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Partial Purification and Characterization of $(Na^+ + K^+)$-ATPase from Garfish Olfactory Nerve Axon Plasma Membrane

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Summary. The $(Na^+ + K^+)$-ATPase of garfish olfactory nerve axon plasma membrane was purified about sixfold by treatment of the membrane with sodium dodecyl sulfate followed by sucrose density gradient centrifugation. The estimated molecular weights of the two major polypeptide components of the enzyme preparation on sodium dodecyl sulfate gels were 110,000 and 42,000 daltons, which were different from those of the corresponding peptides of rabbit kidney $(Na^+ + K^+)$-ATPase. No carbohydrate was detected in the 42,000-dalton component either by the periodic acid-Schiff reagent or by the more sensitive concanavalin A-peroxidase staining procedure. The molecular properties of the garfish $(Na^+ + K^+)$-ATPase, such as the K_m for ATP, pH optimum, energies of activation, Na and K ion dependence and vanadium inhibition, were, however, similar to those of the kidney enzyme.

The partially purified garfish $(Na^+ + K^+)$-ATPase was reconstituted into phospholipid vesicles by a freeze-thaw-sonication procedure. The reconstituted enzyme was found to catalyze a time and ATP dependent $^{22}Na^+$ transport. The ratio of $^{22}Na^+$ pumped to ATP hydrolyzed was about 1; under the same reconstitution and assay conditions, eel electroplax $(Na^+ + K^+)$-ATPase, however, gave a $^{22}Na^+$ pumped to ATP hydrolyzed ratio of nearly 3.

Key words: Axon membrane, $(Na^+ + K^+)$-ATPase, polypeptide components, enzyme reconstitution, garfish olfactory nerve

The sodium and potassium-activated adenosine triphosphatase, $(Na^+ + K^+)$-ATPase, is an integral membrane protein responsible for the active translocation of sodium and potassium ions across mammalian cell membranes. The enzyme has been shown to span the entire plasma membrane [26] and to require the presence of lipids for its catalytic activity [38]. The $(Na^+ + K^+)$-ATPase has been purified from mammalian kidney [19, 25, 27], duck salt gland [18], dogfish rectal gland [16], eel electroplax [32], and brine shrimp [33]. Analysis of these preparations by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis has shown that the $(Na^+ + K^+)$-ATPase consists of two polypeptide components, a large catalytic subunit (α) with a molecular weight of 90,000 to 100,000 and a small glycoprotein subunit (β) of 38,000 to 60,000. A proteolipid of about 12,000 mol wt has also been found as a component of pig kidney $(Na^+ + K^+)$-ATPase [10]. The purified $(Na^+ + K^+)$-ATPase has been reconstituted into phospholipid vesicles in several laboratories [1, 12, 15, 34], and the reconstituted enzyme shown to catalyze ATP-dependent, ouabain inhibitable Na^+ and K^+ transport, thus providing direct evidence that the $(Na^+ + K^+)$-ATPase is, in fact, the sodium pump.

Our interest in the biochemical characterization of axon plasma membrane led to the finding that the $(Na^+ + K^+)$-ATPase of garfish, *Lepisosteus osseus,* olfactory nerve axon membrane has considerably different structural properties as compared to those of $(Na^+ + K^+)$-ATPase isolated from other sources. $(Na^+ + K^+)$-ATPase from an axon plasma membrane has never been purified and chemically characterized before. In this report, we describe the partial purification of $(Na^+ + K^+)$-ATPase from garfish nerve axon membrane and the characterization of its polypeptide components as well as some of its molecular properties. We also report our initial result on the reconstitution of the partially purified $(Na^+ + K^+)$-ATPase into phospholipid vesicles and its $^{22}Na^+$ transport properties. A brief report on the

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polypeptide components of garfish axon $(Na^+ + K^+)$-ATPase has been presented [24].

Materials and Methods

Preparation of axon membrane

The procedures used for the isolation of olfactory nerve from long-nosed garfish and of axon plasma membrane from the nerve have been described before [5]. The axon plasma membrane was suspended in 0.25 M sucrose, 5 mM Tris-HCl, pH 7.4, at a protein concentration of 3 to 4 mg/ml and frozen at −30 °C in 1- or 2-ml aliquots in glass vials. The frozen membrane was stored for up to one year without change of polypeptide pattern on SDS-polyacrylamide gels or loss of $(Na^+ + K^+)$-ATPase activity.

Partial Purification of $(Na^+ + K^+)$-ATPase from Axon Plasma Membrane

The procedure for the purification of $(Na^+ + K^+)$-ATPase from axon plasma membrane was based on the SDS method of Jørgensen [19] as modified by Hopkins et al. [18]. The frozen membrane was thawed quickly and diluted with 7 vol of cold 1 mM EDTA, 20 mM Tris-HCl, pH 7.4, and centrifuged at 27,000 rpm in a Beckman 30 rotor for 60 min. The pellet was suspended in 3 mM ATP, 2 mM EDTA, 20 mM Tris-HCl, pH 7.5, to a protein concentration of 1.2 to 1.5 mg/ml. Approximately 80–85% of the membrane protein was recovered in this washing procedure. To the washed membrane suspensions was added a solution containing 2 mg/ml SDS, 3 mM ATP, 2 mM EDTA, 20 mM Tris-HCl, pH 7.5, over a period of 2 min with constant stirring with a magnetic stirring bar so that the final protein concentration was 0.14 mg/ml and SDS concentration was 0.20 mg/ml. The treated membrane was recovered by centrifugation and the pellet resuspended in 0.25 M sucrose, 2 mM EDTA, 20 mM Tris-HCl, pH 7.5, to a protein concentration of about 0.4 mg/ml. The detergent treated membrane (2.4 ml) was layered over a discontinuous gradient made from 12, 10 and 10 ml of 30, 15 and 10% (wt/vol) sucrose solution, respectively, and centrifuged for 3 hr at 25,000 rpm in a Beckman SW 27 rotor at 4 °C. The sucrose solution contained 1 mM EDTA, and 20 mM Tris-HCl, pH 7.5. Two membrane fractions were seen after the centrifugation. A broad, diffuse band was present above the 15–30% sucrose interface (upper fraction). The other membrane fraction was narrower and denser and appeared at the 15–30% sucrose interface (lower fraction). A light brown translucent pellet, which showed no $(Na^+ + K^+)$-ATPase activity was found at the bottom of the tube. The upper and the lower fractions were removed and diluted with 1.5 and 3 vol, respectively, of 2 mM EDTA, 20 mM Tris-HCl, pH 7.5. The membrane fractions were recovered by centrifugation and suspended in 0.25 M sucrose, 2 mM EDTA, 20 mM Tris-HCl, pH 7.5, at a protein concentration of 0.6 to 1.0 mg/ml. The purified $(Na^+ + K^+)$-ATPase preparations were kept at 0 °C for up to 10 days without significant loss of enzyme activity. $(Na^+ + K^+)$-ATPase from rabbit kidney outer medulla was prepared essentially according to the procedure of Jørgensen [19].

