies, which was detected with protein A conjugated to colloidal gold of a different size than used in detecting the first antibody. The grids were shortly incubated with free protein A after labeling with the first gold marker. After thorough rinse with PBS the grids were lightly negatively stained with 1% uranyl acetate and observed in a JEOL 100CX transmission electron microscope.

Three control groups were introduced for each immunolabeling: in groups 1 and 2 the specific antisera were replaced by pre-antisera and normal rabbit immunoglobin fragments, respectively; and in group 3 preabsorption controls were performed by pre-incubation of each antiserum with the specific oligopeptide against which it was raised.

3. RESULTS

Negative staining showed that essentially all membrane fragments exhibited the two-dimensional crystalline arrays characteristic of Na,K-ATPase, thus confirming the identity of the individual membrane fragment.

After single labeling with each antibody a fraction of the membranes was labeled while another fraction was unlabeled (Figs. 2 and 3). With antibodies L25, L16 and L21, the majority were labeled while a minority were unlabeled. Labeling with L28 gave the opposite result, the majority being unlabeled and a minority labeled. Specific labeling was absent in all control groups (Fig. 4).

In double-labeling experiments, the faces of most membranes were co-labeled with the N-terminal antibody L25(1–13) and the C-terminal antibody L21(1002–1016) while gold particles were absent on the faces of some adjacent (flipped) membranes (Fig. 5); labeling with antibodies L25 and L16(815–828) gave the same result (Fig. 6). However, double-labeling experiments showed that L25 and L28(889–903) did not label the same membrane faces (Fig. 7).

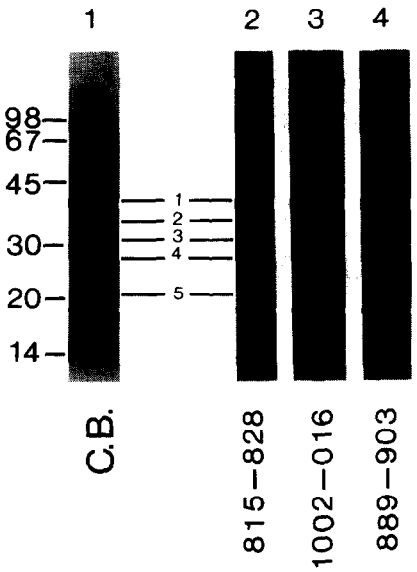


Fig.1. SDS-PAGE electrophoresis of tryptic digest and immunoblot analysis of Na,K-ATPase membranes. Purified Na,K-ATPase (1 mg protein/ml) was treated with trypsin (0.3 mg/ml) for 20 min at 20°C in a medium containing 10 mM KCl, 1 mM EDTA, 20 mM HEPES (pH 7.4), and 100 mM mannitol. Proteolysis was stopped by addition of trypsin inhibitor (at a weight ratio to trypsin of 5:1). The membrane fraction was pelleted 3 times at 100,000 × *g* for 30 min in the Beckman Airfuge (each pelleting being followed by resuspension with the buffer) and subjected to SDS-PAGE according to Laemmli [21] on a 12% polyacrylamide gel, cross-linked with 0.4% bisacrylamide. After gel electrophoresis sample components were blotted onto a poly- vinylidene difluoride membrane (Immobilon, Bio-Rad), using a 10 mM CAPS (3 cyclohexylamino-1-propanesulfonic acid) buffer, pH 11, containing 10% (v/v) methanol. Immunoreactive bands were detected after incubation of separate lanes with C-terminal antibodies (dilution 5,000–10,000), followed by treatment with peroxidase-conjugated goat anti-rabbit γ-globulin and staining by incubation with aminoethylcarbazole and H_2O_2. Lane 1 represents the Coomassie blue (C.B.) stained part of the tryptic digest, and lanes 2, 3 and 4 show immunoreactive bands (labelled as bands 1–5), arising after reaction with L16, L28 and L21 (against the oligopeptides 815–828, 889–903 and 1002–1006, respectively).

Table I

Oligopeptides of Na,K-ATPase α-subunit used for production of sequence-specific antibodies

Antibody	Amino acids (No. in sequence)	Sequence[a]
L25	1–13	GRDKYEPAAVSEH-Cys
L16	815–828	Cys-LAYEQAESDIMKRQ
L28	889–903	Cys-NDVEDSYGQQWTYEQ
L21	1002–1016	Cys-IRRRPGGWVEKETYY

[a] Additional N-terminal or C-terminal cysteine residues, used for coupling to keyhole limpet hemocyanin, are indicated as Cys.

