Solubilization and Purification of Artemia salina (Na,K)-activated ATPase and NH₂-terminal Amino Acid Sequence of Its Larger Subunit*

Michiaki Morohashi and Masaru Kawamura†

From the Department of Biology, Faculty of Science, Chiba University, Chiba, Japan 280

Membrane bound (Na,K)-ATPase partially purified from the nauplius larva of the brine shrimp, Artemia salina, was solubilized with the non-ionic detergent C₁₂Es in the presence of KCl. The addition of KCl was essential for protecting the enzyme against inactivation. With solubilization the enzyme could then be purified to apparent homogeneity. Electron microscopic observation of the purified enzyme revealed a homogeneous population of particles with a diameter of approximately 4 nm.

The larger (α) subunit of the enzyme formed double bands on sodium dodecyl sulfate-polyacrylamide gels. NH₂-terminal sequence analysis of the α subunit revealed the possible presence of two isoforms of (Na,K)-ATPase. At the third position a small but distinct amount of lysine was found in addition to glycine, suggesting that the two forms are different from each other at least at the third residue. The NH₂-terminal sequence determined is as follows. NH₂-Ala-Lys-Gly (Lys)-Lys-Gln-Lys-Lys-Gly-Lys-Asp-Leu-Asn-Glu-Leu-Lys-Lys-Glu-Leu-Asp-Ile-Asp-Phe-His-Lys-Ile-Pro- The sequence is abundant in hydrophilic amino acids, especially lysine, and is quite different from those of vertebrate enzymes reported so far.

The sodium and potassium-activated adenosine triphosphatase has been isolated from plasma membranes of a variety of tissues: the outer medulla of mammalian kidney (1–5), rectal gland of the dogfish (6, 7), electroplax of the electric eel (8), and the brine shrimp (9). The two forms of the brine shrimp are slightly different in NH₂-terminal amino acid sequence, which indicates that the two forms are the products of different genes. We also describe a procedure for solubilization and further purification of the (Na,K)-ATPase. Upon solubilization with C₁₂Es, the addition of KCl is effective in purifying the active brine shrimp enzyme.

EXPERIMENTAL PROCEDURES

Preparation of Membrane-bound (Na,K)-ATPase—Partial purification of (Na,K)-ATPase from brine shrimp nauplii was carried out as described by Peterson and Hokin (11) with minor modifications. The dry cysts (50 g) were immersed in 60% strength seawater and incubated at 28 °C for 24 to 32 h with aeration. The harvested nauplii were homogenized in a Waring blender containing 150 ml of homogenizing medium (250 mM sucrose, 2 mM EDTA (Tris salt), 2 mM 2-mercaptoethanol, 0.1 mM PMSF, and 50 mM imidazole/HCl, pH 7.2). The solubilized enzyme had specific activity of 450 to 590 pmol of P₃₂₀/mg/h (37 °C), and it showed two closely moving bands near M₉₀₄₀₀ and M₁₀₀,₀₀₀ with a few other minor bands on SDS-polyacrylamide gel electrophoresis as described by Peterson and Hokin (11).

Solubilization of SDS-treated Enzyme with C₁₂Es—The SDS-treated enzyme was in membrane bound form and was solubilized with the non-ionic detergent C₁₂Es. Unless otherwise specified, solubilization was carried out as follows. To 5 ml of 3 mg/ml SDS-treated enzyme in the medium containing 80 mM KCl, 250 mM sucrose, 2 mM EDTA (Tris salt), 2 mM 2-mercaptoethanol, 0.1 mM PMSF, and 50 mM imidazole/HCl, pH 7.2, was gradually added at an equal volume of 6 mg/ml C₁₂Es at 4 °C with gentle stirring. The final concentrations of the enzyme and the detergent were 1.5 and 3 mg/ml, respectively. After standing for 20 min at 25 °C, the mixture was centrifuged at 105,000 × g for 60 min at 4 °C. The resulting supernatant was recovered and concentrated to 2 ml by a membrane filter (Amicon YM-5). The solubilized enzyme thus obtained was then applied to a

---

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom reprint requests should be addressed.

