Biochimica et Biophysica Acta, 1059 (1991) 157–164

ADONIS 0005272891001923

BBABIO 43457

The 'lysine cluster' in the N-terminal region of Na^+/K^+-ATPase α-subunit is not involved in ATPase activity

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(Received 31 January 1991)

Key words: ATPase, Na^+/K^+-; Amino-terminus; Lysine cluster; Deletion mutant; (*R. catesbiana*)

The α-subunit of the Na^+/K^+-ATPases from several animal species have markedly similar amino acid sequences. However, the N-terminal sequences of the α-subunit are rather divergent except for lysine-rich sequences, the 'lysine cluster'. Here we report that the α-subunit from frog (*Rana catesbiana*) has an N-terminal sequence with the 29 amino acid residues shorter than that of the *Xenopus* α-subunit deduced from its cDNA and hence lacks the 'lysine cluster'. Nevertheless, the *Rana* enzyme still exhibits ATPase activity. The ATP-dependent Na^+ transport activity of the *Rana* enzyme was similar to that of the dog enzyme, which contains the 'lysine cluster'. Moreover, the *Torpedo* α-subunits deprived of the 'lysine cluster' by means of two gene deletions showed the same Na^+/K^+-ATPase activities as that of the wild type when expressed in *Xenopus* oocytes from their mRNAs. These results strongly suggest that the 'lysine cluster' in the N-terminal region of the α-subunit is not involved in the ATPase and ion transport activities. Since an active α-subunit was translated in *Xenopus* oocytes from mRNA lacking the N-terminal region including the 'lysine cluster', these regions were proved not to function as a membrane insertion signal sequence.

Introduction

The sodium- and potassium-activated adenosine triphosphatase (Na^+/K^+-ATPase), purified from the plasma membranes of a variety of mammalian tissues and the other sources, is composed of a large subunit (α) and a small subunit (β). The α-subunit contains the binding sites for Na^+, K^+ [1], nucleotide [2], cardiac glycoside [3] and vanadate [4]. The complete amino-acid sequences of the α-subunits of Na^+/K^+-ATPase from brine shrimp (*Artemia*) [5], *Drosophila* [6], *Torpedo* [7], sheep [8], chicken [9], rat [10], and man [11] have been deduced from the sequences of their cDNAs. The amino-acid sequences of the α-subunits from the various animals are markedly similar to each other, but the N-terminal regions are divergent in their length and amino acid composition. However, in the N-terminal region, a lysine-rich sequence, the 'lysine cluster', is exceptionally well conserved among these α-subunits [12–16].

Shull et al. [8] suggested that the hydrophilic N-terminal domain including the 'lysine cluster' functions as an ion-selective barrier that controls access of ions to cation-binding sites. This 'lysine cluster' is also conserved in the N-terminus of the other cation-transporting ATPases, such as the H^+/K^+-ATPase [17] and the H^+-ATPase [18]. Although there is no similarity in this region between the Na^+/K^+-ATPase and the Ca^{2+}-ATPase, the latter contains another cluster of four charged glutamic acid residues instead of the 'lysine cluster' [19]. Jørgensen and Collins [20] described that the tryptic cleavage at the specific lysine residue in the N-terminal region alters both the binding of ions and the conformational transitions. (For *Xenopus* α-subunit, the residue corresponds to the 38th lysine. Hereafter, the number of the amino-acid residue indicates the position from the initiation methionine of the *Xenopus* α-subunit unless otherwise specified.) Thus, researchers insisted that the N-terminal region including the 'lysine cluster' is functionally important, despite

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its divergence. Recently, Baxter-Lowe et al. [5] claimed that the N-terminal region of the α-subunit is not essential for ATPase activity, based on the observation that the deduced sequence of the α-subunit of the *Artemia* (San Francisco Bay Brand) Na^+/K^+-ATPase lacks 20–23 N-terminal residues from the initiation methionine which are present in other α-subunits. Morohashi and Kawamura also reported that the α-subunit from the *Artemia* (Tetra Brand) enzyme lacks 19 N-terminal residues [16]. The *Artemia* sequences are determined in those of the different brand, and then any differences in the N-terminal sequence are observed among them. However, these shorter α-subunits still contain the 'lysine cluster'. Thus the functional role of the N-terminal region of the α-subunit, particularly the 'lysine cluster', is still ambiguous.

Here, we describe the lack of the 29 N-terminal residues including 'lysine cluster' in the α-subunit of the *Rana* kidney Na^+/K^+-ATPase that is capable of catalyzing ATP-dependent translocation of Na^+ ions. Moreover, the *Torpedo* enzymes lacking the 'lysine cluster', the gene deletion products, showed the same Na^+/K^+-ATPase activity as that of the wild-type enzyme.

