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AN ANION-STIMULATED ADENOSINE TRIPHOSPHATASE FROM THE POTASSIUM-TRANSPORTING MIDGUT OF THE LARVA OF *HYALOPHORA CECROPIA*

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SUMMARY

1. Some properties of an adenosine triphosphatase (EC 3.6.1.4.) present in 40000 × *g* sediment of the midgut epithelium of the larva of *Hyalophora cecropia* are described.

2. The ATPase has an optimum activity at pH 8.7 and is sensitive to anions. In the presence of magnesium, bicarbonate and to an even greater degree borate and selenite enhance the activity compared to halogen ions, while thiocyanate acts as an inhibitor. When magnesium is replaced by calcium no stimulation by bicarbonate is found.

3. Alkali metal ions have only slight influence on the activity. With bicarbonate or borate as anion, potassium and rubidium are slightly more stimulating than sodium and lithium.

4. The alkaline-phosphatase activity (EC 3.1.3.1) of the preparation with *p*-nitrophenylphosphate as substrate, has a pH optimum of 10.0 and an anion sensitivity which is less pronounced and different to that of the ATPase.

5. Imidazole gives a lower ATPase activity than buffer compounds with a primary amino group.

INTRODUCTION

The isolated midgut from the larva of *Hyalophora cecropia* transports potassium actively from the haemolymph to the lumen[1]. The potassium transport is completely independent of sodium and is not inhibited by ouabain[2] contrary to active sodium transport in vertebrates. In the intact animal pH of the haemolymph is around 7.0 and of the intestinal contents around 10.6. The total CO_2 of the haemolymph is around 5 mM and of the intestinal contents around 50 mM (B. O. TURBECK AND S. NEDERGAARD, unpublished observations).

The present study deals with an ATPase derived from the epithelium of the *H. cecropia* midgut and was undertaken to see if a correlation could be found between some of the physiological characteristics of this tissue and the properties of the ATPase.

EXPERIMENTAL PROCEDURES

Preparation of ATPase

The tissue used for the preparation of the ATPase was the epithelium from the midgut of mature 5th instar larva of the *H. cecropia*. The larvae were reared indoors in Copenhagen on willow leaves and used when they weighed 8–12 g. They were anaesthetized with solid CO_2 and the midgut was dissected out. The gut was opened by a longitudinal section and the viscous peritrophic membrane was removed together with the intestinal contents. The gut was then placed under a slight pressure between two microscope slides and frozen on a block of solid CO_2. A few minutes later the gut was thawed and the epithelium removed from the muscular coat with a fine brush and transferred to the homogenizing medium kept at 0° in amounts sufficient to yield an approx. 10% (w/v) suspension. By this procedure it was possible to prevent the muscular tissue from contaminating the homogenate; this was confirmed histologically on several occasions. Homogenization was performed with a teflon–glass homogenizer using a medium consisting of 250 mM sucrose, 30 mM histidine, and 5 mM EDTA (pH 7.6). The crude homogenate was first centrifuged at 800 $\times$ *g* for 10 min and the sediment discarded. The resulting supernatant was then centrifuged at 40000 $\times$ *g* for 10 min. Sediment from this centrifugation was washed in the homogenizing medium and centrifuged again in the same way. This procedure was repeated and the final sediment was homogenized, yielding a suspension of about 3 mg protein per ml. Aliquots of this suspension were placed in small test tubes and stored at —20°. The ATPase activity did not fall appreciably in the course of several months. A pellet of the 40000 $\times$ *g* sediment used in the ATPase experiments was fixed in OsO_4 and embedded in vestopal. Electron microscopy showed that the preparation contained mitochondria some intact but most disintegrated. A lighter (microsomal) fraction was prepared by centrifugation of the supernatant from the 40000 $\times$ *g* fraction at 90000 $\times$ *g* for 2 h.