The abbreviations used are: SDS, sodium dodecyl sulfate; (Na,K)-ATPase, sodium and potassium-activated adenosine triphosphatase phosphohydrolase (EC 3.6.1.3); C₁₂Es, octoethylene glycol mono-n-dodecyl ether; PTH, phenylthiohydantoin; PMSF, phenylmethylsulfonyl fluoride; HPLC, high-performance liquid chromatography.

1 The abbreviations used are: SDS, sodium dodecyl sulfate; (Na,K)-ATPase, sodium and potassium-activated adenosine triphosphatase phosphohydrolase (EC 3.6.1.3); C₁₂Es, octoethylene glycol mono-n-dodecyl ether; PTH, phenylthiohydantoin; PMSF, phenylmethylsulfonyl fluoride; HPLC, high-performance liquid chromatography.
column of Bio-Gel A-1.5m, 200 to 400 mesh (2.1 × 100 cm); equilibrated with 150 mg/ml C12Es, 40 mM KCl, 1 mM EDTA, 1 mM 2-mercaptoethanol, 0.1 mM PMSF, and 25 mM Tris/HCl, pH 7.2. The gel filtration was carried out at 4 °C at a flow rate of 6.2 to 6.5 ml/h.

**Isolation of Subunits of (Na,K)-ATPase**—The purified enzyme was denatured in 5% SDS and 2% 2-mercaptoethanol and layered on a Bio-Gel A-1.5 m column (1.4 × 90 cm), equilibrated with 0.1% SDS, 0.1 M Tris/HCl, pH 7.4, and 1 mM EDTA. The protein was eluted with the same buffer at a flow rate of about 4 ml/h at room temperature. The fractions were analyzed by SDS-polyacrylamide gel electrophoresis to verify the positions of the subunits. Fractions containing the subunits were pooled and dialyzed against 0.05% SDS for 2 days at 4 °C. After the dialyzed sample was concentrated to about 1 mg/ml, the proteins were stored at -20 °C. The subunit was homogeneous but the subunit was slightly contaminated with other proteins. No further purification of the β subunit was performed.

**NH2-terminal Sequence Analysis of α Subunit—NH2-terminal sequence analysis was carried out on a Applied Biosystems Model 470 A protein sequenator (18). PTH-derivatives were identified and quantified by using a Spectra Physics SP 8100 HPLC system with a C8 column (4.6 × 300 mm).

**Other Methods**—The activity of (Na,K)-ATPase was determined as the rate of release of inorganic phosphate in the presence of 160 mM NaCl, 40 mM KCl, 10 mM MgCl2, 50 mM imidazole/HCl, pH 7.2, and 3 mM ATP disodium salt. For the controls, the rate of release of inorganic phosphate in the presence of 1 mM ouabain was determined and subtracted from the above. To start the reaction, 50 μl of enzyme suspension was added to 450 μl of reaction mixture and the reaction was terminated by the addition of 1 ml of 2 N HCl containing 1.25% ammonium molybdate. Inorganic phosphate was determined by the method of Peterson (19). Protein was determined by the method of Lowry (20) modified by Peterson (21) with bovine serum albumin as a standard. Phospholipids were determined by measuring organic phosphorus content according to the ultramicro method of Bartlett (22). SDS-polyacrylamide gel electrophoresis was carried out by the method of Laemmli (23) using a separating gel of 8.75%. Electron microscopy was done as described elsewhere (24) using negative staining with uranyl acetate. The specimens were examined in a JEOL 100 CX electron microscope at 80 kV.

**Materials**—Dried brine shrimp cysts were obtained from Tetra Brand and stored at -20 °C. Instant ocean sea salts were the products of Nippon Jisei Sango Co., Ltd., Tokyo. C12Es was purchased from Nikko Chemicals, Tokyo. All other chemicals were obtained from various sources as analytical grade reagents.