Materials and Methods

Materials. Frogs (*Rana catesbiana*) were obtained from Saitama Experimental Animal Center. ^{22}Na (3.7 GBq/mg Na) was purchased from Amersham International. Soybean phospholipids (Asolectin) purchased from Associated Concentrates, Woodside, NY, were partially purified according to Kagawa and Racker [21]. All other reagents used were analytical grade.

Preparation of membrane-bound Na^+/K^+-ATPase. Partial purification of Na^+/K^+-ATPase from frog kidney was carried out by the procedures of deoxycholate (DOC)/sodium dodecyl sulfate (SDS) treatment essentially as described by Jørgensen [22] and modified by Hayashi et al. [23]. 35 coupled kidneys were obtained from *Rana*. The kidneys were minced with scissors and homogenized with a Polytron (Kinematica, PT10-35) in 10 vol. of a homogenizing medium containing 250 mM sucrose, 50 mM imidazole-HCl (pH 7.4), 5 mM EDTA, and 1 mM dithiothreitol (DTT). The homogenate was centrifuged at $3500 \times g$ for 10 min at 4°C. The supernatant was pooled and filtered through cheesecloth and then centrifuged at $55\,000 \times g$ for 30 min at 4°C. The pellet (microsomal fraction containing plasma membrane) was suspended in the homogenizing medium. The resulting crude microsome fraction was further purified by repeated centrifugation at $3500 \times g$ for 5 min to pellet non-microsomal proteins. The supernatant containing the microsomes was stored at −20°C until they were subsequently treated with DOC and SDS. The microsomal fraction (5.5 mg/ml) was incubated with 3 mg/ml of DOC containing 500 mM NaCl, 40 mM KCl, 25 mM imidazole-HCl (pH 7.0), 1 mM EDTA-Tris, and 10% (w/v) sucrose at 4°C for 30 min and then centrifuged at $100\,000 \times g$ for 30 min. Glycerol (20%) was added to the supernatant prior to the dilution in an equal volume of buffer A (25 mM imidazole-HCl (pH7.4) and 1 mM EDTA-Tris). This suspension was kept on ice for 45 min and then centrifuged at $100\,000 \times g$ for 90 min at 4°C. The resulting pellets were washed and resuspended in the buffer A (DOC-enzyme). The 1.4 mg/ml of DOC-enzyme was further treated with 0.45 mg/ml of SDS for 30 min at room temperature and purified by centrifugation on a discontinuous sucrose gradient as described by Jørgensen [22]. The interface layer between 15 and 30.5% (w/v) of sucrose which contains the enzyme was recovered (DOC/SDS-enzyme).

Isolation of the α-subunit from Na^+/K^+-ATPase. The purified *Rana* enzyme was denatured in a solution containing 2% SDS and 1 mM EDTA, and the α-subunit was isolated from the enzyme by gel filtration chromatography with a column of SepharoseCL-4B (2.5×83 cm), equilibrated with 0.1% SDS, 100 mM imidazole/HCl (pH 7.4) and 1 mM EDTA at room temperature. The fractions including the α-subunit were pooled and concentrated in a dialysis tube covered with poly(vinylpyrolidone). The purity of the protein was assessed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

Amino acid sequence analysis. N-terminal sequence of the α-subunit was analyzed by a gas phase protein sequenator (Applied Biosystems Model 470A system). The isolated α-subunit (110 μg, 1 nmol) was directly applied on the sequenator or after transblotting onto a poly(vinyldiphenylfloridone) (PVDF) membrane. In the latter method, the Coomassie-blue-stained band corresponding to the α-subunit was carefully cut out from the membrane and applied on the sequenator without polybrene treatment [24].

ATPase assay. The ATPase activity of the membrane-bound enzyme was measured in a medium containing 100 mM NaCl, 10 mM KCl, 5 mM $MgCl_2$, 3 mM ATP (sodium salt), and 50 mM Tris-HCl (pH 7.4) at 37°C. The reaction was initiated by adding enzyme (8 μg/ml) to the assay medium, and it was terminated by the addition of 1 M H_2SO_4 containing 1.25% ammonium molybdate. The P_i was determined by the method of Fiske and SubbaRow [25]. ATPase activity coupled to the ion transporting activity of the reconstituted enzyme was measured in a medium consisting of 118 mM Hepes-Tris (pH 7.4), 1 mM EDTA-Tris, 2 mM $MgCl_2$, 2 mM NaCl and 0.1 mM ouabain. Ouabain was added to inhibit the non-incorporated enzyme. ATPase activities of the reconstituted enzyme was determined by measuring P_i using the above method in the presence of 5% SDS added after the enzyme reaction.

