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Na^+,K^+-REQUIRING ATPase

V PREPARATION AND ASSAY OF A SOLUBILIZED Na^+-STIMULATED ADP–ATP EXCHANGE ACTIVITY

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SUMMARY

Stimulation by Na^+ of the ADP–ATP exhange activity is observed in a fraction solubilized from pig-kidney membrane preparations. The solubility of this activity is indicated by the lack of sedimentation at 195 000 × *g*, and the lack of inhibition by a non-ionic detergent.

Addition of serum albumin and a lower pH of the assay for the exchange activity significantly enhanced the Na^+-stimulated portion of the exchange.

Conditions for solubilizing the fraction and for its dialysis, are crucial in obtaining active fractions.

The Na^+-stimulated portion of the exchange activity is specific for ATP. Mn^{2+}, but not Ca^{2+}, can replace Mg^{2+}. This Na^+-stimulated exchange appears to represent a solubilized fraction from the $(Na,^+K^+)$-ATPase.

INTRODUCTION

Subcellular fractions containing Na^+,K^+-activated ATPase (ATP phosphohydrolase, EC 3.6.1.3) also contain an ADP–ATP exchange activity which is not stimulated by Na^+ (refs. 1–4). Conditions for Na^+-stimulation of the ADP–ATP exchange activity of the microsomal fraction from the electric organ of the eel were first reported by FAHN *et al.*[5,6]. In the first method used to elicit Na^+-stimulation, low $MgCl_2$ concentration (0.2 mM) is added in the assay, the second method requires pretreatment of the enzyme with a specific inhibitor (*e.g.*, *N*-ethyl maleimide[6] or arsenite together with 2,3-dimercaptopropanol[7]). The Na^+-stimulation of the exchange activity at low $MgCl_2$ concentration has also been shown by STAHL[8,9] and SWANSON[10] in brain microsomes, and by our laboratory using fractions prepared from pig kidney (F. E. HOSSLER AND R. RENDI, unpublished results). FAHN *et al.*[5]

Abbreviations: MES, 2-(*N*-morpholino)ethanesulfonic acid; EGTA, ethyleneglycol bis-(β-aminoethyl ether) *N,N'*-tetraacetic acid.

concluded that the Na^+-stimulated exchange is a likely component of the (Na^+,K^+)-ATPase They also suggested that the exchange not stimulated by Na^+ is a contaminant of the microsomal preparation and is not involved in the (Na^+,K^+)-ATPase However, solubilized ADP–ATP exchange activity was not stimulated by Na^+ (refs 3, 4, 8) The data of STAHL *et al* [3] indicate that most of the ADP–ATP exchange activity of brain microsomes is not a part of the Na^+,K^+-requiring ATPase

I wish to report that extraction of a fraction prepared from pig kidney with NaI solubilizes an ADP–ATP exchange activity which is stimulated by Na^+ in a modified assay procedure The solubility of the exchange activity is indicated by the lack of sedimentation at 195 000 × *g*, and the lack of inhibition by a non-ionic detergent

EXPERIMENTAL

The materials and methods were the same as previously described[4], with the following modifications Crystalline bovine serum albumin was obtained from Calbiochem, Los Angeles, Calif

Assay for exchange activity

50 μmoles of Tris–acetate buffer (pH 7 15), 1 5 μmoles of $MgCl_2$, 0 15 μmole of ethyleneglycol bis-(β-aminoethyl ether) *N*,*N*′-tetraacetic acid (EGTA), 300 μg of serum albumin, 1 5 μmoles of Tris–ATP and 0 5 μmole of Tris–[^{14}C]ADP (90 000 counts/min per μmole) in a final volume of 0 5 ml were incubated with appropriate amounts of enzyme for 20 min at 37° When needed, 5 μmoles of NaCl were added The reaction was stopped by heating in a boiling-water bath for 2 min