Assays

The ATPase medium is described in detail in the legend of each experiment, but the basic medium contained 5 mM ATP, 5 mM $MgCl_2$, 25 mM Tris–HCl, and about 15 μg enzyme protein in a final volume of 600 μl. In some cases 25 mM 2-amino-2-methyl-1-propanol was used instead of Tris–HCl. Owing to the high pK of this buffer substance (pK_a about 10) it is convenient for use in the study of anion activation at pH 8.7 as the amine can be titrated to this value with the acid corresponding to the acid of choice (*e.g.* HCl or H_2CO_3), avoiding other cations in the medium. The incubation was carried out in a water bath at 25° for 30 or 60 min and the reaction stopped by the addition of 350 μl trichloroacetic acid (10%, w/v). The orthophosphate released was determined by an extraction procedure described by Vestergaard-Bogind[3]. Less than 20% of the substrate was split during the incubation time. All assays were run in duplicate and blanks were run with each determination. Thiocyanate was precipitated with Ag^+ as it interferes with the orthophosphate assay in concentrations exceeding 50 mM. Selenite in concentrations higher than about 10 mM interfered to some extent, but corrections were made by using phosphate standards with appropriate concentrations of selenite.

Protein was determined according to the method described by LOWRY *et al.*[4].

p-Nitrophenylphosphatase activity was determined in a medium containing 5 mM *p*-nitrophenylphosphate, 5 mM $MgCl_2$, 50 mM 2-amino-2-methyl-1-propanol, and about 15 μg of enzyme protein in a final volume of 600 μl. The incubation was carried out at 25° for 20 min and the reaction stopped by addition of 5 ml 0.2 M NaOH. The amount of *p*-nitrophenol liberated was determined spectrophotometrically at 410 mμ.

RESULTS

pH dependence

The optimum pH for the ATPase activity in chloride medium was found to be 8.7 (Fig. 1). The following substrates were tested in the same medium: ADP, AMP and *p*-nitrophenylphosphate, for all of which the enzyme had an optimum pH of 10.0. If the rates of hydrolysis of these substrates at pH 10.0 are related to that of ATP at 8.7, the following percentages were found: ADP, 70%; AMP, 65%; and *p*-nitrophenylphosphate, 130%.

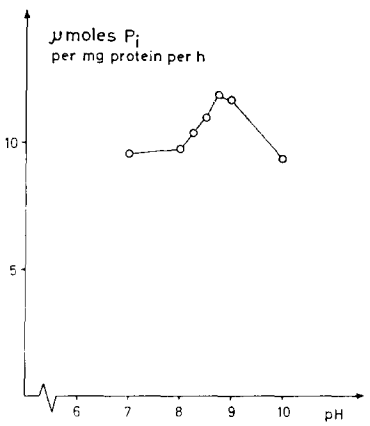

Fig. 1. ATPase activity as function of pH in chloride medium. 5 mM ATP, 5 mM $MgCl_2$, 25 mM Tris–HCl, 100 mM KCl.

Effect of buffer substance

Table I shows that the activity of the ATPase is dependent on the buffer. The three buffers Tris–HCl, 2-amino-2-methyl-1-propanol, and glycine all have a primary amino group. The ratio of activity in bicarbonate/activity in chloride is approximately the same for these three buffers. Compared with this group of buffers imidazole inhibits the ATPase, and the inhibition is greatest in bicarbonate medium. With borate buffer the ratio is lower than unity. This may be explained by additive effects of borate and bicarbonate (see Fig. 2).

Anions

The effects of some anions in concentration of 40 mM on the ATPase activity in Tris–HCl buffer are shown in Table II. Compared with the halogen ions, the ATPase is stimulated in order of increasing effect by bicarbonate, borate, and selenite,

TABLE I

EFFECT OF VARIOUS BUFFERS ON ATPase ACTIVITY

The incubation mixture contained 5 mM ATP, 5 mM $MgSO_4$, 25 mM buffer adjusted to pH 8.7 with HCl or KOH. KCl or $KHCO_3$ was added to a final concentration of 100 mM of either Cl^- or HCO_3^-, and pH was rechecked. $KHCO_3$ was prepared from KOH and titrated to pH 8.7 with CO_2.