Preparation of reconstituted liposomes with Na^+/K^+-ATPase. Proteoliposomes were reconstituted with the *Rana* enzyme by the method of Karlish and Pick [26]. The purified enzyme at a protein concentration of 1 mg/ml, 125 mM Hepes-Tris (pH 7.4), 5 mg/ml cholate (sodium salt), 0.02 mM EDTA (sodium salt), and 110 mM KCl were sequentially added and mixed. The mixture was left on ice for 5 min and then centrifuged at $500\,000 \times g$ for 10 min (Beckman TL-100 Ultracentrifuge). The supernatant was mixed with sonicated liposomes (25 mg/ml of Asolectin) by a Branson sonicator. The mixture was quickly frozen in a solid CO_2/ethanol bath, thawed at room temperature, and then sonicated briefly. To remove cholate, the proteoliposome suspension was passed through a column of Sephadex G-50 (0.8×20 cm) which was pre-equilibrated with 125 mM Hepes-Tris (pH 7.4). The fractions that eluted at the void volume were collected and concentrated by centrifugation.

Na^+ transport assay. Na^+ influx into the reconstituted proteoliposomes was measured in 125 mM Hepes-Tris (pH 7.4) as described by Karlish and Pick [26]. The proteoliposomes were preincubated for 2 min at 30°C. The transport assay was initiated by mixing 100-μl aliquots of the proteoliposomes with 900 μl of the pre-warmed assay medium containing 118 mM Hepes-Tris (pH 7.4), 1 mM EDTA-Tris, 2 mM $MgCl_2$, 2 mM $^{22}NaCl$ ($1.8 \cdot 10^5$ Bq/ml), and 0.1 mM ouabain. After incubation for 4.5 min at 30°C, 3 mM of ATP (Tris salt) was added. Aliquots (100 μl) of the suspension were withdrawn at appropriate times and passed through membrane filters (Sartorius, 0.45 μm pore size), that had been soaked in the stopping solution (10 mM Hepes-Tris (pH 7.4) containing 150 mM NaCl). The filters were washed with two 4 ml aliquots of the stopping solution, dried, and then counted for radioactivity by a gamma counter (Packard 5650).

Construction of the deletion mutants of the α-subunit. The plasmids pSPT(α) and pSPT(β) containing the cDNA encoding *Torpedo* α and β subunits, respectively, were constructed by previously described methods [27]. Two deletion mutants, pSPT(Δ K28) and pSPT(Δ T29), were constructed from pSPT(α). Fig. 1 is a schematic representation of the construction of these mutants. The DNA fragment was deleted at the *Eco*RI site in the vector and at the *Afl*II site (nucleotides 223) in the coding region from pSPT(α) and followed by purification of the larger fragment. The mutant pSPT(Δ K28) was replaced by synthetic DNAs, 5′-AATTCAAACTTTTATTTATAATCTAGTCGCATTTGGAAGGAAAATGAAGACTACGGATCTAGATGAAC-3′ and 3′-GTTTGAAAATAAATATTAGATCAGCGTAAACCTTCCTTTTACTTCTGATGCCTAGATCTACTTGAATT-5′, and pSPT(Δ T29) was also replaced by 5′-AATTCAAACTTTTATTTATAATCTAGTCGCATTTGGAAGGAAAATGACTACGGATCTAGATGAAC-3′ and 3′-GTTTGAAAATAAATATTAGATCAGCGTAAACCTTCCTTTTACTGATGCCTAGAT-CTACTTGAATT-5′. The constructed mutants pSPT(Δ K28) and pSPT(Δ T29) correspond to deletion mutants of the *Xenopus* α-subunit which lack the nucleotides encoding the first 29 and 30 amino acid residues, respectively, from the initiation methionine deduced from the cDNA [28].

Expression of the deletion mutant genes. mRNAs were synthesized in vitro by using SP6 RNA polymerase [29,30]. Microinjection of mRNAs into *Xenopus* oocytes, identification of translation products, and preparation and ATPase assay of microsomes from oocytes were carried out as described previously [27].

Other methods. Protein was determined by the method of Lowry et al. [31], as modified by Peterson [32], with bovine serum albumin as the standard. SDS-PAGE was carried out by the method of Laemmli [33] using a 10% separating gel. Immunoblotting was performed with the procedure described by Burnette [34]. After SDS-PAGE, the gel was transblotted onto the PVDF membrane by a semi-dry blotting system (Bio-Rad Laboratories) at 10 V for 60 min. The blotting membrane was rinsed in a medium containing 500 mM NaCl and 20 mM Tris-HCl (pH 8.0) (TBS) and then blocked with TBS containing 3% gelatin at 37°C for 30

