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PARTIAL PURIFICATION OF A SOLUBLE ($Na^+ + K^+$)-DEPENDENT ATPASE FROM RABBIT KIDNEY

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SUMMARY

A deoxycholate-solubilized ($Na^+ + K^+$)-dependent ATPase from rabbit renal cortex has been stabilized in buffers containing glycerol and purified about 50-fold by agarose gel filtration and glycerol density gradient centrifugation. The ATPase is ouabain-sensitive and requires phospholipid for maximum activity.

The optimal Na^+/K^+ ratio is about 10:1, and the optimal pH is 7.1. CTP and ITP, but not ADP or GTP, serve as alternate but less effective substrates. The partially purified ATPase contains K^+-activated *p*-nitrophenylphosphatase activity which is not accelerated by phospholipid.

INTRODUCTION

One of the obstacles to understanding the mechanism of ($Na^+ + K^+$)-dependent ATPase (ATP phosphohydrolase, EC 3.6.1.3) in active transport of cations across biological membranes[1,2] has been the failure to purify and stabilize the enzyme in soluble form. Recent reports from UESUGI *et al.*[3] and KAHLENBERG *et al.*[4] have indicated that a Lubrol-solubilized preparation from beef brain can be stabilized by ATP and Na^+ or K^+, but subsequent efforts toward purification were hindered by loss of activity.

TANAKA AND STRICKLAND[5] described the preparation of a deoxycholate-solubilized, phospholipid-activated ATPase from a membrane fraction of beef brain. An earlier report from this laboratory[6] showed that such a soluble preparation from rat kidney cortex also required phospholipid for maximum activity and that the requirement was best fulfilled by phosphatidylserine. The present communication describes the stabilization, partial purification, and properties of a soluble, ouabain-sensitive, ($Na^+ + K^+$)-dependent ATPase from rabbit kidney cortex. The final preparation possesses high specific activity and retains the phospholipid requirement.

EXPERIMENTAL PROCEDURE

Solutions and reagents

Solutions used in the purification procedure were of the following composition. Buffer A20: 50 mM Tris–maleate, 1 mM dithiothreitol, 1 mM EDTA, 20% (v/v)

glycerol (pH 7.2, 3°); Buffer A50: 50% (v/v) glycerol, *plus* other reagents as listed for Buffer A20.

ATP, *p*-nitrophenylphosphate, and phosphatidyl-L-serine were obtained from Nutritional Biochemicals Corp. Tris(hydroxymethyl)aminomethane (Trizma base), Tris–maleate, ouabain, dithiothreitol, ADP, CTP, ITP, *p*-nitrophenol, and bovine serum albumin were purchased from Sigma Chemical Co. GTP was obtained from Calbiochem and sodium deoxycholate from Mann Research Laboratories. 8% agarose (Bio-Gel A-1.5 m, 100–200 mesh) was obtained from Bio-Rad Laboratories, blue dextran from Pharmacia, and reagents for polyacrylamide electrophoresis from Canalco. All other materials were reagent grade.

Analytical methods

ATPase activity was determined in a reaction mixture containing 50 mM Tris–acetate buffer (pH 7.1, 37°), 3 mM $MgCl_2$, 100 mM NaCl, 10 mM KCl, 2 mM ATP–Tris (pH 7.0), 1 mM dithiothreitol, 0.15 mM phosphatidylserine, *plus* 1 mM ouabain as indicated. After incubation at 37° for 20 min as previously described[6], P_i was determined by the method of FISKE AND SUBBAROW[7]. Ouabain-sensitive, $(Na^+ + K^+)$-dependent ATPase activity ($(Na^+ + K^+)$-ATPase) was defined as the difference between values obtained without ouabain and those obtained in the presence of 1 mM ouabain (Mg^{2+}-ATPase). Since commercial phosphatidylserine activated the enzyme as well as preparations purified by column chromatography[8] or preparative thin-layer chromatography[9], unpurified phospholipid was used routinely. Aqueous emulsions of phosphatidylserine were prepared by drying a chloroform–methanol solution at 25° under a stream of N_2 and homogenizing in an all-glass Potter–Elvehjem apparatus.

p-Nitrophenylphosphatase activity was estimated in a mixture containing 50 mM Tris–acetate buffer (pH 7.1, 37°), 3 mM $MgCl_2$, 1 mM dithiothreitol, and 2 mM *p*-nitrophenylphosphate, in a final volume of 2 ml. Additions of 10 mM KCl, 0.15 mM phosphatidylserine, or 1 mM ouabain were made as indicated. The reaction was initiated by the addition of enzyme and was terminated after 20 min at 37° by adding 2 ml cold 10% (w/v) trichloroacetic acid. Following centrifugation, aliquots of the supernatant were mixed with 3 ml 1 M Tris in a final volume of 5 ml, and the $A_{405\ m\mu}$ was determined. K^+-activated *p*-nitrophenylphosphatase activity was calculated by subtracting values obtained in the presence of Mg^{2+} alone from those obtained in the presence of both Mg^{2+} and K^+.

