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COMPARISON OF SOURCES OF A PHOSPHORYLATED INTERMEDIATE IN TRANSPORT ATPase

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SUMMARY

1. The relationship between the enzymatic activity and the phosphorylated intermediate of the ($Na^+ + K^+$)-dependent ATPase (EC 3.1.6.3) from 11 species and 6 tissues was tested.

2. The range in specific activity of the ATPase was more than 400-fold, yet the range in the ratio of the ($Na^+ + K^+$)-ATPase activity to the phosphorylated intermediate was only 2-fold, and the range in the ratio of the (K^+)-acylphosphatase (EC 3.6.1.7) activity to the ($Na^+ + K^+$)-ATPase activity was also only 2-fold.

3. The ($Na^+ + K^+$)-ATPase of all sources tested required Na^+ for phosphorylation and its dephosphorylation was greatly accelerated in the presence of K^+.

4. The turnover of the phosphorylated enzyme at 0° in the absence of K^+, estimated by washout of ^{32}P, showed a range of 3-fold.

5. The phosphorylated enzymes yielded identical ^{32}P-labeled peptides after digestion with pepsin (EC 3.4.4.1) or pronase and electrophoresis. The ^{32}P-labeled peptides of the ($Na^+ + K^+$)-ATPase of all sources tested were sensitive to hydroxylamine.

6. The results indicate that the mechanism of the ($Na^+ + K^+$)-dependent ATPase is likely to be similar in most animal species and tissues.

INTRODUCTION

[^{32}P]ATP phosphorylates membrane preparations having transport ATPase activity, specifically ($Na^+ + K^+$)-ATPase (EC 3.6.1.3). The terminal phosphate group of ATP appears to be transferred to an acyl group on a protein. Na^+ stimulates formation in the presence of Mg^{2+}; K^+ stimulates breakdown to P_i. The phosphorylated material is thought to be an intermediate in the active transport of Na^+ and K^+. Evidence supporting these conclusions in one or more respects has come from at least 10 laboratories using similar techniques with material from 7 species and 3 tissues[1,2]. To test further the relationship between the enzymatic activity and the phosphorylated material we varied the species of animal and the tissue from which the membrane preparation was made. We tested the effect of Na^+ and K^+ on the amount of the

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phosphorylated material, we estimated the turnover directly and indirectly, and we partially characterized chemically the phosphorylated material from 11 species and 6 tissues. An associated K^+-dependent acylphosphatase (EC 3.6.1.7) activity[3–6] was also tested.

METHODS

Membrane preparations were made according to POST AND SEN[7] except that those from erythrocytes were made according to SEN AND POST[8] and those from the electroplax of the electric eel were made according to ALBERS AND KOVAL[9]. ($Na^+ + K^+$)-ATPase activity was estimated according to POST AND SEN[7]; K^+-dependent acylphosphatase activity was estimated according to BADER AND SEN[3]. By definition one unit of activity releases 1 μmole P_i per min at 37° under standard conditions. Phosphorylation of the membranes with [^{32}P]ATP in the presence of Na^+ and Mg^{2+} at 0° was according to POST AND SEN[10]. [^{32}P]Intermediate was estimated by subtracting background phosphorylation appearing when Na^+ was replaced by K^+ in the reaction mixture. The turnover number at 37° was estimated from the ratio of ($Na^+ + K^+$)-ATPase activity to [^{32}P]intermediate because the amount of the [^{32}P]intermediate was the same at 0° and 40° (see ref. 11). The rate constant of the turnover of the phosphorylated material at 0° was estimated according to POST, *et al.*[11]. At zero time excess unlabeled ATP was added to the phosphorylating system and acid was added at 5 sec. The turnover was estimated from the loss of radioactivity with the assumption that the loss was exponential with time. The phosphorylated material was solubilized by peptic digestion (pepsin, EC 3.4.4.1) and isolated by paper electrophoresis according to BADER, SEN AND POST[12]. Treatment of the soluble fragments with 0.1 M NH_2OH at pH 6.5 was for 15 min at 23°.

RESULTS AND DISCUSSION

Table I shows a correlation between the ($Na^+ + K^+$)-ATPase activity, the phosphorylated material, and the (K^+)-acylphosphatase activity. The range in specific activity of the ATPase was more than 400-fold, yet the range in the ratio of the ($Na^+ + K^+$)-ATPase activity to the intermediate, expressed as turnover number at 37°, was only 2-fold (disregarding the electroplax for the moment) and the range in the ratio of the (K^+)-acylphosphatase activity to the ($Na^+ + K^+$)-ATPase activity was also only 2-fold. Direct estimation of turnover of the intermediate at 0° by washout of radioactivity showed a range of 3-fold. We have encountered a similar variability in preparations made only from guinea-pig kidney over a period of 2 years. In an additional test (not shown), every preparation except that of the erythrocyte lost at least 80% of its radioactivity within 5 sec after addition of 16 mM KCl at 0°. The determinations in this table were done twice. In all cases except the erythrocyte, the background radioactivity (without Na^+) was less than 20% of the maximal values. The estimates of the enzyme activities of the electroplax preparation are probably low. At 37° this preparation is unstable; it is usually assayed at 26° (see ref. 9). A calculation of a correction for this instability raised the turnover at 37° to 16000 min^{-1}.