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis of axon membrane and $(Na^+ + K^+)$-ATPase preparations was carried out according to the procedure of Fairbanks et al. [8]. Gels were stained for protein with Coomassie blue [8]. The presence of glycoproteins on gels was detected by periodic acid-Schiff method as described by Fairbanks et al. [8] with the following modifications. Periodic acid oxidation was carried out in 1% sodium metaperiodate in 0.35% nitric acid, pH 1.6, for 2 hr. Schiff reagent was prepared by the "traditional method" [28] and stored at 5 °C. After Schiff reagent treatment, the gels were rinsed several times in 0.1% sodium metabisulfite in 0.01 M HCl until the rinse solutions failed to turn pink upon addition of formaldehyde. The gels were then transferred to water to intensify the color of the glycoprotein bands. The color remained stable for several months. As an alternative method for identification of glycoproteins, concanavalin A-peroxidase staining procedure as modified by Wood and Sarinana [39] and Olden and Yamada [31] was also applied. Ovalbumin was used as a marker of glycoprotein to check the specificity and sensitivity of the staining procedures. Coomassie blue stained gels were scanned at 550 nm and periodic acid-Schiff stained gels at 560 nm with a Beckman Acta II spectrophotometer equipped with a gel scanner.

Phosphorylation of $(Na^+ + K^+)$-ATPase and Identification of the Phosphorylated Polypeptide Component

The purified enzyme was phosphorylated and electrophoresed according to the procedures of Grefrath and Reynolds [13] and of Fairbanks et al. [8], respectively. The gels were then washed with a solution containing 25% isopropyl alcohol and 10% acetic acid for 8 hr with several changes and sliced into 2.5-mm thick sections. The individual slices were incubated in 1 ml of H_2O_2 (30%) at 60 °C overnight, and the radioactivity was estimated in a Beckman LS-230 liquid scintillation spectrometer.

$(Na^+ + K^+)$-ATPase Assay

In order to expose any latent $(Na^+ + K^+)$-ATPase activity, the enzyme preparations were first preincubated at a protein concentration of either 0.2 or 0.5 mg/ml for 30 min at room temperature in solutions containing either 0.1 or 0.2 mg SDS/ml, respectively, and 3 mM ATP, 2 mM EDTA, 20 mM Tris-HCl, pH 7.5. Aliquots of the enzyme preparation containing from 5 to 60 µg protein were then assayed for $(Na^+ + K^+)$-ATPase activity in a final 1-ml volume of reaction medium containing 130 mM NaCl, 20 mM KCl, 3 mM $MgCl_2$, 3 mM ATP and 30 mM histidine-HCl, pH 7.5. The reaction medium was equilibrated at 37 °C for 5 min in a water bath and the reaction was initiated by the addition of the enzyme preparation. The reaction was terminated by the addition of 0.1 ml of 5% SDS. Depending on the level of enzyme activity, the reaction time was varied from 5 to 20 min. A maximum of 20% of the total ATP in the reaction medium was hydrolyzed under these conditions. The rate of hydrolysis of ATP was linear with respect to time of incubation and enzyme concentration. Inorganic phosphate (P_i) was measured by the method of Fiske and Subbarow [9]. $(Na^+ + K^+)$-ATPase activity was taken as the difference in P_i released in the absence and presence of 1 mM ouabain in the reaction medium. Activity measured in the presence of 1 mM ouabain was considered the Mg^{2+}-ATPase activity. In order to study the effect of temperature on $(Na^+ + K^+)$-ATPase activity, the enzyme was assayed at temperatures ranging from 0 to 40 °C. Protein was determined by the method of Lowry et al. [29], using bovine serum albumin as the standard.

Lipid Extraction and Analysis

Lipids were extracted by the method of Bligh and Dyer [4] and analyzed by standard procedures [6, 35, 40].

Reconstitution of $(Na^+ + K^+)$-ATPase into Phospholipid Vesicles

The freeze-thaw-sonication procedure [17, 23] used for the reconstitution of garfish $(Na^+ + K^+)$-ATPase preparation into phospholipid vesicles was the following. Acetone-washed soybean phospholipids (asolectin, 90 mg) in 1 ml of a medium containing 150 mM K_2SO_4, 40 mM β-mercaptoethanol and 100 mM imidazole-H_2SO_4, pH 7.4, in a test tube, were flushed with N_2, covered with parafilm and sonicated at room temperature in a bath-type sonicator for 20–25 min. The $(Na^+ + K^+)$-ATPase preparation was washed and suspended in 0.25 M sucrose. Aliquots containing 225 µg enzyme protein were added to phospholipid vesicles (9.0 mg) in a final volume of 0.2 ml of medium containing 75 mM K_2SO_4, 50 mM Na_2SO_4, 50 mM imidazole-H_2SO_4, pH 7.4, 20 mM β-mercaptoethanol and 0.04 M sucrose. The protein-lipid mixture was quickly frozen in dry ice/acetone for 2 min, thawed at room temperature for 15 min, and sonicated for 1 min at room temperature in bath-type sonicator.