Protein concentrations were measured according to LOWRY *et al.*[10], using crystalline bovine serum albumin as standard. Organic phosphorus was determined by the method of AMES[11], and deoxycholate was determined according to MOSBACH *et al.*[12].

Purification of soluble $(Na^+ + K^+)$-dependent ATPase

All operations were performed at 0–3°. Frozen rabbit kidneys, obtained from Pel-Freez Biologicals, Inc., served as a convenient source of enzyme if used within one month after slaughter. Kidneys were thawed in 0.25 M sucrose and the renal cortex homogenized in the same medium in an all-glass Potter–Elvehjem homogenizer. Preparation of a membrane fraction, treatment with 0.33% deoxycholate, and 35–60% $(NH_4)_2SO_4$ fractionation were performed essentially as described by TANAKA AND STRICKLAND[5]. The final $(NH_4)_2SO_4$ precipitate was suspended in Buffer A20 to give 5–6 mg protein/ml and centrifuged at 100 000 × *g* for 1 h. The supernatant,

which contained the enzyme referred to as "soluble", was dialyzed overnight against 50–100 vol. of Buffer A20.

The dialysate was concentrated 4-fold using Centriflo membrane filters supplied by Amicon Corp. Approx. 2 ml of concentrated enzyme (10–15 mg protein/ml) were then applied to a 25 mm × 360 mm column of 8% agarose which had been packed using Buffer A20. Upward flow of the same buffer was maintained at 10 ml/h with a Mariotte flask, and 3-ml fractions were collected. Void volume was determined as the elution volume of blue dextran. All of the $(Na^+ + K^+)$-dependent ATPase activity and approx. 25% of the protein eluted in the void volume (Fig. 1).

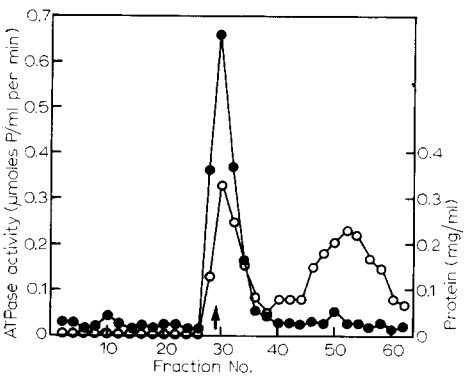


Fig. 1. Gel filtration of $(NH_4)_2SO_4$ fraction of $(Na^+ + K^+)$-dependent ATPase on 8% agarose. ATPase activity was measured in complete medium (see EXPERIMENTAL PROCEDURE). 81% of the applied ATPase activity was recovered. The arrow indicates the void volume. ●—●, ATPase; ○–○, protein.

Active fractions of the 8% agarose eluate were pooled and concentrated with Centriflo filters. The glycerol concentration was then diluted to 4% (v/v) with 1 mM EDTA–1 mM dithiothreitol, and the enzyme solution was reconcentrated. Approx. 0.5 ml (1–2 mg protein/ml) was layered over linear gradients (30 ml) of 5–20% (v/v) glycerol prepared in 50 mM Tris–maleate, 1 mM EDTA, 1 mM dithiothreitol (pH 7.2,

TABLE I

PURIFICATION OF $(Na^+ + K^+)$-DEPENDENT ATPase

Assay conditions are described in the text.

Step	*Volume (ml)*	*Protein (mg)*	*Specific activity (μmoles P/mg per min)*		*Recovery (%)*	*Purification (fold)*	*B/A*
			Mg^{2+}-ATPase (A)	*$(Na^+ + K^+)$-ATPase (B)*			
Homogenate	300	4470	0.125	0.088	100	1.0	0.7
Membrane fraction	36.0	421	0.219	0.092	9.9	1.0	0.4
$(NH_4)_2SO_4$ fraction	15.4	89.6	0.071	0.410	9.4	4.7	5.8
Agarose eluate	38.6	15.5	0.157	1.51	6.0	17.2	9.6
Glycerol gradient fraction	105	2.2	0.56	4.88	2.7	55.5	8.7

3°) over a 4-ml cushion of Buffer A50. Following centrifugation in a SW-25.1 rotor for 14 h at 24000 rev./min, fractions were collected dropwise from the bottom of the tubes. Fractions with high specific activity were pooled and used in experiments described below. For long-term storage, the purified enzyme solution was dialyzed for 36 h against Buffer A50 and stored at −20°.