**RESULTS**

**Solubilization of Brine Shrimp (Na,K)-ATPase with C12Es**—A non-ionic detergent, C12Es, first utilized for solubilization of the (Na,K)-ATPase from rectal glands of Squalus acanthias (25). C12Es solubilized an active brine shrimp (Na,K)-ATPase. To determine the optimal detergent concentration for solubilization, the concentration of C12Es was increased from 0 to 9 mg/ml at a fixed concentration of the SDS-treated enzyme preparation (1.5 mg/ml) in the presence or absence of 40 mM KCl (Fig. 1). (Na,K)-ATPase activity was decreased with increasing amounts of C12Es added in the absence of KCl (Fig. 1, open circles). However, the addition of 40 mM KCl to the medium protected the enzyme against inactivation (closed circles) and more than 80% of the (Na,K)-ATPase activity of the original membrane bound SDS-treated enzyme was retained even at the highest concentration of C12Es tested (9 mg/ml). The presence of NaCl was also effective in protection of the (Na,K)-ATPase although KCl seemed to be more effective than NaCl (Fig. 2).

(The (Na,K)-ATPase activity was recovered in the supernatant when centrifuged at 105,000 × g for 60 min after treatment with C12Es, indicating that the enzyme was essentially solubilized (Table 1, Fig. 3). Since the amount of residual activity was, however, dependent on the medium used for solubilizations, as mentioned above, the amount of activity recovered in the supernatant was higher in the sample treated with NaCl or KCl (closed circles).)

**FIG. 1. Effect of C12Es on the membrane bound brine shrimp (Na,K)-ATPase.** SDS-treated (Na,K)-ATPase from membranes was treated with varied concentration of C12Es at a protein concentration of 1.5 mg/ml in a medium containing 125 mM sucrose, 1 mM EDTA (Tris), 1 mM 2-mercaptoethanol, 0.05 mM PMSF, 25 mM imidazole/HCl, pH 7.2, in the presence (Ο) or absence (Ο) of 40 mM KCl. After standing for 20 min at 25 °C, each sample was diluted 10.5-fold with 25 mM imidazole/HCl, 1 mM EDTA (Tris). Residual activities were measured at 25 °C for 3 min by using aliquots (50 μl) of the diluted samples in reaction mixtures of 500 μl.

**FIG. 2. Protection of (Na,K)-ATPase activity by NaCl (Ο) or KCl (Ο) against inactivation with C12Es.** The enzyme was treated as described in the legend to Fig. 1. The activity of untreated enzyme was taken as 100%.

<table>
<thead>
<tr>
<th>Additives Residual activity (%)</th>
<th>Protein solubilized (%)</th>
<th>Activity solubilized (%)</th>
<th>Specific activity (a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 mg/ml C12Es</td>
<td>41</td>
<td>58</td>
<td>32</td>
</tr>
<tr>
<td>3 mg/ml C12Es + 40 mM NaCl</td>
<td>86</td>
<td>54</td>
<td>65</td>
</tr>
<tr>
<td>3 mg/ml C12Es + 40 mM KCl</td>
<td>86</td>
<td>48</td>
<td>62</td>
</tr>
</tbody>
</table>

(a) The activity of untreated membrane-bound enzyme was taken as 100%.

(b) The fraction recovered in the supernatant after centrifugation at 105,000 × g for 60 min.

(c) μmol Pi/mg of protein/h at 25 °C.
Purification of Artemia salina (Na,K)-ATPase

FIG. 3. Electrophoretograms of the fractions obtained during solubilization of brine shrimp (Na,K)-ATPase. Gel m represents the membrane bound SDS enzyme. Gel s represents the supernatant fraction obtained after the centrifugation of C₁₂E₈-solubilized enzyme. Gel p represents the pellet fraction. The positions of the α₁, α₂, and β subunit bands are indicated.

in the presence of either KCl or NaCl than in its absence. When the enzyme was treated at a protein concentration of 1.5 mg/ml with 3 mg/ml C₁₂E₈ in the presence of 40 mM KCl, activity and protein were recovered in the supernatant to the extent of about 60 and 50% of the original membrane bound enzyme, respectively. The specific activity of the solubilized enzyme was 451 μmol of P₃₀₄/mg/h at 25 °C (349 μmol of P₃₀₄/mg/h for membrane bound enzyme). As shown in Fig. 3, a large fraction of the (Na,K)-ATPase subunits were recovered in the supernatant leaving the contaminating proteins largely in the pellet. Treatment with C₁₂E₈ in the presence of KCl or NaCl is, therefore, effective in the purification of the brine shrimp (Na,K)-ATPase.

