PREPARATION OF HIGHLY ACTIVE ($Na^+ + K^+$)-ATPase FROM THE OUTER MEDULLA OF RABBIT KIDNEY

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Summary

It is described how ($Na^+ + K^+$)-ATPase from the outer medulla of rabbit kidney was purified to a specific activity of 881 ±25 μmoles Pi/mg protein per hr at 37 C. The procedure consists of treatment of a microsomal fraction with deoxycholate and subsequent fractionation by differential centrifugation and sucrose gradient centrifugation. The "purity" of the preparation was estimated to be 31-61%.

Introduction

In cell homogenates the ($Na^+ + K^+$)-activated enzyme system (($Na^+ + K^+$)-ATPase) is firmly bound to subcellular particles, and a pure preparation of the enzyme system has not been obtained (1). The highest specific activity of ($Na^+ + K^+$)-ATPase reported is 200 - 300 μmoles Pi/mg protein per hr for preparations from mammalian tissues (2, 3, 4) and 360 - 516 μmoles Pi/mg protein per hr for preparations from the electroplax of eels (5, 6).

In a crude microsomal fraction from the outer medulla of rat kidney the activity of ($Na^+ + K^+$)-ATPase after treatment with deoxycholate was above 200 μmoles Pi/mg protein per hr (7). In similar preparations from rabbit kidney the activity was even higher, and an effort was therefore made to purify the enzyme from this source further.

Experimental

Tissue from the dark red outer medulla and from the outer cortex was obtained by dissection of transverse sections of rabbit kidneys placed on a block of frozen 0.03 M histidine, 0.25 M sucrose, pH 7.2 (20 C), (h-s). For preparation of the microsomal fraction homogenates (10 w/v % in h-s) were centrifuged at 7,000 x g for 15 min. The sediment was resuspended by

homogenization in h-s and centrifuged again at 7,000 x g for 15 min. The combined supernatants were centrifuged at 48,000 x g for 30 min in a Sorval RC-2B centrifuge. The pellet was resuspended by homogenization in h-s to a concentration of 2.5 mg protein per ml and was stored at - 25 C in 1.5 ml aliquots. The procedures for incubation with deoxycholate, enzyme analysis, and differential centrifugation of the microsomal fraction are described in the legends. Sucrose density gradients of 4 ml ranging from 10 to 45 g sucrose per 100 ml were prepared using a mixing chamber (8). 0.5 ml of the enzyme preparation was layered over the gradient and centrifuged at 20,000 rpm for 120 min in a swinging bucket rotor (Christ No 9630) of the model Omega ultracentrifuge. For sampling of resultant bands for enzyme and protein analysis the tubes were placed in a block of lucite and punctured from the side at 4 mm intervals. 10 - 11 fractions of 0.4 ml were collected. Density measurements were made with an Abbe, PCI refractometer at 20 C. Protein was determined by the micro-Kjeldahl method or by the method of Lowry et al. (9) after precipitation and wash with 5% TCA at 0-4 C. The Lowry method was standardized with micro-Kjeldahl determinations, and bovine serum albumin (Armour) was used as working standard.

Results and discussion

Table I shows that the specific activity of the $(Na^+ + K^+)$-ATPase is about 3 fold higher in the microsomal fraction from the outer medulla than in the preparation from the outer cortex. Furthermore, that incubation with deoxycholate and EDTA leads to a 5-6 fold increase in the activity of $(Na^+ + K^+)$-ATPase, whereas it has practically no effect on the Mg^{++}-ATPase. This effect of deoxycholate agrees with previous results of studies on preparations from kidney (4, 10, 11) and brain (2, 4).

The optimal conditions for treatment of the microsomal fraction with deoxycholate were evaluated in a series of experiments in which the time course and the concentration dependence of the activation by deoxycholate of the $(Na^+ + K^+)$-ATPase were examined. It was found that 0.6 mg deoxycholate

TABLE I

THE EFFECT OF INCUBATION WITH DEOXYCHOLATE ON THE ACTIVITY OF $(Na^+ + K^+)$-ATPase IN THE MICROSOMAL FRACTION FROM THE OUTER MEDULLA AND THE OUTER CORTEX OF RABBIT KIDNEY.