The purification procedure is summarized in Table I.

Polyacrylamide gel electrophoresis

Enzyme preparations were mixed slowly with 9 vol. of acetone at −20°, stirred for 10 min at 0°, and centrifuged at 10000 × *g* for 5 min. The precipitates were dissolved in phenol–acetic acid–water (2:1:1, w/v/v) containing 4 M urea and submitted to electrophoresis on 5% polyacrylamide gels in the system of TAKAYAMA *et al.*[13], as modified by WIDNELL AND UNKELESS[14]. The lower electrode served as the cathode. Gels were stained in 1% amido schwarz in 7% acetic acid for 1.5 h and destained in several changes of 7% acetic acid.

RESULTS AND DISCUSSION

Stabilization of enzyme

The instability of $(Na^+ + K^+)$-dependent ATPase upon release from membrane preparations has hindered attempts at its purification[3,4]. We have observed that the $(NH_4)_2SO_4$ fraction of the enzyme, suspended in either 50 mM Tris–maleate alone, or 50 mM Tris–maleate *plus* 1 mM EDTA and 1 mM dithiothreitol (pH 7.2, 3°), lost 80–100% of its ouabain-sensitive enzymatic activity upon storage for 3 days at 3°. If, however, the solution contained 20% (v/v) glycerol, 70% of the original ouabain-sensitive ATPase activity was retained after 3 days. If the glycerol concentration was increased to 50%, full activity was maintained after storage for 24 days. Glycerol was thus recognized to be an effective stabilizing agent, with no requirement for substrate or activating cations. Glycerol, at concentrations of up to 10.5%, had no effect on the assay of $(Na^+ + K^+)$-dependent ATPase. However, dithiothreitol increased activity substantially and was used routinely.

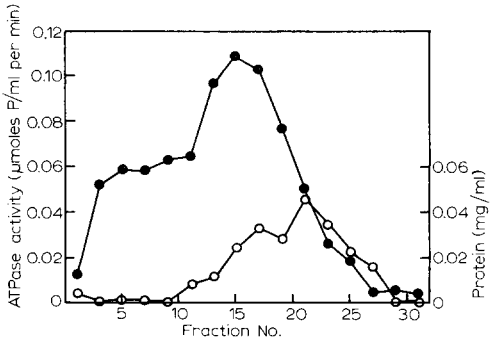


Fig. 2. Glycerol density gradient centrifugation of $(Na^+ + K^+)$-ATPase eluted from agarose column. ATPase activity was measured as in Fig. 1. ●—●, ATPase; ○—○, protein.

Purification of enzyme

The preparations described in this report had a specific activity of 4.5–6.5 μmoles P/mg protein per min at 37°, representing a 50- to 100-fold purification. Previously, the purest known solubilized ($Na^+ + K^+$)-dependent ATPase, with a specific activity of 2.2, was prepared from beef brain by KAHLENBERG *et al.*[4]. A particulate enzyme from rabbit kidney medulla, recently described by JØRGENSEN AND SKOU[15], had a maximum specific activity of 14.6 after a 19-fold purification. Our preparation thus appears to be one of the purest so far reported. Polyacrylamide electrophoresis of fractions at various stages of purification confirmed that significant purification had been achieved. The final preparation showed two major bands *plus* a small amount of non-migratory material (Fig. 3). Since denaturing conditions were employed for

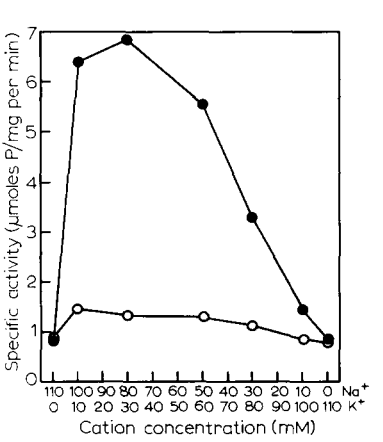
Fig. 3. Polyacrylamide gel electrophoresis of preparations at various stages of purification. Experimental conditions were as described in the text. A, membrane fraction; B, $(NH_4)_2SO_4$ fraction; C, glycerol gradient fraction.

electrophoresis, neither the excluded material or the bands can be associated with enzymatic activity. However, it is evident that the purification procedure removed a considerable number of non-ATPase proteins. Thus, the observed increase in specific activity appears to be due primarily to actual purification, and not to activation of the enzyme system by deoxycholate, which has been observed with particulate preparations from kidney[15–17].