Buffer	*μmoles P_i liberated per mg protein per h*		*Ratio activity in $KHCO_3$/ activity in KCl*
	in KCl	*in $KHCO_3$*	
Tris–HCl	33	88	2.7
2-Amino-2-methyl-1-propanol	30	77	2.6
Glycine	33	92	2.8
Imidazole	16	29	1.8
Borate	69	61	0.9

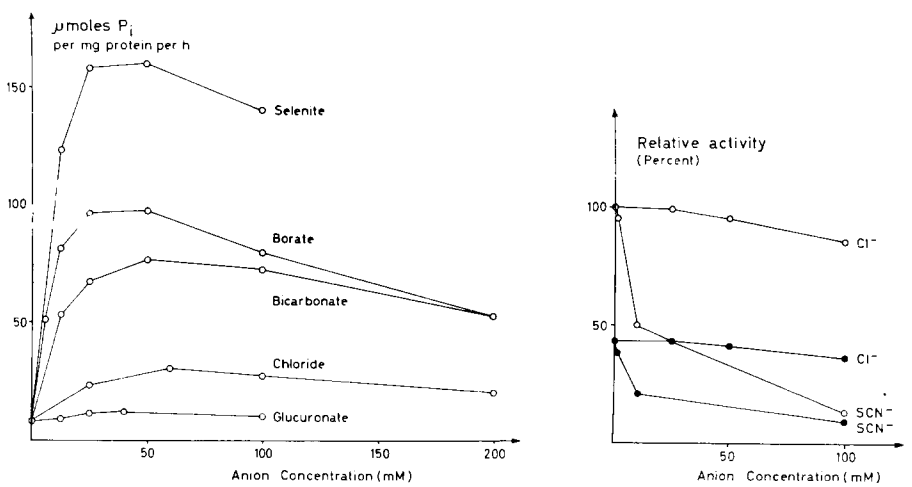

Fig. 2. Stimulation of ATPase by different anions. 5 mM ATP-disodium salt, 5 mM $MgSO_4$, 25 mM Tris–HCl, the cation corresponding to the anions mentioned was in all cases sodium; pH 8.7.

Fig. 3. Thiocyanate and chloride effect on ATPase in chloride or bicarbonate media. 5 mM ATP, 5 mM $MgSO_4$, 25 mM Tris–HCl (pH 8.7) and 100 mM KCl or 100 mM $KHCO_3$. To these substrates varying amounts of KCl or KSCN were added; these additional concentrations are indicated on the abscissa. ●—●, chloride medium; ○—○, bicarbonate medium. Ordinate: % of the activity in 100 mM bicarbonate, no thiocyanate added.

and fairly strongly inhibited by thiocyanate. ATPase activity is shown as a function of the anion concentration in Fig. 2. Maximum activity for bicarbonate is close to 50 mM, for chloride it is slightly higher and for borate and selenite a little lower. The inhibitory effect of thiocyanate is shown in Fig. 3. The inhibition in per cent of the initial activity is the same whether bicarbonate or chloride is present in the medium. Bicarbonate stimulation is still pronounced at 50 mM thiocyanate.

TABLE II

EFFECT OF ANIONS ON ATPASE AND *p*-NITROPHENYLPHOSPHATASE

The ATPase medium contained 5 mM ATP, 5 mM $MgSO_4$, 25 mM Tris–HCl at pH 8.7. The *p*-nitrophenylphosphatase medium contained 5 mM *p*-nitrophenylphosphate, 5 mM $MgSO_4$, 50 mM 2-amino-2-methyl-1-propanol at pH 10.0. The same batch of enzyme was used for the two series. The anion concentration of the added salt was 40 mM. Sodium salts were used throughout. The pH was rechecked after addition of the salt.