Assay for adenylic kinase

This activity was measured in two ways (a) by omitting the ATP from the above assay system and adding it after stopping the reaction, (b) by determining the amount of AMP formed in the usual assay by cutting out the spots of AMP separated by paper electrophoresis, and measuring it spectrophotometrically

Partial purification of Na^+,K^+-requiring ATPase activity from pig kidney

As previously described[4], at Step IV the pellet is then dialyzed overnight against 1 mM ADTA and 2 mM mercaptoethanol (brought to pH 8 60 with Tris base at 30°) The suspension is centrifuged 60 min at 195 000 × *g* and the supernatant discarded The pellet and fluffy layer are suspended in 0 02 M Tris buffer (pH 7 6) and 0 1 mM EDTA and the fraction is quick frozen This fraction will be called hereafter EDTA-residue

NaI treatment

This was carried out on the EDTA-residue as described by NAKAO *et al* [11] with a modified solution containing 6 7 mM EDTA, 4 mM ATP, 6 7 mM $MgCl_2$, 66 mM cysteine, 9 mM KCl, 10% glycerol and 2 M NaI The suspension was diluted with 10% glycerol to a NaI concentration of 0 8 M, then centrifuged for 20 min at 41 500 × *g* For each ml of supernatant, 575 mg of $(NH_4)_2SO_4$ are added The precipitate is left to form for at least one day at 4° The precipitate is then collected in the cold,

and dissolved in a small volume of 0 02 M 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (pH 6 5), with 0 5 mM $MgCl_2$ and 0 1 mM EGTA and dialyzed overnight against 200 vol of the same buffer at 4° The material which precipitates overnight is removed by centrifuging for 10 min at 12 000 × *g* and the clear supernatant is retained (hereafter called $(NH_4)_2SO_4$ fraction)

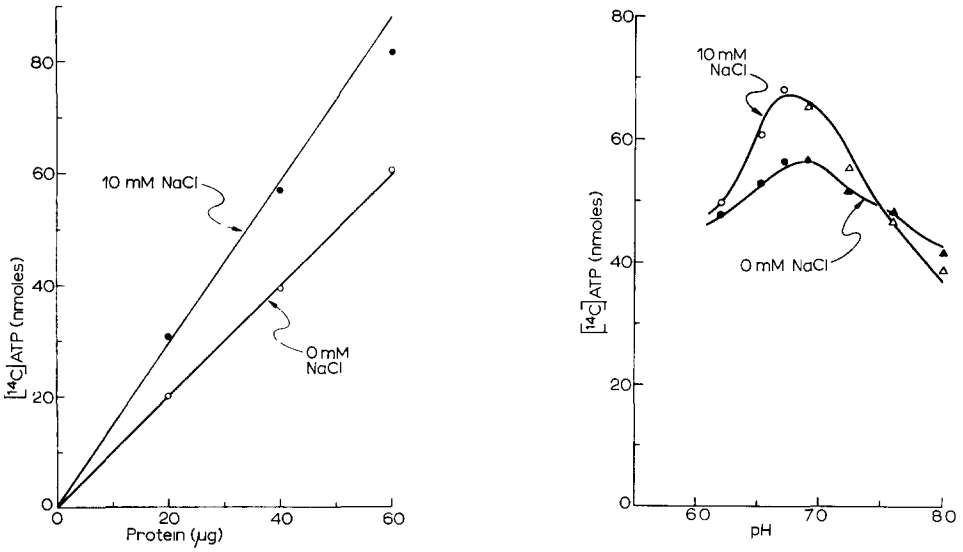

Fig 1 Effect of enzyme concentration on ADP–ATP exchange in the presence or absence of NaCl Assay as in EXPERIMENTAL

Fig 2 Effect of pH on exchange in the presence or absence of NaCl Assay as in EXPERIMENTAL with 36 μg protein of $(NH_4)_2SO_4$ fraction ○ and ●, MES buffer, △ and ▲, Tris–HCl buffer