Assays of Reconstituted $(Na^+ + K^+)$-ATPase

Aliquots of proteoliposomes (25 µg protein) were assayed at 37 °C in a medium containing 50 mM imidazole-H_2SO_4, pH 7.4, 75 mM K_2SO_4, 50 mM Na_2SO_4, 20 mM β-mercaptoethanol and either 4.5 mM ATP, 4.5 mM $MgCl_2$ and $^{22}NaCl$ (60 cpm/nmol) in a total volume of 0.115 ml for ATP-dependent Na^+ uptake by the method of Gasko et al. [11] or 4 mM [γ-^{32}P]-ATP (4.1×10^4 cpm/µmol) and 5 mM $MgCl_2$ in a total volume of 1.0 ml for $(Na^+ + K^+)$-ATPase activity by the method Nelson et al. [30].

Results

The garfish olfactory nerve axon plasma membrane had an average $(Na^+ + K^+)$-ATPase activity of 7.6 µmol P_i/mg protein/hr (Table 1). When the membrane (0.5 mg protein/ml) was incubated in the presence of a low concentration of SDS (0.2 mg/ml) prior to the $(Na^+ + K^+)$-ATPase assay [21] the activity increased almost three times, presumably due to the unmasking of latent enzyme sites. The $(Na^+ + K^+)$-ATPase activity measured this way represented 100% in calculations of purification and recovery. The Mg^{2+}-ATPase activity increased about twice upon incubation with SDS.

Sodium Dodecyl Sulfate Treatment of the Membrane and Purification of the $(Na^+ + K^+)$-ATPase

To optimize the concentrations of detergents and membrane proteins, axon membrane at various protein concentrations was treated with increasing concentrations of SDS at room temperature for 30 min and then assayed for $(Na^+ + K^+)$-ATPase activity. The results so obtained are shown in Fig. 1. At all concentrations of membrane protein the enzyme activity increased to a maximum and then decreased as the SDS concentration increased. The concentration of SDS needed for maximum activation was dependent on the protein concentration. Higher specific activities were obtained when the concentrations of membrane and SDS were low. However, at low membrane concentrations, the enzyme was more sensitive to changes in SDS concentration. Since about 0.2 mg SDS/ml gave the highest specific activity, this detergent concentration was chosen for purification of the enzyme from the axon membrane. To more accurately determine a final membrane protein concentration for detergent treatment, axon membrane preparations at varying protein concentrations were then incubated in solutions containing 0.2 mg SDS/ml for 30 min at room temperature and the membranes recovered by centrifugation. The treated membranes were then assayed for $(Na^+ + K^+)$-ATPase activity and protein concentration. These results are shown in Fig. 2. The

Table 1. Specific activity and recovery of $(Na^+ + K^+)$-ATPase and of Mg^{2+}-ATPase from garfish olfactory nerve axon plasma membrane[a]

Preparation	Protein (mg)	$(Na^+ + K^+)$-ATPase activity[b]			Mg^{2+}-ATPase activity[b]		
		Sp. Act. (µmol P_i/mg/hr)	Total Act. (µmol P_i/hr)	Recovery (%)	Sp. Act. (µmol P_i/mg/hr)	Total Act. (µmol P_i/hr)	Recovery (%)
Membrane							
−SDS	100	7.6 (27) (3.5–11.3)	760		6.6 (10) (3.4–9.4)	660	
+SDS	100	22.0 (11) (15.6–32.2)	2200	100	14.5 (4) (7.3–17.8)	1450	100
Purified $(Na^+ + K^+)$-ATPase							
Upper fraction	4.2 (6) (2.9–5.8)	139.0 (7) (90.9–228)	584	26.5	12.8 (6) (9.4–19.3)	53.8	3.7
Lower fraction	14.8 (6) (11.0–21.2)	110.0 (7) (66.4–139)	1640	74.5	11.4 (6) (4.7–16.7)	168.0	11.6

[a] Purification was according to the procedure in Materials and Methods. Upper and lower fractions refer to the material collected at the 10/15% and 15/30% sucrose interfaces, respectively, after density gradient centrifugation.

[b] Values are expressed as the average of (n) number of determinations with the range indicated in parentheses

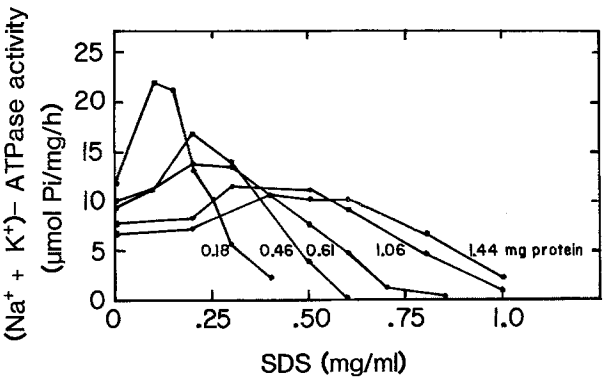

Fig. 1. The influence of protein concentration on the activation of $(Na^+ + K^+)$-ATPase by SDS. Axon plasma membrane was washed with 20 vol of 1 mM EDTA, 20 mM Tris-HCl, pH 7.5, centrifuged for 30 min at 28,000 rpm in a Beckman 30 rotor, and suspended (2 mg protein/ml) in 0.25 M sucrose, 1 mM EDTA, 20 mM Tris-HCl, pH 7.5. Aliquots of the membrane suspension were incubated in 1 ml containing 3 mM ATP, 1 mM EDTA, 20 mM Tris-HCl, pH 7.5, at protein concentrations as shown in the figure, in the presence of SDS at concentrations as indicated on the abscissa. After 30 min incubation at room temperature, 0.2-ml aliquots of the mixture were transferred to the $(Na^+ + K^+)$-ATPase assay medium, and enzyme activity was measured