The elution pattern of the solubilized ($Na^+ + K^+$)-dependent ATPase from 8% agarose (Fig. 1) is consistent with the gel filtration behavior of other solubilized ATPase preparations[3,4]. The somewhat disperse distribution of activity in the glycerol density gradient following centrifugation (Fig. 2) may represent varying degrees of protein aggregation.

Properties of purified $(Na^+ + K^+)$-dependent ATPase

The properties of soluble $(Na^+ - K^+)$-activated ATPase from rabbit kidney cortex, with respect to effects of cation concentration and pH, are in good agreement with results reported for particulate preparations isolated from mammalian kidney[15–18]. Results from experiments in which Na^+ and K^+ concentrations were varied while holding total concentration constant indicated that the presence of both cations is necessary for stimulation of ATPase activity (Fig. 4). Optimal activity was observed at a Na^+/K^+ ratio of between 10:1 and 8:3. Ouabain depressed ATPase activity in the presence of both Na^+ and K^+, but had no effect with either cation alone.



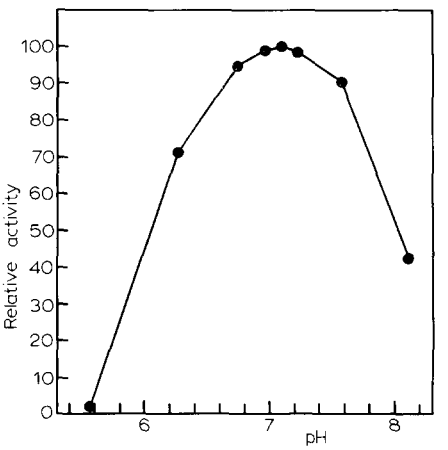


Fig. 4. Effect of $[Na^+]/[K^+]$ ratio on ATPase activity. ●—●, complete medium with indicated $[Na^+]$ and $[K^+]$; ○—○, *plus* 1 mM ouabain.

Fig. 5. Effect of pH on ouabain-sensitive $(Na^+ + K^+)$-dependent ATPase activity measured in 50 mM Tris–maleate buffers. Other components of the assay medium were as described in the text. Measurements of pH were made during the incubation.

TABLE II

SUBSTRATE SPECIFICITY OF TRIPHOSPHATASE ACTIVITY

All nucleotides were assayed at a concentration of 2 mM.

Nucleotide	*Phosphatidyl-serine*	*Specific activity (μmoles P/mg per min)*	
		Mg^{2+}-dependent	*$(Na^+ + K^+)$-dependent*
ATP	−	0.40	2.28
	+	0.63	4.87
ADP	−	0.13	0
	+	0.14	0
CTP	−	0.53	0.48
	+	0.24	3.73
ITP	−	0.27	0.13
	+	0.43	0.78
GTP	−	0.48	0
	+	0.26	0.06

The dependence of ouabain-sensitive ATPase activity on pH is shown in Fig. 5. Maximum activity was observed at pH 7.1.

CTP and ITP, but not ADP or GTP, served as alternate but less effective substrates for the purified ATPase (Table II). The hydrolysis of CTP and ITP was inhibited by ouabain and accelerated by phosphatidylserine.

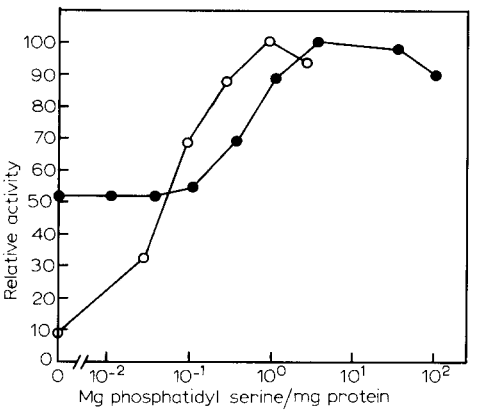


Fig. 6. Effect of phosphatidylserine/protein ratio on ouabain-sensitive ATPase activity of $(NH_4)_2SO_4$ fraction (○—○) and glycerol gradient fraction (●—●). Optimal phospholipid concentrations were 150 and 15 μM, and maximum ATPase activities were 0.33 and 4.58 μmoles P/mg protein per min, for the $(NH_4)_2SO_4$ fraction and glycerol gradient fraction, respectively.