RESULTS

Fig 1 indicates that the $(NH_4)_2SO_4$-fraction contains an enzyme catalyzing a Na^+-stimulated exchange which is roughly linear with protein concentration In thirty active preparations of $(NH_4)_2SO_4$-fraction, the Na^+ stimulation varied between 10 and 90%

The data of Fig 1 were obtained after the development of the assay for the exchange activity reported in EXPERIMENTAL Table I shows the effect of serum albumin and EGTA on the exchange activities, omission of EGTA reduces Na^+-stimulated exchange, without affecting the activity measured in the absence of Na^+ The stimulation by EGTA is small but reproducible Serum albumin alone, or in combination with EGTA, increased both exchange activities, especially the Na^+-stimulated Another important variable in the assay proved to be the pH of the incubation medium (Fig 2) At pH higher than 7 4, inhibition of the exchange by Na^+ is observed With different preparations, the pH optimum of the Na^+-stimulated exchange varied between 6 9 and 7 15 In all cases Na^+ inhibition was observed at pH 7 6 Below pH 6 8, MES buffer could not be replaced by imidazole or *N*-Tris-(hydroxymethyl)methyl-2-aminoethanesulfonate buffer Tris–HCl buffer and Tris–acetate buffer gave essentially the same results

TABLE I

EFFECTS OF SERUM ALBUMIN AND EGTA ON THE EXCHANGE REACTION IN THE $(NH_4)_2SO_4$ FRACTION
Assay as in EXPERIMENTAL with 28 μg protein

	[^{14}C]ATP formed (nmoles)	
	−NaCl	+NaCl
1 Complete system	51.1	64.2
2 −EGTA	52.3	62.3
3 −EGTA and serum albumin	43.1	45.7

The Na^+-stimulated exchange activity of the intact ATPase is very sensitive to the Mg^{2+} concentration[5] Fig 3 shows that the optimum Mg^{2+} concentration is 3 mM for both exchange activities

Table II shows the specificity of substrates and activators of the reaction Exchange is observed in the absence of Na^+ when ITP or GTP replaces ATP No stimulation by Na^+ is observed when ATP is replaced by these nucleotidetriphosphates (1–3, Table II) Mg^{2+} is required for the reaction, it can be replaced to a certain extent by Mn^{2+}, but not by Ca^{2+} (4–7, Table II) Na^+ cannot be replaced by either K^+ or Li^+ (8–10, Table II)

The effect of various inhibitors has been tested on the exchange activity (Table III) Strophanthin inhibits somewhat the Na^+-stimulated exchange but not the exchange observed in the absence of Na^+ This inhibition is small but reproducible Since the concentration of strophanthin is quite high (0.1 mM) it is not clear if the inhibition by this compound is specific Triton X-100, at a concentration that inhibits the (Na^+,K^+)-ATPase by 90%, does not inhibit exchange activity either in the presence or absence of Na^+ This fact suggests that the Na^+-stimulated exchange enzyme(s) is not significantly bound to essential lipids

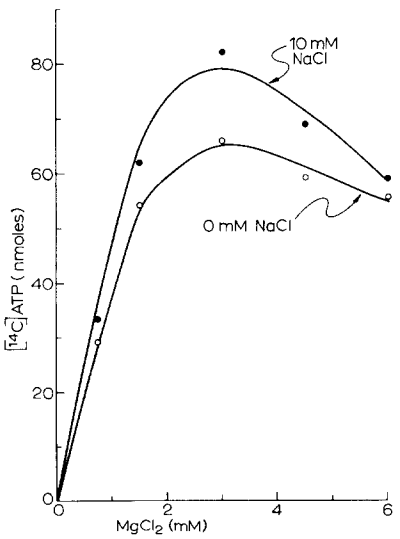

Fig 3 Effect of $MgCl_2$ concentration on exchange in the presence or absence of NaCl Assay as in EXPERIMENTAL with 33 μg protein of $(NH_4)_2SO_4$ fraction

