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Immunochemical identification of exposed regions of the Na^+,K^+-ATPase α-subunit

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A polyclonal antibody against the Na^+,K^+-ATPase holoenzyme was prepared. This antibody recognized native Na^+,K^+-ATPase and inhibited its activity. The peptide fragments corresponding to various regions of the Na^+,K^+-ATPase α-subunit, which were synthesized from the cDNA, were immunoprecipitated with the antibody, and the M32-D75, M158-D197 and M470-V552 fragments (the latter included K508, a putative ATP binding site) were identified as the epitopes. The M267-I442 fragment, which included a phosphorylation site at D376, and the C-terminal one-third of the α-subunit from M615 to the C-terminus, were not detected using this antibody. These results suggest that at least three regions on the Na^+,K^+-ATPase α-subunit, M32-D75, M158-D197 and M470-V552, cover its exposed regions, and that some of them are essential for ATPase activity.

Na^+,K^+-ATPase; Antibody; Immunoprecipitation; cDNA

1. INTRODUCTION

Na^+,K^+-ATPase (EC 3.6.1.3) actively transports sodium ions from the cytoplasmic to the extracellular side, and potassium ions in the opposite direction via the significant affinity changes for the cations that occur during the ATP hydrolysis reaction [1,2]. This enzyme comprises α- and β-subunits [2]. The former, the catalytic moiety, has been proposed to possess seven or eight transmembrane segments, namely M1–M8 from the N-terminus, and three exposed hydrophilic domains on the cytoplasmic side (N-terminus to M1, M2–M3 and M4–M5) on the basis of hydropathy analysis of its primary structure [3–5]. If these hydrophilic regions are located on the surface of the Na^+,K^+-ATPase molecule and are involved in its enzymatic activity, they would be expected to function as epitopes to the Na^+,K^+-ATPase-directed antibody. Therefore, we prepared an antibody which recognized native Na^+,K^+-ATPase and inhibited its activity, and then identified the epitopes by immunoprecipitating the peptide fragments corresponding to various regions of the α-subunit, which were synthesized from the cDNA, with the antibody. We expected this method to produce more precise data than those obtained by Farley et al. [6], who used the Western blotting method combined with proteolysis. The results obtained indicate that the proposed model is essentially correct and, furthermore, that K508, which is present in some putative ATP binding sites, is located on the surface of the Na^+,K^+-ATPase molecule, whereas other such sites and the phosphorylation site may be located in its interior.

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The amino acid positions quoted in this paper are based on those of the amino acids of the human Na^+,K^+-ATPase α-subunit [9].

2. EXPERIMENTAL

2.1. *Antibody preparation*

Canine kidney Na^+,K^+-ATPase, which comprises α_1- and β_1-subunits, was prepared [7]. 1 ml of 0.47 mg/ml purified enzyme with specific activity higher than 25 μmol P_i/min/mg was emulsified in 1 ml of complete Freund's adjuvant and injected into the footpads of Japanese white rabbits weekly for 4 weeks. Each animal was bled during the fifth week. The antiserum was applied to a protein-A column (Wako Chemicals). The immunoglobulin G (IgG) was eluted with 0.1 M sodium citrate (pH 3.0) containing 0.15 M NaCl, neutralized with 3 M imidazole, and concentrated by ultrafiltration.

2.2. *Measurement of Na^+,K^+-ATPase activity*

An aliquot of Na^+,K^+-ATPase (1 μg) with a specific activity of 13–15 μmol P_i/min/mg was preincubated for 1 h at 37°C with 0–100 μg IgG in 0.2 ml reaction mixture without ATP (100 mM NaCl, 10 mM KCl, 4 mM $MgCl_2$, 50 mM imidazole-HCl, pH 7.4, and 1 mM EDTA). The ATP hydrolysis reaction was started by adding 0.8 ml of the reaction mixture, including 2.5 mM ATP to the preincubated mixture. After incubation for 10 min at 37°C, the liberated P_i was measured by the method of Fiske-Subbarow [8].

2.3. *Plasmid construction*

2.3.1. pSNα-36

This plasmid was constructed by inserting the 819-bp *Pst*I fragment of pSNα-100, which encodes the whole α_1-subunit of human Na^+,K^+-ATPase [9], into the *Pst*I site of pSP 64, and linearizing with *Ava*II.