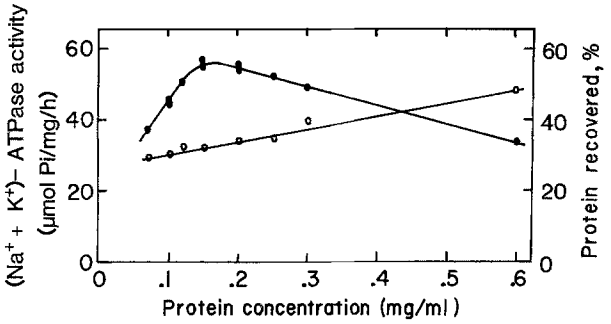

Fig. 2. The effect of protein concentration on the purification of $(Na^+ + K^+)$-ATPase from axon plasma membrane by SDS. Axon plasma membrane was washed as described in legend of Fig. 1, suspended (2 mg protein/ml) in a solution containing 3 mM ATP, 2 mM EDTA, 50 mM imidazole-HCl, pH 7.5, and divided into aliquots containing 1 mg protein. The above ATP buffer and SDS (1 mg/ml in ATP buffer) were added to the membrane to make all samples 0.2 mg SDS/ml and protein concentrations as indicated on the abscissa. After a 30-min incubation at room temperature, the samples were diluted with 2 vol of 1 mM EDTA, 25 mM imidazole-HCl, pH 7.5, centrifuged for 60 min at 27,000 rpm in a Beckman 30 rotor, and the pellet was suspended in the same buffer to a protein concentration of 0.5 mg/ml. The samples were assayed for protein (○) and $(Na^+ + K^+)$-ATPase (●) as described in Materials and Methods

highest specific activity was obtained when the protein concentration of the membrane was about 0.14 mg/ml. For protein concentrations below 0.14 mg/ml, the specific activity and the total activity of the preparation decreased. Therefore, a final detergent concentration of 0.2 mg SDS/ml and a protein concentration of 0.14 mg/ml were selected for the purification of $(Na^+ + K^+)$-ATPase from axon plasma membrane. An increase in pH of the incubation medium up to 9.0 or addition of EDTA to the incubation medium up to a 10 mM concentration did not result in any further increase in specific activity of the enzyme preparation; however, a decrease in pH below 7.5 resulted in a decrease in specific activity.

Table 2. Chemical composition of garfish olfactory nerve axon plasma membrane and purified $(Na^+ + K^+)$-ATPase fractions[a]

	Axon membrane	$(Na^+ + K^+)$-ATPase	
		Upper fraction	Lower fraction
Protein, % wt	38.4 (6) (36.0–40.9)	14.4 (4) (14.0–14.6)	19.8 (4) (19.4–20.4)
Total lipid, % wt	61.6 (6) (59.1–64.0)	85.6 (4) (85.4–86.0)	80.2 (4) (79.6–80.6)
Lipid/protein, wt.ratio	1.61 (6) (1.44–1.78)	5.98 (4) (5.86–6.13)	4.07 (4) (3.91–4.16)
Phospholipid, % of total lipids	72.9 (5) (72.4–73.3)	71.4 (4) (70.5–72.3	71.5 (4) (70.0–73.3)
Cholesterol, % of total lipids	27.1 (5) (26.7–27.6)	28.6 (4) (27.7–29.5)	28.5 (4) (26.7–30.0)
Phospholipid/cholesterol mole ratio	1.34 (5) (1.31–1.38)	1.24 (4) (1.19–1.30)	1.26 (4) (1.16–1.37)

[a] Values are expressed as the average of (*n*) number of experiments with the range indicated below in parentheses.

Centrifugation of the detergent-treated membrane over a discontinuous sucrose gradient (10/15/30% wt/vol) yielded two membrane fractions. The 10/15% sucrose interfacial membrane material (upper fraction) appeared as a diffuse band, whereas the material at the 15/30% sucrose interface (lower fraction) appeared as a dense narrow band. About 1% of the original membrane protein was recovered as a pellet at the bottom of the tube and had no measurable $(Na^+ + K^+)$-ATPase activity. The $(Na^+ + K^+)$-ATPase activity was found associated with both the upper and the lower membrane fractions (Table 1). The upper fraction had a $(Na^+ + K^+)$-ATPase activity of 139 μmol P_i/mg protein/hr, and this activity represented a 6.3-fold purification over that of the detergent activated axon membrane. The enzyme activity of the lower membrane fraction was about 20% less than that of the upper fraction. About 19% of the original membrane protein was recovered in the two membrane fractions. The Mg^{2+}-ATPase activity of these fractions was about 8–10% of the total initial ATPase activity.

Chemical Composition

The lipid content of both enzyme fractions increased considerably (Table 2). Thus the total lipid to protein

ratio of the upper and lower fractions were 6 and 4, respectively, as compared to 1.6 for the starting membrane. However, the phospholipid to cholesterol ratio of the enzyme preparations was similar to that of the starting material, suggesting that neither phospholipid nor cholesterol was preferentially removed to any appreciable extent during the purification procedure.

Phosphatidylcholine (44%) and phosphatidylethanolamine (31%) were the major classes of phospholipids. The other phospholipids were phosphatidylserine (12%) phosphatidylinositol (3%) and sphingomyelin (10%). These five lipids together accounted for more than 99% of the total phospholipids. The phopholipid composition of the enzyme preparations was similar to that of axon plasma membrane [5].