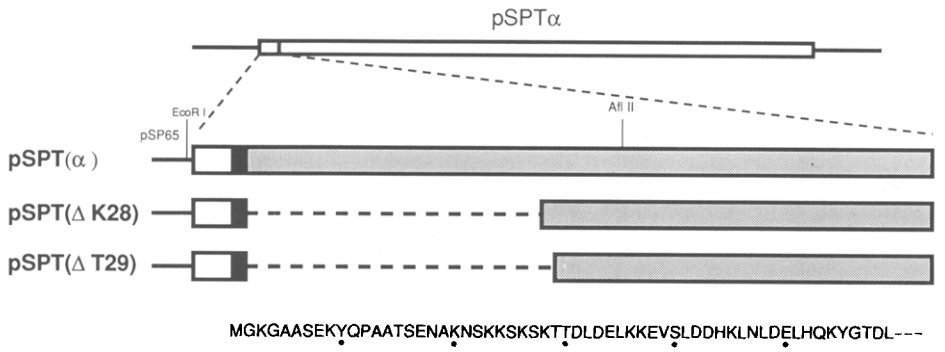

Fig. 1. Construction of the deletion mutant from the *Torpedo* Na^+/K^+-ATPase α gene in the plasmid pSPT(α).

min. The blocked membrane was soaked in antisera (×50) against the dog kidney Na^+/K^+-ATPase, subsequently soaked in horseradish peroxidase (HRP)-conjugated goat-anti-rabbit IgG (Bio-Rad Laboratories, ×2000) as the second antibody at 37°C for 60 min, and then developed with a color-development reagent (4-chloro-1-naphthol) to visualize the protein bands.

Results

Purification of Na^+/K^+-ATPase from the Rana kidney microsome

To determine the optimal detergent concentration for solubilization of the enzyme from *Rana* kidney microsomes, 5 mg/ml of the fraction was incubated with various concentrations (1–5 mg/ml) of DOC. DOC at 3 mg/ml was optimum, while higher DOC concentrations reduced the enzyme activity (data not shown). Then, the DOC-enzyme at a protein concentration of 1.4 mg/ml was treated routinely with 0.45 mg/ml of SDS. The SDS-enzyme was recovered from a discontinuous sucrose gradient at the interface layer between 15% (w/v) and 30.5% (w/v) sucrose. The final preparation had a specific activity of 600–800 μmol P_i/mg protein/h ($n = 3$; activity yield = 9–11%), which was relatively lower than that of dog kidney (1500–2000 μmol P_i/mg protein/h). The SDS-PAGE pattern of the *Rana* enzyme showed some bands and two bands with molecular masses of 110 kDa and 66 kDa, respectively, were identified as Na^+/K^+-ATPase by immunoblotting (Fig. 2). The former was identified to be the α-subunit and the latter to be the β-subunit when the SDS-PAGE separated proteins were subjected to immunoblotting with antisera against dog kidney Na^+/K^+-ATPase (lane 2 in Fig. 2). Since the α-subunit migrated as a single band even on a 5% gel (data not shown), on which two isoforms of the α-subunit (α1 and α2) are clearly distinguished by a difference in electrophoretic mobility [35], the *Rana* enzyme was homogeneous and not digested into peptide fragments by unknown proteinases during the purification procedure.

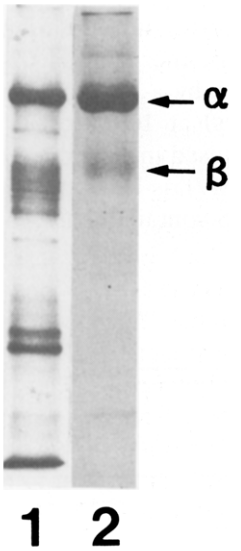

Fig. 2. Immunoblotting of the Na^+/K^+-ATPase from *Rana* kidney. The blots were prepared from samples electrophoresed in 10% polyacrylamide gels. Lane 1 on the left side was stained with Coomassie brilliant blue. Lane 2was soaked in an antiserum against the dog kidney Na^+/K^+-ATPase, followed by staining with HRP-conjugated goat-anti-rabbit IgG as the second antibody.

Isolation of the α-subunit from the purified Na^+/K^+-ATPase

The α-subunit was isolated by either gel-filtration after solubilization with SDS or blotting to a PVDF membrane after SDS-PAGE. Fig. 3 shows the separation of the α-subunit of the *Rana* kidney Na^+/K^+-ATPase by Sepharose CL-4B gel-filtration in the presence of SDS. As shown in the SDS-PAGE pattern of Fig. 3, the fractions under the bar (fraction Nos. 126–132) were collected as the purified α-subunit and were not contaminated with any other proteins. The fraction peaking at No. 154 was identified as the β-subunit by SDS-PAGE. The small peak eluting before the α-subunit was contaminated by a protein of about 200 kDa, and the peak eluting after the β-subunit fractions was contaminated with phospholipid. The fractions containing the pure α-subunit were pooled, concentrated to a minimum volume (1 mg in 500 μl) for amino-acid sequence analysis, and stored at 4°C until used.