4. DISCUSSION

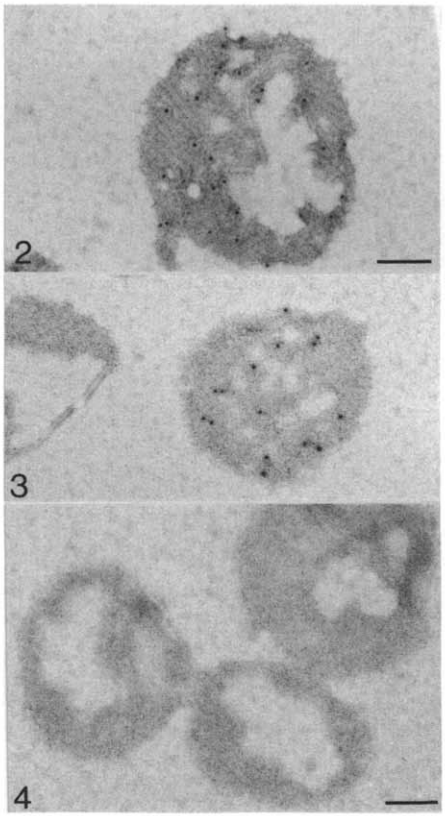
Immunogold double-labeling at the electron microscope level allows direct demonstration of the location of epitopes on isolated Na,K-ATPase membranes and provides topological information about the α subunit, including sidedness of epitopes.

In this study, the N- and C-termini as well as epitopes 815–828 and 889–903 were for the first time directly

co-localized by immunoelectron microscopy on Na,K-ATPase membranes. Based on the generally accepted fact that the N-terminus is localized on the cytoplasmic side [1,2,5,6,8,10,19], double-labeling of epitopes on Na,K-ATPase membranes demonstrated that the C-terminus as well as the epitope 815–828 were also on the cytoplasmic side, whereas 889–903 was identified on the opposite, extracellular side.

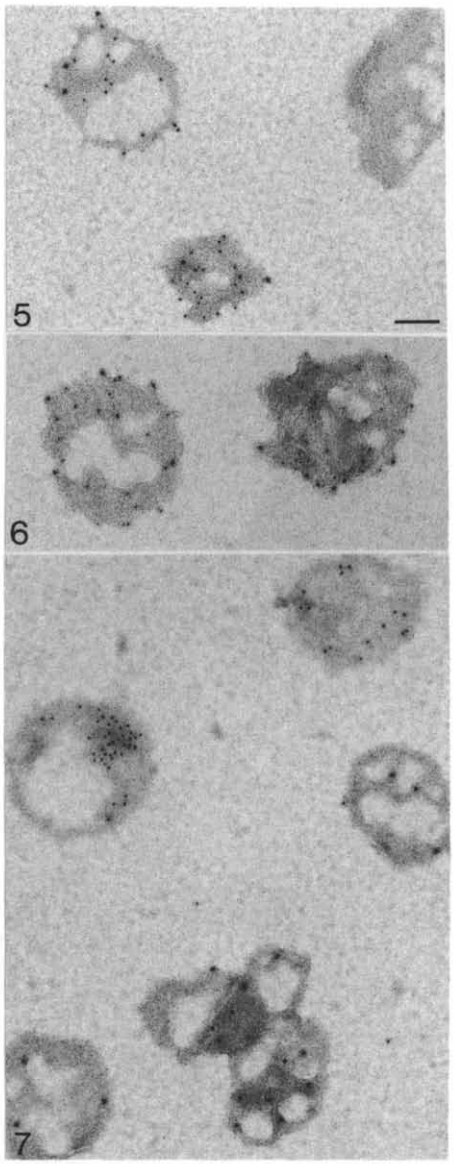
Using monoclonal antibodies to label a pig kidney embryonic cell line by fluorescent microscopy, Ovchinnikov et al. [20] located by fluorescence microscopy peptides 804–841 and the C-terminus on the extracellular and 887–904 on the cytoplasmic side, opposite to our present result. However, because intact cultured cells were used it is difficult in the quoted experiments to exclude cross-reactions between the antibodies and epitopes other than α subunit epitopes on the plasma membrane.

The cytosolic location of the Leu^{815}–Gln^{828} is consonant with the presence of the two transmembrane segments (M5 + M6), corresponding to the preceding, broad hydrophobic peak in the hydropathy plot, and is supported by evidence for cytosolic exposure of the amino acid residue Asn^{831} at which trypsin cleaves to produce the 19 kDa C-terminal fragment [8]. The present observations also suggest that there is only one transmembrane segment (M7) between Gln^{828} and Asn^{889} because it is highly unlikely that such a short polypeptide spans the lipid bilayer three times. On the other hand, it is difficult to decide whether there are one



Figs. 2 and 3. Electron micrographs of Na,K-ATPase membranes immunolabeled with L25(1–13) and L16, respectively. The primary antibodies are detected with 5 nm gold conjugated to protein A. In Fig. 3 adjacent membrane is unlabeled. Bar = 0.1 μm.