Polypeptide Components

A photograph and a densitometric tracing of a Coomassie blue-stained gel of an upper fraction ($Na^+ + K^+$)-ATPase preparation are shown in Fig. 3*a*. Two major and several minor polypeptide bands were seen in the ($Na^+ + K^+$)-ATPase preparation. The apparent molecular weights of the two major components were 110,000 and 42,000. These components corresponded to band II (110,000 daltons) and band VII (42,000 daltons) of starting axon membrane [7], (Fig. 3*b*). The 110,000-dalton polypeptide band was identified as the catalytic subunit of ($Na^+ + K^+$)-ATPase by the sodium-dependent, potassium-sensitive phosphorylation with [γ-^{32}P]-ATP. Comparison of the mobility of the sodium-dependent radioactive peak of the phosphorylated preparation on SDS gels (Fig. 4) with the Coomassie blue-stained polypeptides in gels run concurrently showed that the radioactivity corresponded to the 110,000-dalton polypeptide. The 42,000-dalton polypeptide component of the ($Na^+ + K^+$)-ATPase preparation was not positive to periodic acid-Schiff reagent even when 100 μg protein containing enzyme preparation was electrophoresed and stained. Furthermore, none of the polypeptide components of the enzyme preparation was stained by this reagent. In a control gel, as little as 0.4 μg hexose containing standard ovalbumin (20 μg) could easily be detected by the modified periodic acid-Schiff staining procedure. The concanavalin A-peroxidase staining procedure also did not reveal any glycoprotein component. The average purity of the upper fraction ($Na^+ + K^+$)-ATPase preparation was about 50%, based on the combined fractional content of the two major polypeptide components, as measured by integration of peak areas from densitometric tracing of Coomassie blue-stained gels. In Fig. 5 is shown the densitometric tracings of the polypeptide patterns of

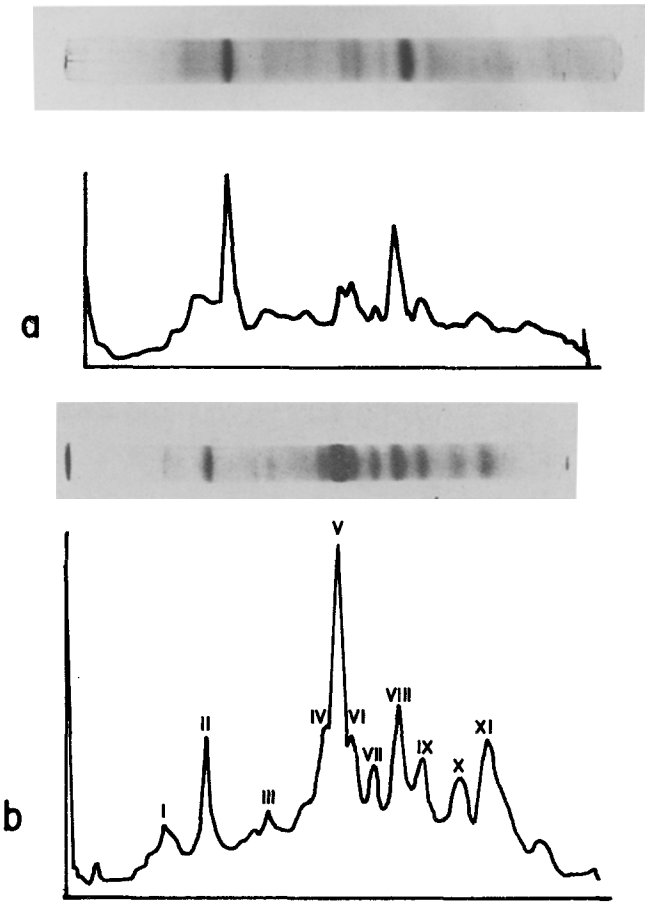

Fig. 3. Densitometric tracings and photographs of Coomassie blue stained SDS gels of the upper fraction ($Na^+ + K^+$)-ATPase preparation and of garfish olfactory nerve axon plasma membrane. Gels were stained with Coomassie blue and scanned at 550 nm. Samples containing 25 to 40 μg protein were prepared and electrophoresed as described in Materials and Methods. Origins are on the left. (*a*): Upper fraction ($Na^+ + K^+$)-ATPase preparation. (*b*): Axon plasma membrane. Above the tracings are shown the photographs of the Coomassie blue stained gels

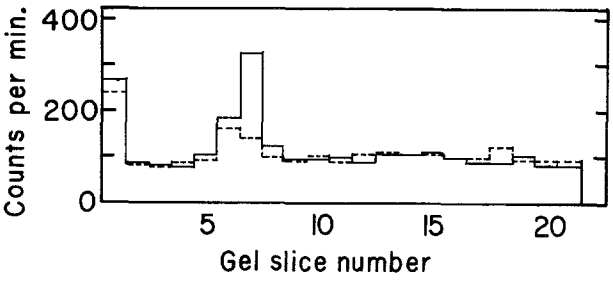

Fig. 4. SDS gel electrophoresis of the phosphorylated axon membrane ($Na^+ + K^+$)-ATPase. Purified ($Na^+ + K^+$)-ATPase (150 μg) from axon membrane was phosphorylated in the absence (——) and presence (-----) of 100 mM KCl according to Grefrath and Reynolds [13] and aliquots containing 50 μg protein electrophoresed according to the procedure of Fairbanks et al. [8]. Bars represent counts per min of individual gel slices. The origin is at the left