N-terminal sequence analysis of the α-subunit

The N-terminal sequence analysis was carried out on the isolated *Rana* α-subunit directly applied onto the protein sequenator; the results are shown in Fig. 4. The sequence of 33 amino-acid residues in the N-terminal region of the α-subunit was determined as follows: NH_2-Lys-Glu-Lys-Asp-Met-Asp-Glu-Leu-Lys-Lys-Glu-Val-Ser-Leu-Glu-Asp-His-Lys-Leu-Ser-Leu-Glu-Glu-Leu-His-Arg-Lys-Tyr-Gly-Thr-Asp-Leu-Thr-. The same result was obtained with the α-subunit blotted onto the PVDF membrane. The initial yield of the PTH-amino acid was calculated to be in the range of 30–40% which was a reasonable yield for a membranous protein [7,16]. These results also suggest that the α-subunit of the *Rana* enzyme is homogeneous and not a mixture of digested and undigested α-subunits. The 33 residues for the N-terminal sequence of the *Rana* α-subunit shows 89% similarity with the sequence from the 30th lysine to the 62th glutamine of the *Xenopus* α-subunit (see Fig. 5), indicating that the N-terminus of the processed *Rana* α-subunit corresponds to the 30th lysine in the sequence of the *Xenopus* α-subunit.

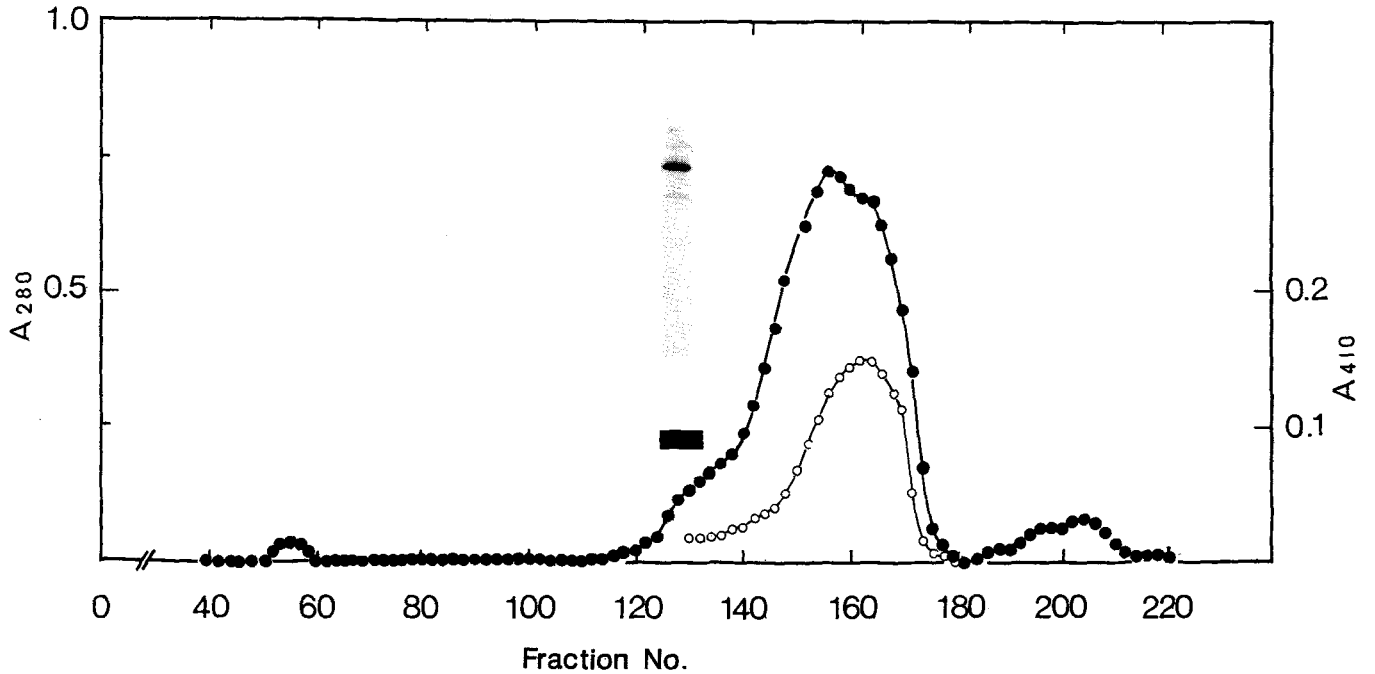

Fig. 3. Separation of the α-subunit by Sepharose CL-4B gel-filtration. The purified enzyme (7.1 mg of protein) was denatured with 2% SDS and applied on the column as described under 'Materials and Methods'. Protein and phospholipid were monitored by measuring the absorbances at 280 nm (•) and 410 nm (○), respectively. The bar indicates the fractions pooled for the α-subunit. Fraction volume, 2.5 ml; flow rate, 0.5 ml/min.