Solubilization of brine shrimp (Na,K)-ATPase was performed hereafter, unless otherwise stated, in a medium containing 125 mM sucrose, 1 mM EDTA (Tris salt), 1 mM 2-mercaptoethanol, 0.05 mM PMSF, 25 mM imidazole/HCl pH 7.2, and 40 mM KCl at 25 °C for 20 min at protein and detergent concentrations of 1.5 and 3.0 mg/ml, respectively.

Care was taken not to measure the enzyme activity at the temperature usually employed (37 °C), especially when solubilized (Na,K)-ATPase was assayed, because thermal instability of the solubilized enzyme has been reported for the enzymes from rectal gland (25) and from porcine kidney (26). As shown in Fig. 4, the solubilized brine shrimp (Na,K)-ATPase was also unstable at 37 °C. Activities were therefore assayed at 25 °C for 3 min or less.

As the supernatant fraction of the solubilized enzyme was still contaminated by a few faint bands near the β subunit, it was chromatographed on a column of Bio-Gel A-1.5m. The result is shown in Fig. 5. The contaminating proteins were eluted either at fractions just before the peak for (Na,K)-ATPase or in fractions near the void volume and were consequently removed from the enzyme fractions. The specific activity was increased up to 650 μmol of P₃₀₄/mg/h.

When the peak fractions were examined in an electron microscope, many particles similar to those observed by Nakao et al. (27) were found and are shown in Fig. 6.

Isolation of α Subunit—Fig. 7 shows the separation of the brine shrimp (Na,K)-ATPase subunits by gel filtration on Bio-Gel A-1.5m in the presence of SDS. The elution pattern was essentially the same as reported by Peterson and Hokin (11), but the peaks were separated much better. Peak 2 contained the α subunit and was not contaminated by other proteins as revealed by SDS-polyacrylamide gel electrophoresis (shown in Fig. 7). The α subunit consisted of two bands, designated by Peterson and Hokin (11) as α₁ and α₂ for the upper and lower bands, respectively, although the two are hardly separated in the electrophoretogram of Fig. 7. Peak 3 contained β subunit with very slight contaminants. Three other major peaks were analyzed by SDS-gel electrophoresis and no significant bands of protein were detected. The pooled fractions indicated by shading in Fig. 7 were dialyzed against 0.05% SDS and stored after concentration on a Millipore immersible CX-10 membrane filter to a minimum volume.

Analysis of NH₂-terminal Sequence of α Subunit—The α subunit in 0.05% SDS was subjected to gas-phase sequence analysis without removal of SDS. The initial yields of PTH-amino acid were rather low (30 to 50%). However, the repetitive yield exceeded 95%.

Two runs were performed using two preparations of α subunit which differed in the relative amounts of α₁ and α₂. One preparation (Sample I) contained subunits α₁ and α₂ in quantities sufficient to distinguish two bands clearly on SDS-polyacrylamide gels, whereas the other (Sample II) contained mostly α₁ with very little α₂, as shown in Fig. 8. We expected that if the two polypeptides were identical in amino acid sequence, only one NH₂-terminal sequence should be obtained from both samples. Since it was not possible to obtain preparations that consisted exclusively of α₁ and α₂, we find this was one of the most fruitful experiments we could perform.

Approximately 2 mmol of Sample I was sequenced. We could determine the sequence of 26 amino acid residues as follows.

NH₂-Ala-Lys-Gly(Lys)-Lys-Gln-Lys-Lys-Gly-Gly-Leu-Leu-Glu-Leu-Lys-Lys-Glu-Leu-Asp-Leu-Asp-Glu-Leu-Glu-Lys-Glu-Leu-Ase-Asp-Phe-His-Lys-Lys-Ile-Pro The amounts of PTH-alanine and PTH-lysine (cycles 1 and 2) recovered were 1170 pmol and 1320 pmol, respectively. The amounts of PTH-derivatives of other amino acids in both cycles were at the level of background. For the third cycle, however, two major PTH peaks were observed. One was PTH-glycine and the other PTH-lysine at 870 and 440 pmol, respectively. PTH-lysine in the third cycle was obtained in a relatively small quantity but we concluded that the lysine did not arise from incomplete cleavage at the second position, based on the following considerations. Lysine is an easily cleavable residue, as has been the usual experience with...
Purification of Artemia salina (Na,K)-ATPase.