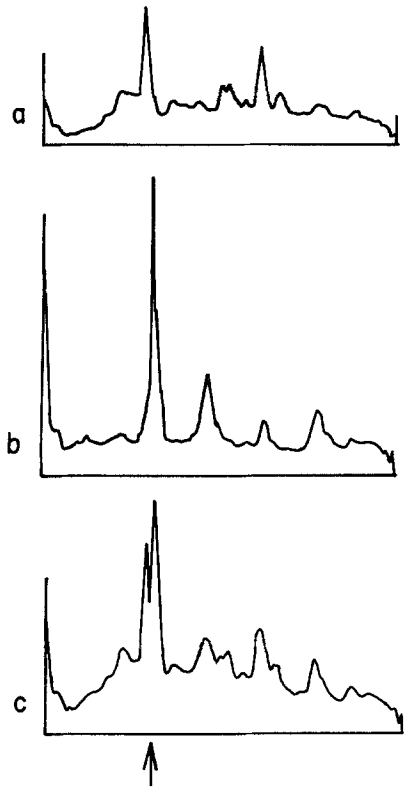

Fig. 5. Densitometric tracings of the polypeptide patterns of purified $(Na^+ + K^+)$-ATPases of garfish axon and rabbit kidney. For purposes of comparison gel scans are included of purified $(Na^+ + K^+)$-ATPase of garfish axon (*a*), rabbit kidney (*b*), and a mixture of axon and kidney (*c*). Gels were stained with Coomassie blue and scanned at 550 nm. Origins are on the left side of the tracings. Arrow indicates the large polypeptide component

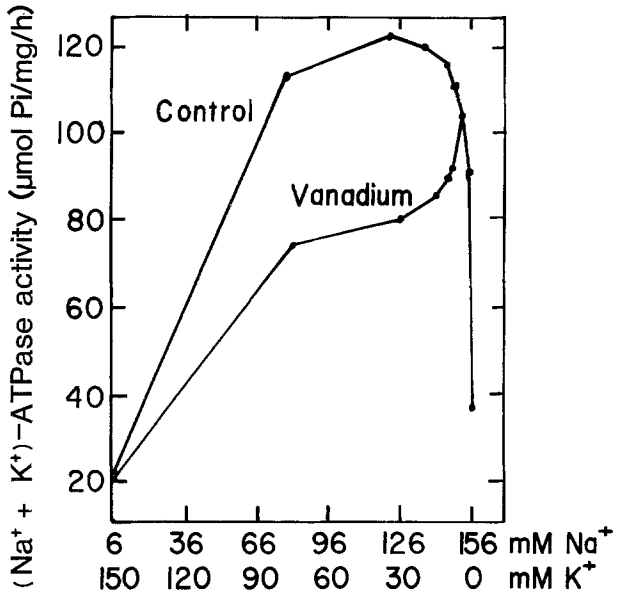

Fig. 6. The effect of vanadium on the sodium and potassium ion dependence of purified axon membrane $(Na^+ + K^+)$-ATPase. Axon membrane $(Na^+ + K^+)$-ATPase was purified as described in Materials and Methods. Before assay, the enzyme was activated with SDS in the presence or absence of 500 mM sodium orthovanadate for 30 min. Aliquots of the activated enzyme were then assayed in media containing various concentrations of Na^+ and K^+ with and without 500 mM sodium orthovanadate

garfish axon $(Na^+ + K^+)$-ATPase (*a*), rabbit kidney $(Na^+ + K^+)$-ATPase (*b*) and a mixture of the two (*c*). The catalytic subunit of garfish $(Na^+ + K^+)$-ATPase was clearly separable from that of the rabbit kidney enzyme. It has a lower electrophoretic mobility and thus an apparent higher molecular weight. The electrophoretic mobility of the small glycoprotein subunit of the kidney enzyme was much different from that of the small polypeptide (42,000 daltons) which co-purified with the catalytic subunit of the garfish $(Na^+ + K^+)$-ATPase. The garfish enzyme preparation did not reveal any polypeptide component comigrating with the 55,000-dalton glycoprotein component of the rabbit kidney $(Na^+ + K^+)$-ATPase.

Kinetic Properties

The dependence of the activity of the purified $(Na^+ + K^+)$-ATPase preparations to Na^+ and K^+ concentration is shown in Fig. 6. At a constant combined concentration of Na^+ and K^+ ions of 156 mM, the enzyme activity was maximum at about 126 mM Na^+ and 30 mM K^+. The activity of the enzyme fell rapidly at K^+ concentration below 20 mM. The membrane $(Na^+ + K^+)$-ATPase also showed a similar Na^+ and K^+ concentration dependence. In the presence of 500 nM vanadium both the purified and the membrane $(Na^+ + K^+)$-ATPase preparations showed reduced activities at all concentrations of Na^+ less than 145 mM and of K^+ greater than 5 mM. A sharp peak of activity occurred around 5 mM K^+. This effect of vanadium to alter the ionic dependence of the enzyme to Na^+ and K^+ concentrations is similar to that reported for $(Na^+ + K^+)$-ATPase of pig kidney [2].

The membrane and the purified $(Na^+ + K^+)$-ATPase preparations had similar pH and temperature profiles and K_m values for ATP. The optimum pH was about 7.5. Arrhenius plots of the $(Na^+ + K^+)$-ATPase activity as a function of temperature showed a transition temperature of about 22 °C for both the membrane and the purified enzyme preparations. The energy of activation of the membrane enzyme was 10.8 Kcal/mol above 22 °C and 20.3 Kcal/mol below