Homology between other Na^+/K^+-ATPase α-subunits

Fig. 5 shows the amino acid sequences of the α-subunits of various animals deduced from their cDNAs. The position of the N-termini of mammalian [13–15] and *Artemia* (Tetra) [16] α-subunit, which have been determined by protein sequencing techniques, are indicated by arrows. The N-terminus of mammalian and *Artemia* (Tetra) enzymes is glycine at the 6th residue, and alanine at the 20th, respectively. Thus it is obvious that these α-subunits are processed differently, but the 'lysine cluster', which is boxed in Fig. 5, is well conserved among them. However, the N-terminal amino-acid residue of the α-subunit of the *Rana* enzyme corresponds to the 30th lysine residue in the sequence of the *Xenopus* α-subunit, and hence, the 'lysine cluster' is apparently deleted.

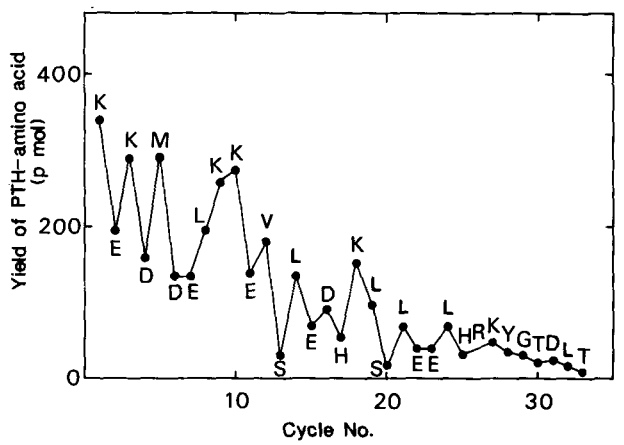

Fig. 4. Recovery of PTH-amino acids at each cycle during the amino-acid sequence analysis of the α-subunit of the *Rana* enzyme (1 nmol) by a gas-phase protein sequenator. HPLC peak heights were converted to pmol using authentic PTH-amino acids as standards.

Active and passive ^{22}Na influx

To estimate the Na^+ translocating activity mediated by the *Rana* enzyme, the purified enzyme was reconstituted into proteoliposomes at a protein to lipid ratio of 1:25 (w/w). As shown in Fig. 6, the influx of the Na^+ into the liposomes was observed when ATP was added. The Na^+/K^+-ATPase activities of the reconstituted liposomes with the *Rana* enzyme and the dog enzyme were 12.8 nmol P_i/mg of protein per min and 37.4 nmol P_i/mg protein per min, respectively. The (K^+ + ATP)-dependent Na^+ influx into the proteoliposomes was 8.8 nmol/ATPase activity per min by the procedure described under 'Materials and Methods'. This flux rate was almost similar to that of the dog kidney enzyme (7.2 nmol/ATPase activity per min), which contains the 'lysine cluster'. For passive transport measurement, ATP (Tris salt) was omitted at the first stage and the passive Na^+ influx at the all stages was indicated by the open circles in Fig. 6.

Expression of the deletion mutant genes of the α-subunit

We further examined whether the product of the 'lysine-cluster'-deleted mutant gene has a ouabain-sensitive ATPase activity using the *Xenopus* oocyte expression system [27]. We constructed two 'lysine clus-