TABLE II

SPECIFICITY OF THE EXCHANGE REACTION IN THE $(NH_4)_2SO_4$ FRACTION

Assay as in EXPERIMENTAL

	[^{14}C]ATP formed (nmoles)	
	−NaCl	+NaCl
1 With 3 mM ATP and 3 mM $MgCl_2$	62 1	68 4
2 With 3 mM ITP and 3 mM $MgCl_2$	15 7	15 8
3 With 3 mM GTP and 3 mM $MgCl_2$	25 3	25 0
4 With 3 mM ATP and 3 mM $MgCl_2$	79 2	93 7
5 With 3 mM ATP and 3 mM $CaCl_2$	28 5	28 3
6 With 3 mM ATP and 3 mM $MnCl_2$	38 3	47 6
7 With 3 mM ATP and 0 mM Me^{2+}	2 3	2 1
8 With 3 mM ATP, and 3 mM $MgCl_2$	57 1	68 9
9 With 3 mM ATP, 3 mM $MgCl_2$ and 10 mM KCl	57 5	—
10 With 3 mM ATP, 3 mM $MgCl_2$ and 10 mM LiCl	54 1	—

TABLE III

EFFECT OF INHIBITORS ON THE EXCHANGE REACTION IN THE $(NH_4)_2SO_4$ FRACTION

Assay as in EXPERIMENTAL with 20 μg protein

	[^{14}C]ATP formed (nmoles)	
	−NaCl	+NaCl
1 Control	16 4	21 0
2 With 0 1 mM strophanthin	16 7	19 4
3 With 0 05 % Triton X-100	16 9	20 4
4 With 10 μM *p*-chloromercuribenzoate	16 0	21 8

Table IV shows enzymatic activities of both non-extracted and extracted fractions The $S_{195\,000}$-fraction is obtained by centrifugation of the $(NH_4)_2SO_4$ fraction for 2 h at 195 000 × *g* The run was terminated without breaking so that the pellet remained compact at the bottom of the tube Approximately 5% of the protein and

TABLE IV

ENZYMATIC ACTIVITIES OF EDTA RESIDUE AND EXTRACTED FRACTIONS

(*a*) Formation of [^{14}C]ATP from [^{14}C]ADP in the absence of ATP in the assay (*b*) Formation of AMP from ADP and ATP

	Protein (mg)	*(Na^+, K^+)-ATPase (total units)*	*ADP–ATP exchange (total units)*		*Adenylic kinase (total units)*			
					(a)		*(b)*	
			−Na^+	+Na^+	−Na^+	+Na^+	−Na^+	+Na^+
EDTA residue	90	93 3	10 0*	12 0*	—	—	—	—
$(NH_4)_2SO_4$ fraction	6	0 012	2 0	2 3	1 8	1 6	1 5	1 5
$S_{195\,000}$ fraction**	4 8	—	1 9	2 2	—	—	—	—

* Assay with 0 2 mM $MgCl_2$

** For preparation, see text

less than 0.1% of the ATPase (assayed as previously described[4]) was recovered in the $(NH_4)_2SO_4$ fraction. The value for ATPase activity is only a maximum value, since no ATPase was actually observed. No ATPase was observed even at pH 7.15 with serum albumin and EGTA, under conditions of the exchange assay used here.

The significance of the percent recovery of the ADP–ATP exchange in the $(NH_4)_2SO_4$ fraction is difficult to assess quantitatively. The reaction had to be determined under different Mg^{2+} concentration (0.2 mM Mg^{2+} for the EDTA-residue[5], and 3 mM Mg^{2+} for the $(NH_4)_2SO_4$ fraction). ATPase activity is known to affect the exchange activity[5]. The EDTA residue contains ATPase activity, while the $(NH_4)_2SO_4$ fraction does not.

More significant are other data on the exchange activity (Table IV). The fact that both exchange activities are completely recovered in the supernatant after centrifugation for 2 h at 195 000 × *g* at pH 6.5 and in the absence of detergent, strongly suggests that both activities are soluble.