The effect of phosphatidylserine on ouabain-sensitive ATPase activity is shown in Fig. 6. The optimal phospholipid/protein ratio for the purified preparation was about 4-fold higher than that for the $(NH_4)_2SO_4$ fraction. This change in ratio which accompanies purification may be interpreted as an index of the specificity of phosphatidylserine for $(Na^+ + K^+)$-dependent ATPase. If the phospholipid interacted equally with the various proteins in the two fractions studied, the optimal phospholipid/protein ratio should not change upon purification. However, if the phospholipid interacted mainly with the $(Na^+ + K^+)$-dependent ATPase, the observed ratio should vary directly with the degree of purification. Our results lead to the latter conclusion.

TABLE III

EFFECT OF PURIFICATION ON RATIO OF PHOSPHOLIPID-ACTIVATED/PHOSPHOLIPID-INDEPENDENT ATPase ACTIVITY

Step	*$(Na^+ + K^+)$-ATPase activity (μmoles P/mg per min)*		*B/A*
	− Phosphatidylserine (A)	*+ Phosphatidylserine (B)*	
Homogenate	0.091	0.088	1.0
Membrane fraction	0.081	0.092	1.1
$(NH_4)_2SO_4$ fraction			
a. Undialyzed	0.050	0.410	8.2
b. Dialyzed	0.123	0.379	3.1
Agarose eluate	0.568	1.51	2.7
Glycerol gradient fraction	2.25	4.88	2.2

The decrease of the ratio phospholipid-activated/phospholipid-independent ATPase activities upon purification (Table III) does not appear to be due to instability of the phospholipid-activated portion. While 92% of the total activity remained after dialysis of the $(NH_4)_2SO_4$ fraction, the ratio of ouabain-sensitive ATPase activity in the presence of phospholipid to activity in its absence decreased from 8.2 to 3.1. Under similar conditions of dialysis, residual deoxycholate was lowered from 0.087 to 0.038 mg/mg protein. Phospholipid might, therefore, activate the enzyme by removing bound detergent, as suggested by EMMELOT AND BOS[19].

TABLE IV

p-NITROPHENYLPHOSPHATASE ACTIVITY IN PARTIALLY PURIFIED ATPase PREPARATIONS

Step	*Ouabain*	*Specific activity (μmoles p-nitrophenol/mg per min)*			
		Mg^{2+}-activated		*K^+-activated*	
		−Phosphatidylserine	*+Phosphatidylserine*	*−Phosphatidylserine*	*+Phosphatidylserine*
$(NH_4)_2SO_4$ fraction, dialyzed	−	0.030	0.026	0.069	0.077
	+	0.028	0.022	0.004	0.004
Glycerol gradient fraction	−	0.05	0.02	1.08	0.88
	+	0.01	0.02	0.14	0.06

The partially purified enzyme contained a highly active K^+-activated, ouabain-sensitive *p*-nitrophenylphosphatase (Table IV), which has been found associated with $(Na^+ + K^+)$-dependent ATPase preparations of lower specific activity[20–22]. Purification of K^+-activated *p*-nitrophenylphosphatase followed that of $(Na^+ + K^+)$-dependent ATPase, with an increase in specific activity of 11.4- and 11.9-fold, respectively from the $(NH_4)_2SO_4$ fraction to the final purified preparation, giving further evidence for close association of the two enzymatic activities. It should be noted that the undialyzed $(NH_4)_2SO_4$ fraction contained an ouabain-sensitive *p*-nitrophenylphosphatase which was not responsive to added K^+. This enzymatic behavior may be attributed to residual NH_4^+, which substitutes for K^+ as an activating cation for the *p*-nitrophenylphosphatase[21].

Phosphatidylserine failed to significantly increase K^+-activated *p*-nitrophenylphosphatase activity in the $(NH_4)_2SO_4$ fraction (Table IV), in contrast with a recent report of TANAKA[22]. In fact, K^+-activated *p*-nitrophenylphosphatase activity in the final purified preparation appeared to be inhibited by phospholipid. On the other hand, phosphatidylserine increased $(Na^+ + K^+)$-dependent ATPase activity of the same preparation by 115%. It thus appears that while phosphatidylserine is required by the ATPase, it is not required for K^+-activated *p*-nitrophenylphosphatase activity, which is thought by some investigators to represent the final step in the mechanism of $(Na^+ + K^+)$-dependent ATPase[2,23].

Our results indicate that $(Na^+ + K^+)$-dependent ATPase from rabbit kidney can be stabilized and purified in soluble form, allowing further investigations on the mechanism and molecular nature of the enzyme.

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