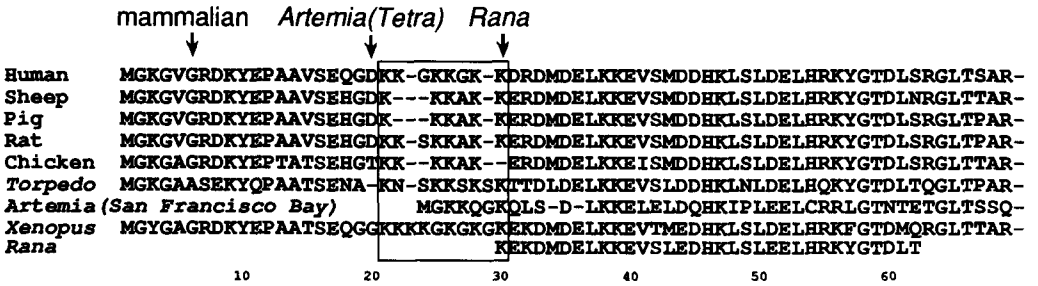

Fig. 5. N-terminal sequences of the Na^+/K^+-ATPase α-subunit deduced from cDNAs which were derived from HeLa cells [11], sheep kidney [8], pig kidney [37], rat kidney [10], chicken kidney [9], *Torpedo* electric organ [7], *Xenopus* kidney [28] and *Artemia* (San Francisco Bay) [5], respectively. The *Rana* enzyme sequence (present results) was aligned with those sequences. The 'lysine clusters' are boxed. The predicted amino-acid sequence was aligned with a gap (−) to obtain maximal homology. The numbers correspond to the position from the initiation methionine of the *Xenopus* α-subunit amino-acid sequence deduced from its cDNA. The arrows indicate the positions of the N-terminal residue revealed by the protein sequence determinations of mammalian [13–15], *Artemia* (Tetra) [16] and *Rana* (present results) enzyme, respectively. Since the *Artemia* sequences are determined in those of a different brand, some differences in the N-terminal sequence are observed among them.

ter' deletion mutant α cDNAs, pSPT(Δ K28) and pSPT(Δ T29). Fig. 1 is a schematic diagram illustrating the construction of these mutants, which deleted the nucleotides coding for amino acid residues from the initiation methionine to the 'lysine cluster'. These two mutants were expressed in *Xenopus* oocytes by microinjection of their mRNA together with mRNA for the β-subunit. The mRNA for the β-subunit is required for the functional expression of the Na^+/K^+-ATPase in the *Xenopus* oocytes [27]. Wild-type pSPT(α) was expressed as a positive control and non-injected oocytes were used in order to assess the endogenous Na^+/K^+-ATPase activity. The polypeptides, which were translated by these mRNAs, were identified by radiolabeling and immunoprecipitation, followed by SDS-PAGE. Fig. 7 shows that the polypeptide with a relative molecular mass of 96 kDa which was immunoprecipitated with antiserum against the α-subunit was produced in the oocytes injected with mRNAs for either deletion mutant and the β-subunit. The mobility of the polypeptide was indistinguishable from that of the wild-type α-subunit.

As shown in Fig. 8, the Na^+/K^+-ATPase activities in the microsomes of oocytes injected with the two deletion mutant mRNAs were 6.5 μmol P_i/mg protein per h for pSPT(Δ K28) and 5.8 μmol P_i/mg protein

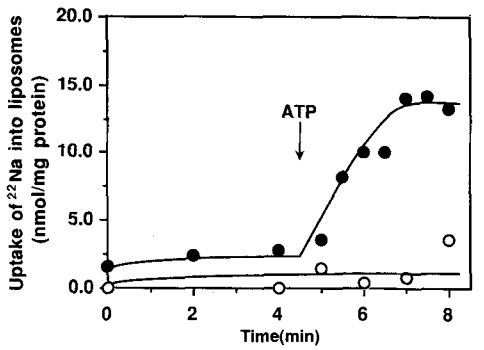

Fig. 6. ATP-dependent ^{22}Na influx into the reconstituted proteoliposomes containing the *Rana* enzyme. The purified *Rana* enzyme (2 mg) was reconstituted into the proteoliposome in a buffer (125 mM Hepes-Tris (pH 7.4)) containing 110 mM KCl and 0.02 mM EDTA. The transport of ^{22}Na was measured in a medium containing 118 mM Hepes-Tris (pH 7.4), 1 mM EDTA-Tris, 2 mM $MgCl_2$, 2 mM $^{22}NaCl$, and 0.1 mM ouabain as described under 'Materials and Methods'. The addition of ATP (Tris salt) is indicated by an arrow. The samples indicated by the open circles are absence of ATP at all stages for measurement of the passive Na^+ influx.