The $(NH_4)_2SO_4$ fraction was checked for adenylic kinase which has ADP–ATP exchange activity. Two different methods of determination demonstrated that considerable amounts of adenylic kinase activity are present (Table IV). However, this activity was not affected by Na^+, therefore is not responsible for the observed Na^+ stimulation of the ADP–ATP exchange. Attempts at separating the adenylic kinase and the Na^+-stimulated exchange activities by $(NH_4)_2SO_4$ fractionation have not been successful.

DISCUSSION

The data presented here indicate that a soluble Na^+-stimulated ADP–ATP exchange activity can be prepared from a pig-kidney membrane fraction by NaI treatment. These results are at variance with those reported by STAHL[8], who reported 35% inhibition of the ADP–ATP exchange activity by 10 mM NaCl in the NaI supernatant from brain microsomes. A likely explanation of this variance is the modification of both the assay procedure and the method of preparation of the enzyme fraction. Major changes in the assay include the addition of serum albumin and a lower pH.

Careful preparation of the enzyme was an important factor in obtaining active fractions. Modifications of the NaI treatment, as noted in the EXPERIMENTAL section, enabled to prepare consistently active fractions. The most important changes of the treatment are the addition of glycerol and KCl. The NaI supernatant was immediately precipitated with $(NH_4)_2SO_4$ in order to decrease the I^- concentration before dialysis. This seemed important since the (Na^+,K^+)-ATPase, itself, is strongly inhibited by I^-. Composition of the dialysis fluid also affects the amount of Na^+-stimulated exchange recovered. Low pH and the presence of Mg^{2+} and EGTA increased recovery. Na^+ stimulation of the exchange could be demonstrated only in fractions dialyzed below 6°. The $(NH_4)_2SO_4$ fraction is tested immediately, since the Na^+-stimulated portion of the exchange is very unstable.

The similarity of Na^+-stimulated portion of the exchange activity in the $(NH_4)_2SO_4$ fraction and that of membrane preparations containing (Na^+,K^+)-ATPase[5–10] is evident. For example (a) the activities of both systems are specific for ATP, (b) in both systems Ca^{2+} cannot replace Mg^{2+}, and (c) K^+ cannot replace Na^+. The

high Mg^{2+} concentration optimal for the Na^+-stimulated exchange in the $(NH_4)_2SO_4$ fraction is similar to some data presented by FAHN *et al*[6] These authors treated microsomes of the electric organ of the eel with *N*-ethylmaleimide and found an increase in the Na^+-stimulated exchange activity The optimum Mg^{2+} concentration for the exchange increased from 0 2 to 3 0 mM after treatment with *N*-ethylmaleimide FAHN *et al*[6] also noticed that the Na^+-stimulated exchange is not inhibited by *p*-chloromercuribenzoate after this treatment whereas the untreated enzyme is sensitive to this inhibitor[5] Similar data indicating a lack of inhibition by *p*-chloromercuribenzoate of the Na^+-stimulated exchange in the $(NH_4)_2SO_4$ fraction is shown in Table III These differences between the exchange activity in native ATPase and in the $(NH_4)_2SO_4$ fraction may be explained on the basis of changes in the enzyme during its release from the bound to soluble state It should be noted that some Na^+ stimulation of the ADP–ATP exchange at 3 mM $MgCl_2$ has been shown by SWANSON[10] using NaI-treated cerebral microsomes

A plausible interpretation of the data reported here is that there are at least two enzymes involved in the ADP–ATP exchange of the $(NH_4)_2SO_4$ fraction Adenylic kinase appears to be the major component (Table IV), but is not related to the Na^+-stimulated exchange This is in keeping with the observation reported by SWANSON[10], that adenylic kinase does not show Na^+ stimulation A quantitatively smaller component is responsible for the Na^+-stimulated ADP–ATP exchange activity From its properties, it closely resembles a Na^+-stimulated exchange activity considered part of this (Na^+,K^+)-ATPase Separation and purification of these two enzymatic activities is in progress

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