In each preparation the phosphorylated material was characterized by peptic

TABLE I

COMPARISON OF TRANSPORT ATPASE, PHOSPHORYLATED INTERMEDIATE AND (K^+)-DEPENDENT ACYLPHOSPHATASE FROM VARIOUS SOURCES

Reaction mixture for ($Na^+ + K^+$)-ATPase in 1 ml: 20 to 30 μg protein, 20 mM imidazole glycylglycine (pH 7.6), 4 mM ATP, 5 mM $MgCl_2$, 125 mM NaCl and 25 mM KCl for 20 min at 37° with subtraction of activity in a control tube in which 0.25 mM ouabain replaced NaCl and KCl. Reaction mixture for ^{32}P-intermediate in 1 ml: 0.4 to 1.0 mg protein, 10 mM imidazole glycylglycine (pH 7.6), 2 mM $MgCl_2$, 16 mM NaCl and 0.04 mM [^{32}P]-ATP for 10 sec at 0° with subtraction of phosphorylation in a control tube in which KCl replaced NaCl. The reaction was stopped with acid. Radioactivity and protein were estimated in the denatured, washed precipitate. Turnover at 0°: the ^{32}P-intermediate was formed in the same way. 5 sec before the acid a 50-fold excess of unlabeled ATP was added. From the loss of radioactivity in the intermediate the turnover was estimated with an assumption of exponential kinetics. Reaction mixture for (K^+)-acylphosphatase in 1 ml: 20–30 μg protein, 20 mM imidazole glycylglycine (pH 7.6), 7 mM acetyl phosphate, 4 mM $MgCl_2$ and 10 mM KCl for 20 min at 37° with subtraction of activity in a control tube in which KCl was replaced by 0.25 mM ouabain. In the case of the erythrocyte the turnover number at 37° was estimated by comparison with that from guinea pig. The yield of [^{32}P]intermediate was estimated from this turnover.

Animal	*Tissue*	*($Na^+ + K^+$)-ATPase (a)* *(units·(mg protein)$^{-1}$)*	*[^{32}P]Intermediate without K^+ (b)* *(μmoles ^{32}P·(mg protein)$^{-1}$)*	*Turnover number at 37° (a/b)* *(min^{-1})*	*Turnover without K^+ at 0°* *(min^{-1})*	*(K^+)-acyl-phosphatase (c)* *(units·(mg protein)$^{-1}$)*	*Ratio (c/a)*
Man	Kidney	0.47	37	12 700	4.1	0.50	1.1
Man	Erythrocyte	0.007	0.6*	12 100*	6.5*	0.01	1.5
Rabbit	Kidney	2.67	299	8 900	4.6	3.73	1.40
Rabbit	Brain	1.18	140	8 400	5.6	1.95	1.65
Dog	Kidney	1.57	94	16 700	5.1	4.27	1.43
Lamb	Kidney	2.44	250	9 800	5.0	1.60	1.02
Guinea pig	Kidney	2.98	265	11 300	5.0	3.25	1.34
Hamster	Kidney	2.73	192	14 200	—	—	—
Gerbil	Kidney	3.07	232	13 200	—	—	—
Goos	Kidney	1.42	128	11 100	4.3	2.22	1.57
Frog	Brain	0.80	87	9 200	3.8	1.00	1.25
Electric eel	Electroplax	3.17*	720	4 400*	10.9	2.67	0.84
Octopus	Gill	0.10	8	12 500	5.0	0.10	1.0
Octopus	Digestive gland	0.17	21	8 100	—	—	—

* See text for discussion.

digestion and paper electrophoresis. Fig. 1 shows that in every case except that of the erythrocyte there appeared three positively charged fragments with similar mobilities at pH 2. In each case the radioactivity in these three fragments moved with P_i when the material was treated with hydroxylamine before electrophoresis. These fragments did not appear when Na^+ was replaced by K^+ in the reaction mixture for phosphorylation. The material shown at the origin for the octopus gill preparation was not sensitive to hydroxylamine. The fragments of each preparation were tested for sensitivity to performic acid. In each case the mobility of the three spots, P4–P6, was reduced in the same way[12,13]. The data are not shown. Incidentally a preparation of electroplax ($Na^+ + K^+$)-ATPase, inhibited with *N*-ethyl-