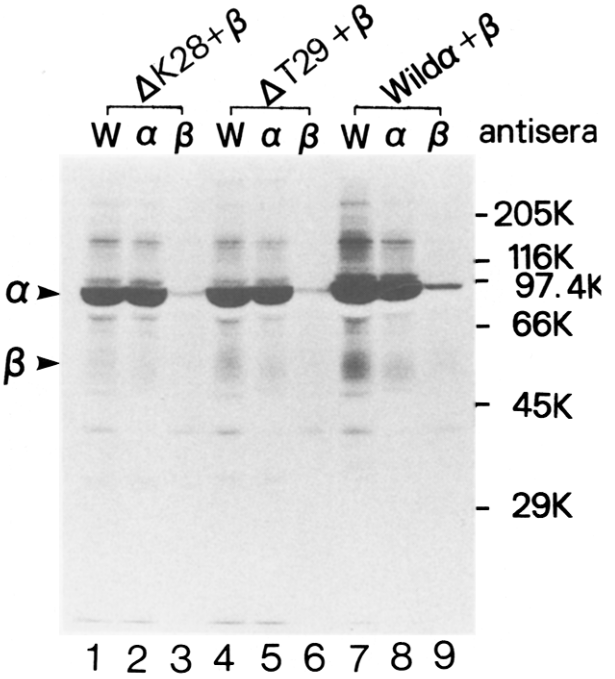

Fig. 7. Autoradiogram of the translation products in *Xenopus* oocytes injected with the mRNAs for the 'lysine cluster' deletion mutants. The mRNAs for the deletion mutants were transcribed by SP6 RNA polymerase and the resulting mRNAs were co-injected into the *Xenopus* oocytes with wild-type β mRNA and labeled with [^{3}H]leucine for 3 days at 19°C. The Triton X-100 extracts of the oocytes were immunoprecipitated with antisera against the the α-subunit (α; lanes 2, 5 and 8), and the β-subunit (β; lanes 3, 6 and 9) from *Torpedo* electric organ and a mixture of both antisera (W; lanes 1, 4 and 7).

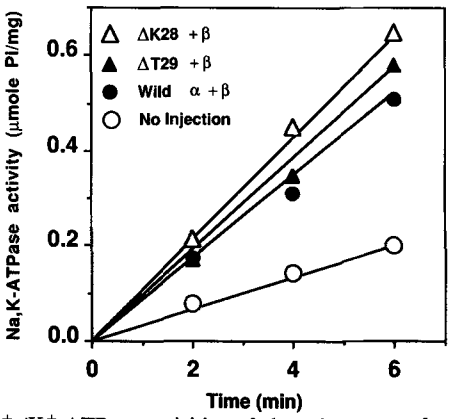

Fig. 8. Na^+/K^+-ATPase activities of the microsomes from oocytes injected with the mutant mRNAs. The Na^+/K^+-ATPase activities in the microsomal fractions from the *Xenopus* oocytes that were injected with mRNAs from pSPT(Δ K28) (△), pSPT(Δ T29) (▲), and wild-type pSPT(α) (●) are shown. These mRNAs were injected together with mRNA for the β-subunit. The ATPase activity of non-injected oocytes is also shown (○).

per h for pSPT(Δ T29). These activities were almost identical with that in the microsomes of the oocytes injected with wild-type α mRNA (5.1 μmol P_i/mg protein per h).

Discussion

The *Rana* enzyme with Na^+- and K^+-dependent ATPase and ion transport activities lacks both the first 29 amino acid residues including the 'lysine cluster' (Fig. 5). Furthermore, the enzymes from the deletion mutants that lacked the 'lysine cluster' showed the same Na^+- and K^+-dependent ATPase activities as the wild type (Fig. 8). These results strongly indicate that the 'lysine cluster' in the N-terminal region of the α-subunit is not necessary for both the Na^+/K^+-ATPase and the ion transport activities.

Although the *Rana* enzyme has shorter N-terminal polypeptide than those of other species, the cleavage at that region of the *Rana* enzyme by some proteinases is improbable from two lines of evidence. First, the α-subunits isolated by two different methods gave the same sequence. Second, the initial yield of the PTH-amino acid derivatives was calculated to be in a sufficient range considering that this enzyme is a membranous protein (Fig. 4). Moreover, the isolated α-subunit of the *Rana* enzyme was deduced to be a homogeneous protein because it migrated as a single band when subjected to SDS-PAGE on a 5% gel. Thus, the isolated α-subunit from the mature form of *Rana* enzyme was not cleaved during the analytical procedure, and yet lacked the 'lysine cluster'.

In contrast to the proposal by Shull et al. [8], the 'lysine cluster' in the N-terminal region of the α-subunit is not involved in cation translocation. The functional importance of the histidine residue at position 18 (sheep, pig and rat α-subunits) proposed by Jørgensen and Collins [20] is denied because of the absence of this residue in the enzyme of *Rana* and several other species (Fig. 5). The possibility is not ruled out that lysine residues present in the preceding region after the 'lysine cluster' could be involved in a controlling the ion passage. The absence of a 'lysine cluster' in the *Rana* enzyme does not conflict with the proposal by Karlish et al. [36] that essential sites for occlusion and transport of cations are located in a 19 kDa tryptic fragment at the C-terminal region of the Na^+/K^+-ATPase α-subunit. The role of the N-terminal 29 residues including the 'lysine cluster' in organization of the enzyme during its biosynthesis (as a membrane insertion signal peptide) was also denied, since mRNA lacking the N-terminal region including the 'lysine cluster' expressed the enzyme activity in *Xenopus* oocytes.

Acknowledgments

The authors wish to thank Prof. Kei Nagano, in whose department this work was initiated, for his encouragement.

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