2.3.2. pSNα-391

This plasmid was constructed by inserting the 2,225-bp *Rsa*I fragment of pSNα-36 into the *Sma*I site of pSP 64 and linearizing with *Sac*I (pSNα-391a) or *Eco*RI (pSNα-391b).

2 3.3. pSNα-776

This plasmid was constructed by inserting the 3,046-bp *Acc*I fragment of pSNα-100 into the *Acc*I site of pSP 64 and linearizing with *Afl*II.

2.3.4. pSNα-1328

The 2,331-bp *Afl*II fragment of pSNα-776 was blunt-ended with T4 DNA polymerase and then cut with *Eco*RI. The plasmid pSNα-1328 was constructed by inserting the 1,496-bp *Afl*II–*Eco*RI fragment into pGEM-3Zf(+), which was cut with *Sma*I and *Eco*RI. The A^{1411} in the 1,496-bp fragment was displaced with G^{1411} to construct a new initiation codon using the site-directed mutagenesis system (Amersham) and linearized with *Stu*I (pSNα-1328a) or *Sty*I (pSNα-1328b).

2.3.5. pSNα-1819

This plasmid was constructed by inserting the 1,740-bp *Taq*I fragment of pSNα-100 into the *Acc*I site of pSP 64 and linearizing with *Bam*HI.

The linearized plasmids, pSNα-36, -391a, -391b, -776, -1328a, -1328b and -1819, were used as templates to synthesize RNAs that encode the fragments M32-D75, M158-A286, M158-D197, M267-I442, M470-K661, M470-V552 and M615-Y1023 (C-terminus), respectively.

2.4. *Transcription and translation*

The transcription mixture was prepared using a commercial kit (Promega Corp.) supplemented with a cap analog (m^7GpppG), as described elsewhere [10]. Each synthesized RNA (0.1–0.3 μg) was translated in the rabbit reticulocyte lysate or wheatgerm extract system (Amersham) supplemented with 74–148 kBq [^{35}S]methionine (American Radiolabeled Chemicals Inc.) for 2 h at 30 or 25°C, respectively.

2.5 *Immunoprecipitation*

This experiment was performed using the method of Anderson and Blobel [11] as follows. (A) Aliquots of Na^+,K^+-ATPase (20 μg) were incubated with 0–100 μg IgG for 1 h at 37°C in the presence of different combinations of ligands, as described in the legends of Fig 2 and Table I. Then 20 μl protein A–Sepharose CL-4B beads suspended in water in the ratio of 1:1 was added. Each mixture was rocked for 1 h at room temperature. The resultant enzyme–antibody–protein A complex was washed as described by Anderson and Blobel, and then was incubated in 15 μl Laemmli's sample buffer containing 2% SDS [12] at 70°C for 5 min to dissociate the complex. (B) The translation mixture was divided into 12 μl portions, which were mixed with 2 4 μl 24% SDS, 14.4 μl water, 115.2 μl dilution buffer (1 25% Triton X-100, 190 mM NaCl, 6 mM EDTA, 50 mM Tris-HCl, pH 7.4) and 8 μl antiserum, and kept in a refrigerator overnight. Each solution was centrifuged at 15,000 rpm for 3 min in a microcentrifuge (Tomy Seiko Co. Ltd.). The resulting supernatant was mixed with 12 μl protein A–Sepharose CL-4B bead suspension and rocked for 2 h. The peptide–antibody–protein A complex was washed and dissociated as above, except that dissociation conditions were changed to 30°C for 10 min to avoid the aggregation of synthesized peptides.

2.6. *SDS-gel electrophoresis*

Peptides with molecular masses greater than 15 kDa were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using the method of Laemmli with a 10.5 or 13.5% separating gel [12], whereas those with molecular masses below 15 kDa were electrophoresed using the method of Schäger and Jagow [13] with a 12% separating gel without glycerol. After electrophoresis, each gel was stained with Coomassie brilliant blue and destained. The densities of the stained bands were measured with a densitometer coupled to a digitizer (ACI Japan) For fluorography, the gels were soaked in 0.5 M sodium sarythylate, dried and kept at −70°C on X-ray film for 2–7 days.