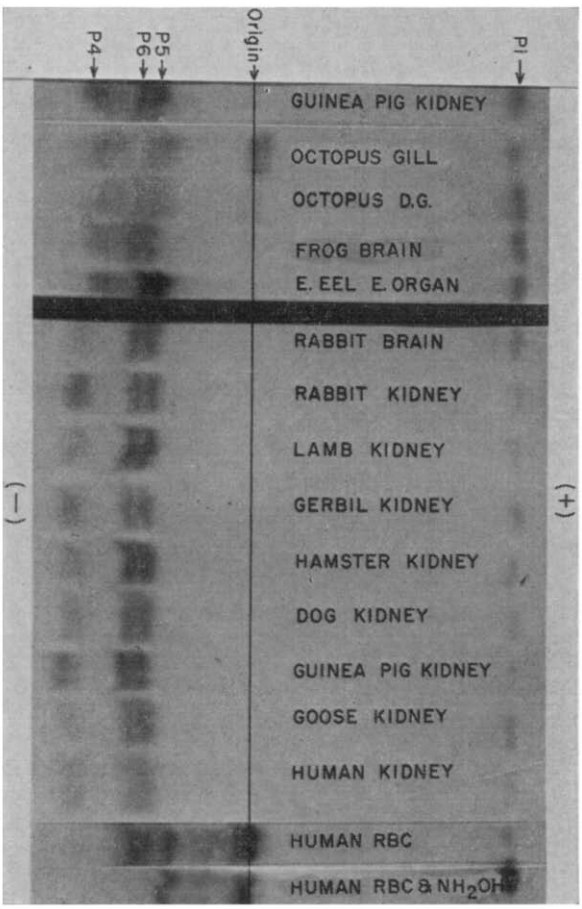

Fig. 1. Comparison of the [^{32}P]intermediate from various sources after solubilization by peptic digestion and electrophoresis. Membrane preparations were labeled with ^{32}P from [^{32}P]ATP in the presence of Mg^{2+} and Na^+. The reaction was stopped with acid. The denatured insoluble membranes were washed 3 times with 20 mM HCl and were digested with pepsin. The soluble products were electrophoresed on paper at pH 2 and a radioautogram was made. The symbols P4, P5 and P6 at the left indicate the 4th, 5th and 6th fragments appearing after peptic digestion of the phosphorylated intermediate in the ($Na^+ + K^+$)-ATPase of guinea-pig kidney membranes[13]. In the case of erythrocyte membranes one aliquot was applied directly to the paper as above. A second aliquot was treated with NH_2OH before application to the paper. Abbreviations: D.G., digestive gland; E, electric; RBC, red blood cells.

maleimide before denaturation[14], showed similar spots with a similar sensitivity to performic acid. This result indicates that inhibition was not due to combination of *N*-ethylmaleimide with a sulfhydryl group on the peptic fragment[12,13] and that this group is probably inaccessible in the native enzyme. In each case, digestion with pronase gave the same prominent spot found earlier[13]. The data are not shown.

In the case of the phosphorylated erythrocyte membranes, Fig. 1 shows radioactivity sensitive to hydroxylamine in the region of fragments marked P5 and P6 and also near the origin. This ^{32}P was replaced by phosphorus from unlabeled ATP with a turnover of about 6.5 min^{-1} at 0°. However, substitution of K^+ for Na^+ yielded almost as much material. In another experiment the radioactive spots were cut out of the electrophoresis paper and counted. The amount of radioactivity in the 'P5–P6' region was reduced by only 40 % when K^+ replaced Na^+ in the reaction mixture. It was not reduced in other regions. When K^+ was added to Na^+ there was a reduction of 30 %. In another similar experiment a $(Na^+ + K^+)$-ATPase preparation from erythrocyte membranes was compared with one from guinea-pig kidney. Except that 6-fold more protein in a 6-fold greater volume was used for the erythrocytes, the preparations were treated identically. The membranes were phosphorylated in the presence of Na^+ or K^+ and the denatured washed precipitate was digested with pronase[13]. The solubilized radioactive material was subjected to electrophoresis at pH 2. After radioautography the spots were cut out of the paper and counted. In addition to P_1 only one positive fragment appeared from guinea-pig kidney and this appeared only with Na^+. From erythrocytes four fragments appeared, only one of which had the same mobility as the one from guinea-pig kidney. Only this fragment showed a reduction in amount with substitution of K^+ for Na^+. The reduction was 31 %. The yield of [^{32}P]intermediate, calculated by difference between the amount with Na^+ and the amount with K^+, was 10.1-fold greater from kidney than from erythrocytes. The total $(Na^+ + K^+)$-ATPase activity in the starting material was 9.4-fold greater with kidney than with erythrocytes. From these data the ratio of turnover numbers at 37° was calculated (Table I). The similarity of the turnover numbers suggests that the [^{32}P]intermediate is the same in erythrocytes and kidney, but its presence is obscured by similar materials which are not functioning as part of a $(Na^+ + K^+)$-ATPase. It may be part of the labile material observed by BLOSTEIN[15].

The similarity in kinetics and character of the phosphorylated material from 14 sources of $(Na^+ + K^+)$-ATPase supports the view that the material is a functional intermediate in active transport and that the mechanism is likely to be similar in most animal species and tissues. The correlation of the (K^+)-acylphosphatase activity with the $(Na^+ + K^+)$-ATPase activity supports the view that this activity is closely associated with the transport system.

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