	Outer medulla		Outer cortex	
	$(Na^+ + K^+)$-ATPase	Mg^{++}-ATPase	$(Na^+ + K^+)$-ATPase	Mg^{++}-ATPase
	μmoles Pi/mg protein per hr			
Fresh	46 ± 8 (6)	97 ± 5 (6)	17 ± 1 (4)	27 ± 2 (4)
DOC	268 ± 8 (6)	88 ± 4 (6)	84 ± 4 (4)	31 ± 1 (4)

Aliquots of the microsomal fractions (approx. 0.25 mg protein) were incubated in 1 ml with 0.6 mg sodium deoxycholate per ml, 2 mM EDTA, 25 mM imidazole (pH 7.0, 20 C) at 20 C. After 30 min, 25 ul was transferred to test tubes (1 ml) containing 3 mM Mg^{++}, 130 mM Na^+, 20 mM K^+, 3 mM ATP (Tris salt), 30 mM histidine (pH 7.5, 37 C). After 10 min at 37 C the reaction was stopped with 100 ul 50% TCA and inorganic phosphate was measured (17). The activity under "Fresh" was measured after parallel control incubations without deoxycholate and EDTA. $(Na^+ + K^+)$-ATPase was calculated as the difference in activity with and without 1 mM ouabain added to the test tubes. Mg^{++}-ATPase is the activity in the presence of 1 mM ouabain. Mean values ± standard error of the means are given.

per ml and 2 mM EDTA gave a maximal enhancement of the activity of $(Na^+ + K^+)$-ATPase within 30 min when the incubation was performed at 20 C and pH 7.0.

Table II shows that an increase in the specific activity of $(Na^+ + K^+)$-ATPase was achieved by differential centrifugation of the microsomal fraction treated with deoxycholate and EDTA (cf. 4). This was due in part to the removal of inactive protein and in part to further fractionation of the microsomal particles. About 40% of the protein in the microsomal fraction was removed with the supernatant, which was devoid of ATPase activity. 42% of the $(Na^+ + K^+)$-ATPase in the microsomal fraction was recovered in the sediment after 25,000 x g, whereas only 18% of the protein and 18% of the

TABLE II

FRACTIONATION OF $(Na^+ + K^+)$-ATPase IN THE MICROSOMAL FRACTION FROM THE OUTER MEDULLA OF RABBIT KIDNEY.

	Total protein	$(Na^+ + K^+)$-ATPase	Mg^{++}-ATPase	$(Na^+ + K^+)$-ATPase	Mg^{++}-ATPase
	mg	µmol.Pi/fract./hr		µmol.Pi/mg prot./hr	
Microsomal fract.	3.12 ± 0.01	848 ± 17	285 ± 2	272 ± 5	90 ± 1
Sed. 5,600 x g	0.95 ± 0.05	307 ± 3	157 ± 8	328 ± 21	166 ± 2
Sed. 25,000 x g	0.56 ± 0.04	356 ± 1	53 ± 3	635 ± 41	96 ± 12
Sed. 105,000 x g	0.31 ± 0.01	94 ± 5	22 ± 2	307 ± 24	71 ± 6
Supernatant	1.31 ± 0.11	0	0	0	0
Recovery	3.13 ± 0.06	757 ± 7	232 ± 4		
Recovery in %	101 ± 2	90 ± 3	82 ± 1		

The microsomal fraction was incubated with 0.6 mg sodium deoxycholate per ml, 2 mM EDTA, 25 mM imidazole (pH 7.0, 20 C) at 20 C for 30 min. 11 ml of the incubation medium was centrifuged for 30 min at each velocity in a type 65 rotor of the L2-65 B Beckman ultracentrifuge at 0-4 C. The sediments were resuspended by homogenization in 4 ml 25 mM imidazole (pH 7.0, 20 C) and ATPase activity was measured as in Table I. The results are averages of 4 separate centrifugations. Mean values ± standard error of the means are given.

ouabain-insensitive Mg^{++}-ATPase were found in this fraction. Accordingly, the specific activity of $(Na^+ + K^+)$-ATPase in the $Sed_{25,000}$ (635 ± 41 µmoles Pi/mg protein per hr) was 2.3 fold higher than in the original microsomal fraction.