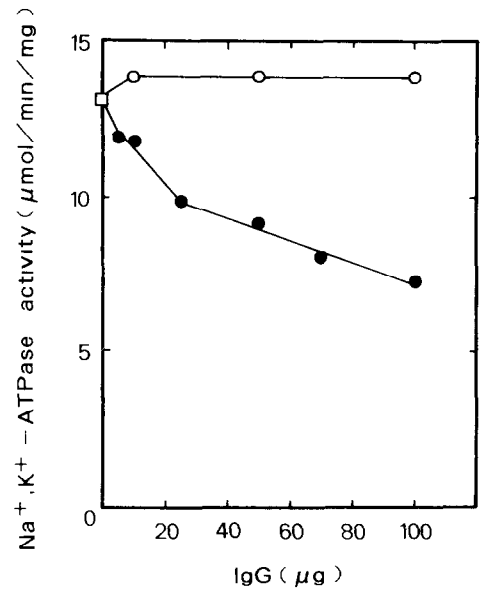

Fig. 1. Effect of IgG on Na^+,K^+-ATPase activity. The experimental procedure is described in section 2. □, no antibody added; ○, IgG from control serum; ●, IgG from antiserum containing the Na^+,K^+-ATPase-directed antibody.

3. RESULTS AND DISCUSSION

3.1. *Effect of the antibody on Na^+,K^+-ATPase*

The IgG isolated from the antiserum raised against Na^+,K^+-ATPase inhibited the ATPase activity (Fig. 1). The amount of IgG that gave half-maximal inhibition of Na^+,K^+-ATPase activity was approximately 100 μg. Fig. 2 shows the binding of the IgG to native Na^+,K^+-ATPase. After incubation of native Na^+,K^+-ATPase with the Na^+,K^+-ATPase-directed antibody, the α-subunit dissociated from the enzyme–antibody complex was observed by SDS-PAGE (Fig. 2, lane 2). The amount of the α-subunit reached half the level of the control α-subunit at approximately 70 μg IgG in the presence of Na^+, K^+ and Mg^{2+} (data not shown). Different combinations of ligands did not significantly change the binding level of the antibody to the enzyme (Table I). These results indicate that the Na^+,K^+-ATPase-directed antibody recognized native Na^+,K^+-ATPase and inhibited its activity, although it could not distinguish between the ligand-induced conformations of the enzyme. Next, we attempted to identify the epitopes to the antibody by immunoprecipitating peptide fragments corresponding to various regions of the α-subunit.

3.2. *Immunoprecipitation of the synthesized peptide fragments with the antibody*

The plasmids encoding various regions of the α-subunit were subcloned, linearized and expressed using an in vitro transcription and translation system. The [^{35}S]methionine-labeled peptide fragments were incubated with the antibody in the presence of 0.4% SDS.

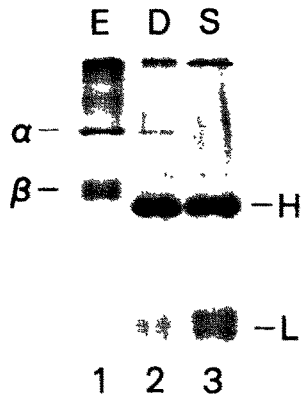

Fig. 2. Binding of the Na^+,K^+-ATPase-directed antibody to native Na^+,K^+-ATPase. The experimental procedure is described in section 2. 20 μg purified Na^+,K^+-ATPase was untreated (E), treated with 20 μg IgG from the antiserum (D) or the control serum (S) in the reaction mixture without ATP, and electrophoresed with a 10.5% separating gel and then stained with Coomassie brilliant blue. α and β denote the α- and β-subunits of Na^+,K^+-ATPase, respectively. H and L denote the heavy and light chains of IgG, respectively.

Table I

Binding of the Na^+,K^+-ATPase-directed antibody to native Na^+,K^+-ATPase in the presence of different combinations of ligands

Ligands	Relative amount of α-subunit
100 mM NaCl	1.0
100 mM KCl	0.88 ± 0.16
10 mM $MgCl_2$	0.98 ± 0.16
10 mM ATP	1.01 ± 0.04
10 mM P_i	1.0 ± 0
1 mM Ouabain	0.84 ± 0.04
100 mM NaCl, 4 mM $MgCl_2$, 2 mM ATP	0.81 ± 0.06
4 mM $MgCl_2$, 2 mM P_i	0.95 ± 0.06
4 mM $MgCl_2$, 2 mM P_i, 1 mM Ouabain	0.80 ± 0.05
100 mM NaCl, 10 mM KCl, 4 mM $MgCl_2$	0.82 ± 0.06

20 μg Na^+,K^+-ATPase was preincubated with 1 mM EDTA, 50 mM imidazole-HCl (pH 7.4) and various ligands, which are listed in the table, for 15 min at 37°C, and then incubated with 20 μg IgG for 1 h at 37°C. The amount of α-subunit dissociated from the enzyme–antibody complexes, which were formed in the presence of various ligands, was compared with that of α-subunit in the presence of 100 mM NaCl. Each value represents the mean ± S.D. of duplicate determinations.