Fig. 5. Gel filtration of C_{12}E_{8}-solubilized brine shrimp (Na,K)-ATPase. C_{12}E_{8}-treated enzyme fraction consisting of 5.5 mg of protein was fractionated by chromatography on a Bio-Gel A-1.5m (200–400 mesh) column (2.1 × 100 cm) at 5 °C as described under "Experimental Procedures." (Na,K)-ATPase activity (○) and phospholipid content (■) in each fraction were measured. The dotted line represents the absorbance at 280 nm. Gels inserted in the figure show the protein patterns of the fractions indicated.

FIG. 5. Gel filtration of C_{12}E_{8}-solubilized brine shrimp (Na,K)-ATPase. The bar indicates 50 nm.

Fig. 6. Electron micrograph of the solubilized brine shrimp (Na,K)-ATPase. The bar indicates 50 nm.

the program used. For example, lysine in the fourth position was cleaved nearly completely, and hence only a background level of lysine was found at the fifth cycle (Fig. 9). This was seen more clearly when the yield of PTH-lysine was followed from the NH_{2} terminus (Fig. 10). The yields of PTH-lysine for cycles 5 and 8 are at the level of background, indicating that the lysines at the fourth and seventh positions were cleaved completely. The yield of PTH-lysine for the third cycle was significantly higher than background. From these results we could assign lysine and glycine as the third amino acid of the α subunit of Sample I. (As will be mentioned below, the yield of lysine at cycle 3 for Sample II was at the level of background shown in Fig. 10 and only glycine was assignable for the third amino acid.) These data were consistent with the results of SDS-gel electrophoresis shown in Fig. 8. It was most likely that the yield of lysine at the third cycle correlated with the appearance of the α2 band on SDS gels.

We could not obviously recognize the existence of multiple major PTH-derivatives in the HPLC traces after the third cycle. Since the background increased with cycle number, it would be impossible to identify minor components, even if they exist.

The NH_{2}-terminal amino acid sequence of the α subunits determined with Sample II was the same as above except that only glycine was in the third position (Fig. 10). These results indicate that the NH_{2}-terminal amino acid sequences of α1 and α2 are very similar but differ at least at the third position, and that the two polypeptides, therefore, are the products of different genes.

The NH_{2}-terminal amino acid sequence of the β subunit was analyzed by using approximately 1 nmol of protein, although it was not homogeneous. We could not find significant amounts of any PTH-derivatives for cycles 1 through 4. The NH_{2} terminus of the β subunit from brine shrimp thus may be blocked.

DISCUSSION

The brine shrimp (Na,K)-ATPase is important for the study of the biogenesis of this enzyme and of interest from a
Purification of *Artemia salina (Na,K)-ATPase*

**Fig. 7.** SDS gel filtration of the brine shrimp (Na,K)-ATPase subunit. The purified enzyme (1.7 mg of protein) was denatured with SDS and fractionated on a column (1.4 X 90 cm) of Bio-Gel A-1.5m (200-400 mesh) as described under “Experimental Procedures.” The shading indicates the fractions pooled for each subunit that provided the patterns on SDS-acrylamide gels shown in the figure.

**Fig. 8.** Electrophoretograms of the α subunits subjected to sequence determinations. The positions of α1 and α2 band are indicated.

Phylogenetic viewpoint. Peterson *et al.* (11, 16, 17) purified the enzyme and obtained a preparation of about 60% purity. It seemed to us, however, that further purification should be achieved to investigate the biogenesis as well as the molecular properties of the (Na,K)-ATPase. They had already made a considerable effort to purify the enzyme, and we could not find any methods to improve the purification of the enzyme in membrane bound form. We, therefore, tried to find conditions that would solubilize an active (Na,K)-ATPase. Since Esmann (25) used the non-ionic detergent C₁₂E₈ for the solubilization of the (Na,K)-ATPase from dogfish rectal grand, the detergent has frequently been utilized to obtain soluble samples of (Na,K)-ATPases (27, 29-31). We first tried to find conditions in which little or no loss of activity occurred upon solubilization with the detergent. Addition of monovalent cations, especially KCl, into the solubilization medium was found to release the active enzyme. The soluble enzyme thus obtained was fairly stable and did not lose activity at least 1 week at 4 °C (data not shown). Glycerol, as well as monovalent cations, protected to some extent (about 70%). The effects of glycerol and monovalent cations were not additive.