A further increase in the activity of $(Na^+ + K^+)$-ATPase was obtained by centrifugation of freshly prepared $Sed_{25,000}$ in a continuous sucrose gradient. This is shown in Fig. 1. The activity in 3 of the 11 fractions collected was higher than in the original $Sed_{25,000}$. On an average the activity of $(Na^+ + K^+)$-ATPase in the fractions with a density of 1.095 to 1.105 g/ml was 881 ± 25 (n = 5) µmoles Pi/mg protein per hr. The activity

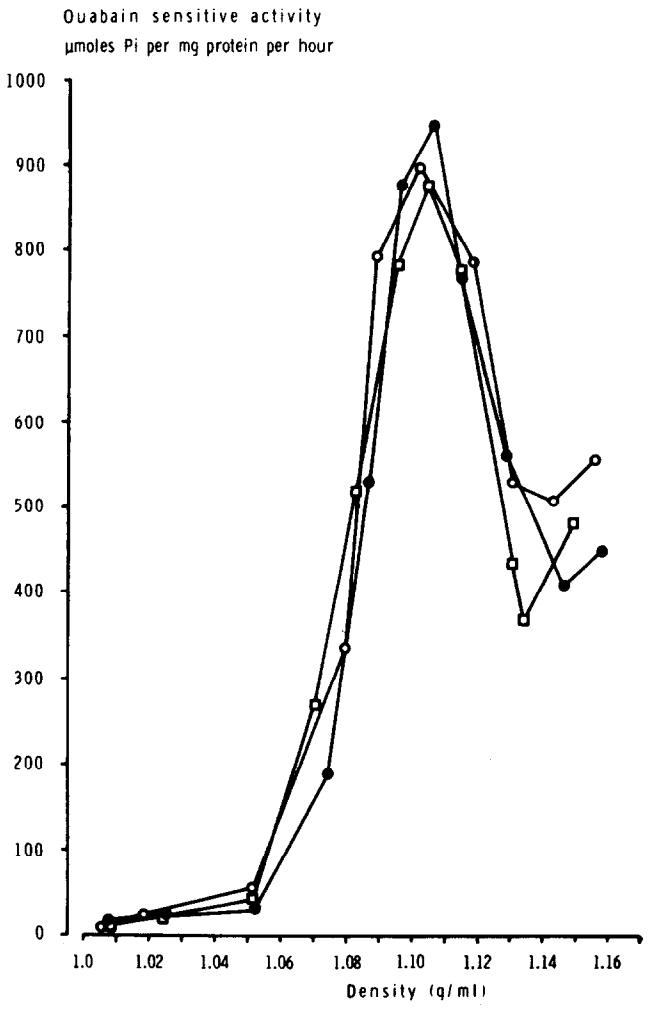

Fig. 1. The distribution of $(Na^+ + K^+)$-ATPase in a continuous sucrose gradient. The resuspended $Sed_{25,000}$ (approx. 0.6 mg protein) was layered over a gradient ranging from 10 to 42 g sucrose per 100 ml. Fractions were collected as described under Experimental and enzyme activity measured as in Table I. The results of 3 separate experiments are shown.

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of Mg^{++}-ATPase in these fractions was 98 ± 4 (n = 5) µmoles Pi/mg protein per hr.

It was thus possible by gradient centrifugation to increase the activity of $(Na^+ + K^+)$-ATPase in the $Sed_{25,000}$ by 1.4 fold, whereas the activity of the Mg^{++}-ATPase remained unchanged.