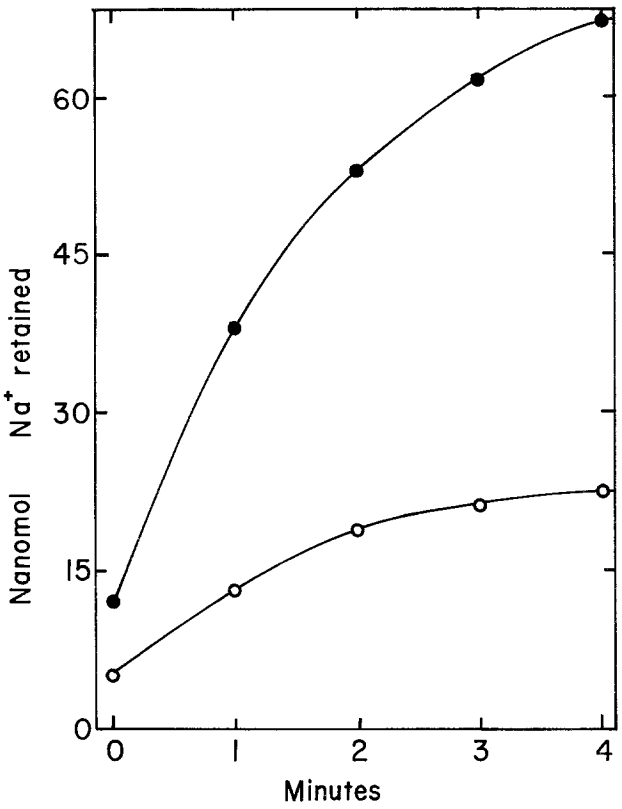

Fig. 7. Time course of the $^{22}Na^+$ uptake by the reconstituted ($Na^+ + K^+$)-ATPase in the presence and absence of ATP. Aliquots of proteoliposome (25 μg protein), prepared as described in Materials and Methods were assayed at 37 °C in a medium containing 50 mM imidazole-H_2SO_4, pH 7.4, 75 mM K_2SO_4, 50 mM Na_2SO_4, 20 mM β-mercaptoethanol, 4.5 mM $MgCl_2$ and $^{22}NaCl$ (60 cpm/nmol) in the absence (○) and presence (●) of 4.5 mM ATP in a total volume of 0.115 ml by the method of Gasko et al. [11]

22 °C. Similar values for energies of activation (11.6 and 22.6 Kcal/mol) were observed for the purified enzyme preparation. The K_m values of the membrane and purified ($Na^+ + K^+$)-ATPase preparations for ATP, as determined from the Lineweaver-Burk plots, were 0.16 and 0.3 mM, respectively.

Properties of Reconstituted ($Na^+ + K^+$)-ATPase

In Fig. 7 is shown the course of uptake of $^{22}Na^+$ at 37 °C by the reconstituted ($Na^+ + K^+$)-ATPase. The $^{22}Na^+$ uptake was time dependent and was stimulated by the presence of ATP. There was a rapid uptake of $^{22}Na^+$ initially, and the rate decreased with time. A specific activity of nearly 56 μmol of $^{22}Na^+$/mg protein/hr was obtained for the reconstituted enzyme preparation with a 1-min assay (Table 3). The ($Na^+ + K^+$)-ATPase preparation without asolectin but taken through freeze-thaw-sonication procedure did not show any $^{22}Na^+$ uptake activity. The $^{22}Na^+$

Table 3. $^{22}Na^+$ transport and ($Na^+ + K^+$)-ATPase activities of Reconstituted garfish nerve ($Na^+ + K^+$)-ATPase

Expt.	Ouabain (μM)	Sp. Act. (μmol Na^+/mg protein/hr)	Sp. Act. (μmol P_i/mg protein/hr)	Na^+ pumped / ATP hydrolyzed
1[a]	0	55.9	233.5	0.24
	50	55.4	59.3	0.93
11[b]	0	25.8	165.6	0.16
	50	19.5	20.2	0.97

[a] 1-min assays with 1 min preincubation at 37°; [b] 4-min assays with 1 min preincubation at 37°.

transport of the reconstituted enzyme was not sensitive to externally added ouabain (50 μM) with the 1-min assay, although it was reduced somewhat in the 4-min assay. About 75% of the ($Na^+ + K^+$)-ATPase activity of the enzyme after the reconstitution procedure was inhibitable by 50 μM ouabain. This is probably due to the low efficiency of incorporation of the enzyme preparation into phospholipid vesicles under the present experimental reconstitution conditions. Only a portion of the enzyme molecules in the reconstituted preparation is properly oriented within the proteoliposomes and therefore involved in the active ion transport. Interestingly, the Na^+ pumping efficiency, i.e., the ratio of $^{22}Na^+$ pumped to ATP hydrolyzed was found to be only about 1, regardless of whether determined from the initial rates of activity or the rates obtained with 4 min of assay. Under the same reconstitution and assay conditions, eel electroplax ($Na^+ + K^+$)-ATPase gave a $^{22}Na^+$ pumped to ATP hydrolyzed ratio of nearly 3. (D. Cohn and E. Racker, *unpublished observations*).

Discussion

Incubation of garfish axon membrane with SDS at optimum protein and detergent concentrations resulted in the removal of more than 80% of the membrane proteins with a corresponding increase in the ($Na^+ + K^+$)-ATPase specific activity. Only a small amount of lipids was removed from the membrane during this treatment as is evidenced by a fourfold increase in the lipid to protein ratio of the enzyme preparation. SDS-treatment of the rabbit kidney membrane during the purification of ($Na^+ + K^+$)-ATPase resulted [19], however, in a parallel loss of both lipids and proteins. An increase in the lipid to protein ratio, similar to what was obtained for axon membrane enzyme preparation, was also reported in the purification of ($Na^+ + K^+$)-ATPase from canine brain microsomes [36].