The resultant peptide–antibody complex was precipitated with protein A–Sepharose CL-4B beads, dissociated with 2% SDS, and analyzed using SDS-PAGE and fluorography. The M32-D75 fragment between the N-terminus and M1, and the M158-A286 and M158-D197 fragments between M2 and M3, were detected with the antibody (Fig. 3, lanes 1–6). The M470-V552 and M470-K661 fragments that included K508, a putative ATP binding site between M4 and M5 [9,14], were also detected (Fig. 3, lanes 7, 8, 11 and 12); however, neither the M267-I442 fragment that included D376, a phosphorylation site [9,15,16], nor that from M615 down to Y1023 (C-terminus) were detected (Fig. 3, lanes 9, 10, 13 and 14). The latter was also not detected with the antibody against the α-subunit, which was isolated from canine kidney Na^+,K^+-ATPase denatured with SDS [10]. The results obtained with the M1-D42 fragment, the N-terminal region, were unclear (data not shown). From the results summarized in Fig. 4, we concluded that the M32-D75, D158-D197 and M470-V552 regions cover the exposed regions of the Na^+,K^+-ATPase α-subunit, and that some of them are essential for ATPase activity. This is supported by the observation that the non-epitope regions, M267-I442 and M615-Y1023, hardly overlap with the sites accessible to trypsin and chymotrypsin during limited proteolysis [9,17,18] (Fig. 4) and, to some extent, is consistent with the conclusions of Farley et al. [6] and Ball and Loftice [19] who

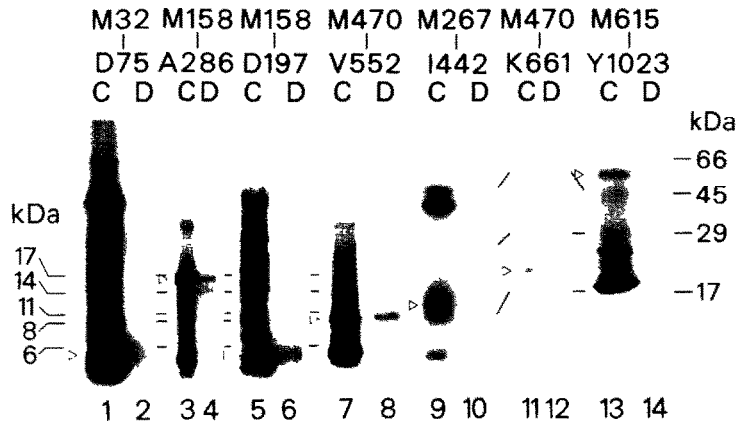

Fig. 3 Immunoprecipitation of the synthetic peptide fragments with the Na^+,K^+-ATPase-directed antibody. The experimental procedure is described in section 2. The translation mixtures were electrophoresed with a 13.5% separating gel before (C) or after (D) treatment with the antibody. The molecular masses of the M32-D75, M158-A286, M158-D197, M470-V552 fragments, which were synthesized in the wheatgerm system, and those of the M267-I442, M470-K661 and M615-Y1023 fragments, which were synthesized in the rabbit reticulocyte lysate serum, were calculated as 5.3, 15, 4.8, 10, 21, 23 and 49 kDa, respectively. Arrowheads indicate the positions of translation products. The positions and molecular masses of standard proteins are indicated in kDa on the vertical axes of left and right sides for lanes 1–8 and for lanes 9–14, respectively.