Fig. 2 shows the $(Na^+ + K^+)$-activation of highly active enzyme

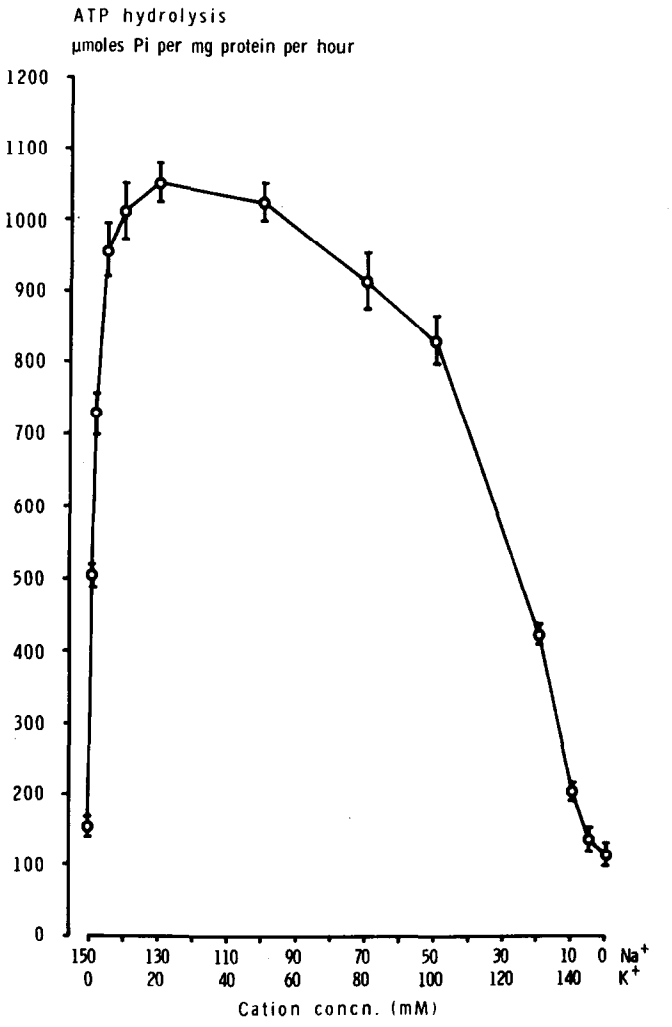

Fig. 2. The activation by $Na^+ + K^+$ of the ATP hydrolysis by fractions collected at a density of 1.095 - 1.105 g/ml after sedimentation of the $Sed_{25,000}$ in a continuous sucrose gradient as in Fig. 1. The ratio between Na^+ and K^+ in the test tubes was varied at a constant ionic strength. ATP 3 mM, Mg^{++} 3 mM. Incubated at 37 C and pH 7.5 for 5 min. The results are means ± standard error of separate determinations on 3 fractions collected from 2 gradients.

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preparations recovered from sucrose gradients. The specific activity of the $(Na^+ + K^+)$-dependent part of the ATPase activity was 934 ± 61 µmoles Pi/mg protein per hr. The shape of the curve is closely similar to that previously reported for enzyme preparations from kidney (4, 11). The optimum was at 130 mM Na^+ + 20 mM K^+, and the stimulation by $Na^+ + K^+$ was about 10 fold.

The specific activity of $(Na^+ + K^+)$-ATPase in the preparations obtained from the outer medulla is thus about 3 fold higher than reported before for preparations from mammalian tissues (2, 3, 4) and 1.7 to 2.4 fold higher than for preparations from the electroplax of eels (5, 6).

From estimates of the molecular activity (1.2×10^4 min^{-1} (12)) and of the molecular weight of $(Na^+ + K^+)$-ATPase the purity of the present preparation can be calculated as the fraction of the total protein, which consists of enzyme. For a molecular weight of 250,000 (13) and 500,000 (14) the purity is 31% and 61%, respectively. Such calculations are, however, of limited value, because there are no measurements of the molecular activity of the enzyme prepared by the method described above and because the exact molecular weight of the enzyme system is unknown.

The highly active $(Na^+ + K^+)$-ATPase from the outer medulla is probably located in the thick ascending limbs of Henle (7, 15), in which Na^+ is transported against a steep electrochemical gradient (16). In view of this, it will be of interest to learn if the higher specific activity is due to a higher molecular activity of $(Na^+ + K^+)$-ATPase in this preparation, to the isolation of membrane fragments with a higher density of enzyme sites per unit membrane area, or if it is due to a better purification of the enzyme system as such.

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REFERENCES

1) Skou, I.C., Physiol. Rev., 54,596(1965).
2) Schoner, W., von Ilberg, C., Kramer, R., and Seubert, W., European J. Biochem., 1,334(1967).
3) Schwartz, A., and Moore, C.A., Am. J. Physiol., 214,1163(1968).

4) Skou, I.C., Biochim. Biophys. Acta, 58,314(1962).
5) Albers, R.W., Koval, G.J., and Siegel, G.J., Mol. Pharmacol., 4, 324(1968).
6) Albers, W., Ann. Rev. Biochem., 36,727(1967).
7) Jørgensen, P.L., Acta Physiol. Scand., 74,11A(1968).
8) Britten, R.J., and Robertis, B.B., Science, 131,32(1960).
9) Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J., J. Biol. Chem., 193,265(1951).
10) Charnock, J.S. and Post, R.L., Austral. J. Exp. Biol., 41,547(1963).
11) Jørgensen, P.L., Biochim. Biophys. Acta, 151,212(1968).
12) Bader, H., Post, R.L., and Bond, E., Biochim. Biophys. Acta, 150, 41(1968).
13) Kepner, G., and Macey, R., Biochim. Biophys. Acta, 163,188(1968).
14) Mizuno, N., Nagano, K., Nakao, T., Tashima, Y., Fujita, M., and Nakao, M., Biochim. Biophys. Acta, 168,311(1968).
15) Schmidt, U., and Dubach, U.C., Pflügers Arch., 306,219(1969).
16) Giebish, G., and Windhager, E.E., Am. J. Med., 36,643(1964).
17) Fiske, C.H., and Subbarow, Y., J. Biol. Chem., 66,375(1925).