Electrophoretic analysis on SDS gels of the $(Na^+ + K^+)$-ATPase preparation from garfish axon membrane has revealed major differences in its polypeptide components, as compared to those of enzyme isolated from other sources. In our electrophoresis system, which is that of Fairbanks et al. [8], the large subunit of axon membrane enzyme was seen as a sharp single band after Coomassie blue staining of the gel. When mixed and electrophoresed together with the rabbit enzyme the large subunit of axon enzyme was clearly separable from that of the kidney enzyme and had a slower electrophoretic mobility. The large catalytic subunit of garfish axon membrane has an apparent molecular weight of 110,000. On SDS gels the corresponding subunit of kidney $(Na^+ + K^+)$-ATPase gave a molecular weight of 95,000. Reports have appeared recently on the presence of two forms of $(Na^+ + K^+)$-ATPase in brine shrimp [33] and in the brains of various vertebrate species [37]. The large catalytic subunits of the enzymes in these preparations appeared as doublets on SDS gels and the potassium-sensitive phosphorylation occurred in both bands. Sweadner [37] also found that, of the two forms, the higher molecular weight one was found in the $(Na^+ + K^+)$-ATPase of axon membrane isolated from the myelinated axons of rat brain and that the other one was found in the non-neural cells of brain. The large catalytic subunit of garfish axon membrane $(Na^+ + K^+)$-ATPase appears to resemble the high molecular weight form of the enzyme reported by Peterson et al. [33] and Sweadner [37], although comparative studies under identical experimental conditions are required for a definite conclusion. We find a difference of about 15,000 daltons between the catalytic subunits of garfish and kidney $(Na^+ + K^+)$-ATPase, while Sweadner [37] reports only a difference of 2000 daltons between the high and low molecular weight forms of the catalytic subunits of brain $(Na^+ + K^+)$-ATPase. The small polypeptide which co-purifies with the large catalytic subunit of the garfish axon membrane $(Na^+ + K^+)$-ATPase appears to differ considerably from that of other $(Na^+ + K^+)$-ATPase preparations in its carbohydrate content and also in its molecular weight. It was not possible to detect carbohydrates in the garfish enzyme on polyacrylamide gels using either the periodic acid-Schfiff reagent or the more sensitive concanavalin A-peroxidase stain for glycoprotein. Negative reactions of a protein to these stains are not sufficient evidence for the absence of carbohydrate. Nonetheless, it is reasonable to conclude that the carbohydrate content of the small subunit of the garfish $(Na^+ + K^+)$-ATPase is considerably less than that for other subunits. The apparent molecular weight of the small subunit of the garfish enzyme, estimated from SDS gels to be 42,000, is less than that of the kidney enzyme [19] but is similar to that of the brine shrimp enzyme [33], Species variation in the molecular weight and carbohydrate content are known to exist in the small subunit of $(Na^+ + K^+)$-ATPase, but the variation appears to be much more pronounced in the case of garfish $(Na^+ + K^+)$-ATPase. It is assumed in this discussion, that the 42,000-dalton polypeptide component in the garfish $(Na^+ + K^+)$-ATPase preparation corresponds to the glycoprotein component of other enzyme preparations, since it co-purifies with the catalytic subunit. Further studies such as NH_2-terminal amino acid analysis of the isolated polypeptide and cross linking of the large and the small peptides are required to show that the 42,000-dalton polypeptide is indeed related to the glycoprotein component found in other $(Na^+ + K^+)$-ATPase preparations.

Based on the fractional content of the large and the small polypeptide components analyzed on SDS gels, the final $(Na^+ + K^+)$-ATPase preparation obtained in the present study is about 50% pure. The highest specific activity found was 228 μmol/mg protein/hr, indicating that the pure enzyme from garfish olfactory nerve will have a maximum specific activity of about 450, far less than the value of 1500 to 2000 reported for other enzyme preparations [14, 16, 18, 20, 27]. A specific activity of about 800 μmol/mg protein/hr was recently reported for the $(Na^+ + K^+)$-ATPase preparations from brine shrimp [33] and the gills of *Anguilla anguilla* [3]; these enzyme preparations, however, were not completely pure. As compared to other enzyme preparations, the garfish enzyme, however, was similar in its molecular properties, such as K_m for ATP, pH optimum, energies of activation, Na^+ and K^+ dependence, and vanadium inhibition. These kinetic properties were similar for both the axon membrane $(Na^+ + K^+)$-ATPase and the purified enzyme, indicating that no alteration had occurred in enzyme properties during the purification.

The present studies show that the garfish nerve $(Na^+ + K^+)$-ATPase can be easily reconstituted into phospholipid vesicles by a freeze-thaw-sonication procedure. The properties, such as the ATP-dependent uptake of $^{22}Na^+$ and the lack of inhibition by externally added ouabain, are what would be expected of $(Na^+ + K^+)$-ATPase oriented inside out with respect to its *in vivo* orientation. The efficiency of reconstitution is about 25% which is similar to or better than those found by others, except that of Goldin [12] who, with a purified renal $(Na^+ + K^+)$-ATPase, has obtained a high efficiency of 95% using the cholate-dialysis procedure [22]. The initial rate of 56 μmol Na^+/mg protein/hr, is fairly high, as compared to those of other reconstituted $(Na^+ + K^+)$-ATPase preparations [12, 15, 34]. In the light of the low effi-

ciency of incorporation, the actual rate of Na^+ transport may be as much as three or four times greater. Therefore, the freeze-thaw-sonication reconstituted enzyme appears to be a suitable enzyme preparation for studying the transport properties of $(Na^+ + K^+)$-ATPase associated with axon plasma membrane. The pumping efficiency, as measured by the ratio of $^{22}Na^+$ pumped to ATP hydrolyzed was only 1, whereas the enzyme from the eel electroplax, reconstituted and assayed at the same conditions, gave a value of nearly 3. Although there is the possibility that we have not achieved the optimum reconstitution and assay conditions, which might have resulted in a low pumping efficiency, the alternate interesting possibility that the garfish nerve $(Na^+ + K^+)$-ATPase pumps Na^+ via a different mechanism cannot be excluded at present.

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