Fig. 4. Preabsorption control. Antibody L25 was pre-incubated with excess amount of oligopeptide against which the L25 was raised. The membranes are devoid of gold-labeling. Bar = 0.1 μm.



Figs. 5–7. Immuno double-labeled Na,K-ATPase membranes. Bar = 0.1 μm. Fig. 5 shows N-terminal (L25, 1–13) and sequence 815–828 (L16) antibodies detected with 5 nm and 10 nm gold conjugated to protein A, respectively, and demonstrates co-labeling of the two epitopes on the same (cytoplasmic) faces of the membranes. Fig. 6 shows N- and C-terminal antibodies (L25 and L21, 1002–1016) detected with 5 nm and 10 nm gold conjugated to protein A, respectively, and co-labeling of the two termini on the same (cytoplasmic) faces of the membranes. Fig. 7 demonstrates N-terminal (L25) and sequence 889–903 (L28) antibodies labeled with 10 nm and 5 nm gold conjugated to protein A, respectively; the two epitopes are located on the opposite sides of the membranes.

or three membrane traverses between Gln^{903} and Tyr^{1016}. As such our data are consistent with an eight- or a ten-transmembrane model for the Na,K-ATPase α subunit.

Acknowledgements: We thank Karen Thomsen, Else-Merete Løcke, and Britt Kristholm for technical assistance. This work was supported by the Danish Medical Research Council and the Danish Biomembrane Research Center.

REFERENCES

[1] Shull, G.E., Schwartz, A. and Lingrel, J.B. (1985) Nature 316, 691–695.
[2] Kawakami, K., Noguchi, S., Noda, M., Takahashi, H., Ohta, T., Kawamura, M., Nojima, H., Nagano, K., Hirose, T., Inayama, S., Hayashida, H., Miyata, T. and Numa, S. (1985) Nature 316, 733– 736.
[3] Ovchinnikov, Yu.A., Modyanov, N.N., Broude, N.E., Petrukhin, K.E., Grishin, A.V., Arzamazova, G.S. and Sverdlov, E.D. (1986) FEBS Lett. 201, 237–245.
[4] Shull, G.E., Greeb, J. and Lingrel, J.B. (1986) Biochemistry 25, 8125–8132.
[5] Herrera, V.L.M., Emanuel, J.R., Ruiz-Opazo, N., Levenson, R. and Nadal-Ginard, B. (1987) J. Cell Biol. 105, 1855–1865.
[6] Ovchinnikov, Yu.A., Arzamazova, N.M., Arystarkhova, E.A., Gevondyan, N.M., Aldanova, N.A. and Modyanov, N.N. (1987) FEBS Lett. 217, 269–274.
[7] Lingrel, J.B., Orlowski, J., Shull, M.M. and Price, E.M. (1990) Prog. Nucleic Acid Res. Mol. Biol. 38, 37–89.
[8] Karlish, S.J.D., Goldshleger, R. and Jørgensen, P.L. (1993) J. Biol. Chem. 268, 3471–3478.
[9] Bayer, R. (1990) Biochemistry 29, 2251–2256.
[10] Antolovic, R., Bruller, H.-J., Bunk, S., Linder, D. and Schoner, W. (1991) Eur. J. Biochem. 199, 195–202.
[11] Modyanov, N., Lutsenko, S., Chertova, E., Etremov, R. and Gulyaev, D. (1992) Acta Physiol. Scand. 146, 49–58.
[12] Green, N.M., Alexander, H., Olsen, A., Alexander, S., Shinnick, T.M. Sutcliffe, J.G. and Learner, R.A. (1982) Cell 28, 477–487.
[13] Harlow, E. and Lane, D. (1988) Antibodies: a Laboratory Manual, pp. 553–612, Laboratory, Cold Spring Harbor, NY.
[14] Capasso, J.M., Hoving, S., Tal, D.M., Goldshleger, R. and Karlish, S.J. (1992) J. Biol. Chem.267, 1150–1158.
[15] Jørgensen, P.L. and Collins, J.H. (1986) Biochem. Biophys. Acta 860, 570–576.
[16] Jørgensen, P.L. (1974) Biochim. Biophys. Acta 356, 36–52.
[17] Skriver, E., Maunsbach, A.B. and Jørgensen, P.L. (1981) FEBS Lett. 131, 219–222.
[18] Mannella, C.A. (1984) Science 224, 165–166.
[19] Felsenfeld, D.P. and Sweadner, K.J. (1988) J. Biol. Chem. 263, 10932–10942.
[20] Ovchinnikov, Yu. A., Luneva, N.M., Arystarkhova, E.A., Gevondyan, N.M., Arzamazova, N.M., Kozhich, A.T., Nesmeyanov, V.A. and Modyanov, N.N. (1988) FEBS Lett. 227, 230–234.
[21] Laemmli, U.K. (1970) Nature 227, 680–685.