The oligomeric structure of the brine shrimp (Na,K)-ATPase, now under investigation in our laboratory by the use of HPLC, was studied by electron microscopy. There were many particles with a diameter of approximately 4 nm, very similar to those observed by Nakao *et al.* (27). Although these authors have also reported a second particle triangular in shape in the higher molecular weight fractions from HPLC, we have not found such material so far in our enzyme fractions.

The NH₂-terminal sequence of the brine shrimp α subunit was determined and compared with others reported thus far. The sequences from the kidney enzyme (32, 33) and the salt gland enzyme (34) are listed in Table II. There is a high degree of conservation among NH₂-terminal sequences of these other enzymes. In contrast, the sequence of the brine shrimp en-
Purification of Artemia salina (Na,K)-ATPase

Fig. 9. HPLC traces from an NH2-terminal amino acid sequence analysis of Sample I shown in Fig. 8. The positions of the PTH-derivatives assigned in the traces for cycles 1 through 5 are indicated by the arrows and three-letter amino acid designations. The peaks indicated by X are due to the reagents used for Edman degradation.

Fig. 10. Yields of PTH-lysine from an NH2-terminal amino acid sequence analysis of Sample I (left) and II (right). HPLC peak heights were converted to picomoles using values from a standard of PTH-lysine.

Table II

NH2-terminal sequences of the a subunit of (Na,K)-ATPase

<table>
<thead>
<tr>
<th>Species</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lamb kidney*</td>
<td>Gly-Arg-Asp-Lys-Tyr-Glu-Pro-Ala-Ala</td>
</tr>
<tr>
<td>Dog kidney</td>
<td>Gly-Arg-Asn-Lys-Tyr-Glu-Pro-Ala-(?)-Ser-Glu</td>
</tr>
<tr>
<td>Duck salt gland†</td>
<td>Gly-Arg-Asn-Lys-Tyr-Glu-Thr-Thr-Ala-(?)-Ser-Glu</td>
</tr>
<tr>
<td></td>
<td>Leu-Asp-Leu-Asn-Glu-Leu-Lys-Lys-Glu</td>
</tr>
<tr>
<td></td>
<td>Leu-Asp-Ile-Asp-Phe-His-Lys-Ile-Pro</td>
</tr>
</tbody>
</table>

* Lamb kidney sequence from Collins and Zot (32).
† Dog kidney sequence from Cantley (33).
‡ Duck salt gland sequence from Hopkins et al. (34).

enzymes is quite different from those sequences. The idea that this fact might represent a difference in sequence of the enzyme between deuterostomia and protostomia is attractive but speculative.

The NH2-terminal sequences of the α subunits, especially the sequence of the brine shrimp α subunit, are hydrophilic, which is consistent with the idea that the NH2-terminus of the α subunit is exposed at the cytoplasmic surface (35). The high concentration of hydrophilic amino acids also indicates that there is no leader sequence retained in these α subunit gene products.

Differences between α1 and α2 have been proposed to be due to differences in glycosylation based on the results of peptide mapping (28) and of gradient SDS-gel electrophoresis (16). These methods appear to be less sensitive to the presence of minor differences in the primary structure. The results shown in this paper clearly demonstrate that α1 and α2 are very similar but not identical and that the two are the products of different genes. The apparent difference in molecular weight is due either to slight differences in amino acid sequence, carbohydrate content, or cleavage at the COOH-terminal end. It is likely that the two forms are present in different cell types in the brine shrimp, as in the rat brain (15).

Acknowledgments—We thank Professor K. Maruyama for his kind hospitality and for providing the facilities for ultracentrifugation and electron microscopy. We are indebted to S. Fukushima and A. Mukunoki of Japan Scientific Instruments Co., Ltd. for their kind help in sequence determinations. Thanks are also due to M. Izawa for her typing of the manuscript.

REFERENCES

Purification of Artemia salina (Na,K)-ATPase