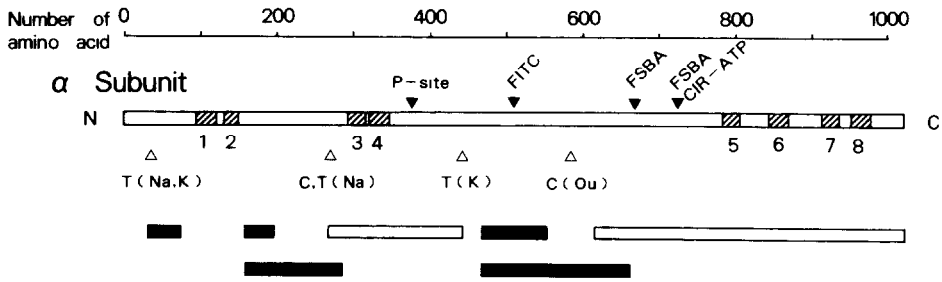

Fig. 4 Epitope map of the Na^+,K^+-ATPase α-subunit The black and white areas represent epitope and non-epitope regions, respectively. The hatched areas are putative transmembrane segments. ▼ denotes the phosphorylation site at D376 (P-site) [9,15,16], the fluorescein isothiocyanate (FITC) binding site at K508, 5′-(*p*-fluorosulfonyl)benzoyladenosine (FSBA) binding sites at C663 and K726, or γ-[4-(*N*-2-chlorethyl-*N*-methylamino)]benzylamide ATP (CIR-ATP) binding sites at D716 and D721 [14,20–22]; △ denotes K37, R269, L273, R445 and near 600, which are the sites accessible to trypsin (T) and chymotrypsin (C) during limited proteolysis in the presence of NaCl (Na), KCl (K) or ouabain (Ou) [9,17,18].

used the chemically synthesized peptides and Na^+,K^+-ATPase-directed antibodies.

In some putative ATP binding sites identified by chemical modification [14,20–22], K508 was included in the M470–V552 fragment which was detected with the antibody (Fig. 4). This suggests that K508 is located on the surface of the Na^+,K^+-ATPase molecule, whereas other such binding sites are in its interior. Furthermore, the phosphorylation site at D376 and the C-terminal one-third of the α-subunit, from M615 to the C-terminus, may also be located in the interior of the molecule, as these fragments were not detected with the antibodies and were not digested with the proteases.

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REFERENCES

[1] Homareda, H., Nagano, Y. and Matsui, H. (1991) J. Biochem. 109, 70–77.
[2] Jørgensen, P.L. (1982) Biochim. Biophys Acta 694, 27–68.
[3] 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.
[4] Shull, G.E., Schwartz, A. and Lingrel, J.B. (1985) Nature 316, 691–695.
[5] Krämer, T., Antolovic, R., Brüller, H.-J., Richter, U., Scheiner-Bobis, G. and Schoner, W (1991) in: The Sodium Pump. Recent Developments (Kaplan, J.H and Weer, P.D., eds.) pp. 253–257, The Rockefeller University Press, New York.
[6] Farley, R.A., Ochoa, G T. and Kudrow, A. (1986) Am. J Physiol. 250, C896–C906.
[7] Hayashi, Y., Takagi, T, Maezawa, S. and Matsui, H. (1983) Biochim. Biophys. Acta 748, 153–167.
[8] Fiske, C. and SubbaRow, Y.H. (1935) J. Biol. Chem. 66, 375–400.
[9] Kawakami, K., Ohta, T, Nojima, H and Nagano, K. (1986) J. Biochem. 100, 389–397.
[10] Homareda, H., Kawakami, K., Nagano, K. and Matsui, H. (1989) Mol. Cell. Biol. 9, 5742–5745.
[11] Anderson, D.J. and Blobel, G. (1983) Methods Enzymol. 96, 111–120.
[12] Laemmli, U.K. (1970) Nature 227, 680–685.
[13] Schägger, H. and Von Jagow, G. (1987) Anal. Biochem. 166, 368–379.
[14] Xu, K. (1989) Biochemistry 28, 5764–5772.
[15] Post, R L. and Kume, S. (1973) J. Biol. Chem. 248, 6993–7000.
[16] Castro, J. and Farley, R.A. (1979) J Biol Chem. 254, 2221–2228.
[17] Jørgensen, P.L. and Farley, R.A. (1988) Methods Enzymol. 156, 291–301.
[18] Jørgensen, P.L. and Collins, J.H (1986) Biochim. Biophys. Acta 860, 570–576.
[19] Ball, W.J. and Loftice, C.D. (1987) Biochim. Biophys. Acta 916, 100–111.
[20] Ohta, T., Nagano, K. and Yoshida, M (1986) Proc. Natl. Acad. Sci. USA 83, 2071–2075.
[21] Ovchinnikov, Y.A., Dzhandzhugazyan, K.N , Lutsenko, S.V., Mustayev, A.A. and Modyanov, N.N (1987) FEBS Lett. 217, 111–116.
[22] Pedemonte, C H. and Kaplan, J.H. (1990) Am. J. Physiol. 258 (Cell Physiol. 27), C1–C23.