Anion	*μmoles P_i liberated per mg protein per h*		*Ratio ATPase to p-nitrophenylphosphatase*
	ATPase	*p-Nitrophenylphosphatase*	
F^-	14	32	0.44
Cl^-	17	22	0.77
Br^-	18	21	0.86
I^-	14	30	0.47
SCN^-	3	20	0.15
NO_3^-	11	22	0.50
SO_4^{2-}	17	17	1.00
CH_3COO^-	14	21	0.67
$C_6H_{11}O_7^-$ (glucuronate)	12	19	0.63
HCO_3^-	54	20	2.7
$H_2BO_3^-$	72	17	4.1
SeO_3^{2-}	171	11	16.0

Magnesium and calcium

The presence of magnesium alone results in a higher activity than calcium alone, and it is only in the presence of magnesium that stimulation by bicarbonate is observed (Table III). In the absence of added magnesium or calcium no activity is observed and there is no stimulative effect after addition of any of the alkali salts mentioned in Table III.

TABLE III

STIMULATION OF THE ATPASE BY Mg^{2+}, Ca^{2+}, K^+, AND Na^+

The medium contained 5 mM ATP–Tris salt, 25 mM 2-amino-2-methyl-1-propanol adjusted with HCl to pH 8.7 and the salts mentioned. The pH was checked after addition of the salts.

Salts added (mM)						*μmoles P_i liberated per mg protein per h*
$MgCl_2$	*$CaCl_2$*	*KCl*	*$KHCO_3$*	*NaCl*	*$NaHCO_3$*	
5						19
5		10				23
5			10			57
5				10		21
5					10	55
	5					9
	5	10				7
	5		10			8
	5			10		10
	5				10	9

Alkali metal ions

In the presence of magnesium and bicarbonate the stimulative effect of potassium was greater than of sodium. This was found with every batch of enzyme and in both Tris and 2-amino-2-methyl-1-propanol buffers. With chloride as anion no such constant difference could be demonstrated. Fig. 4 shows the influence of four alkali metal bicarbonates on the ATPase activity and it can be seen that the results for rubidium and potassium are similar as are those of lithium and sodium.

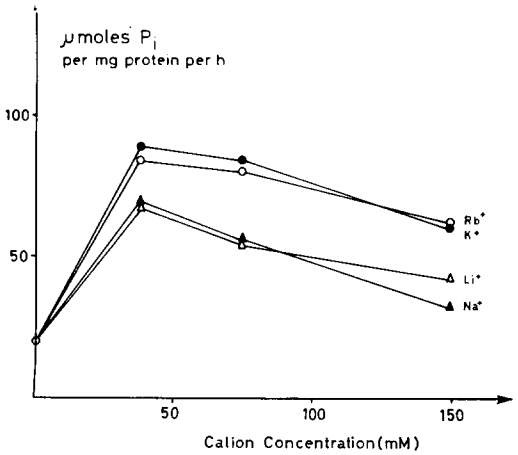

Fig. 4. Influence of alkali metal bicarbonates on ATPase. 5 mM ATP-2-amino-2-methyl-1-propanol salt, 5 mM $MgSO_4$, 25 mM 2-amino-2-methyl-1-propanol adjusted to pH 8.7 with CO_2. The alkali metal bicarbonates were all prepared from the corresponding hydroxides by titration with CO_2 to pH 8.7.

In a medium containing 5 mM ATP, 5 mM $MgCl_2$, 25 mM Tris–HCl and pH 7.4 (in the range of (Na^+-K^+)-activated ATPases) the ATPase activity was determined for various proportions of KCl and NaCl with the sum of the two concentrations 110 mM. The activity was found to be uneffected by the Na^+/K^+ ratio. With NaCl 85 mM and KCl 25 mM in the same system the addition of 1 mM ouabain was without effect. These experiments were also performed with the lighter (microsomal) fraction of the homogenate and in this case too no (Na^+-K^+)-activated ATPase could be demonstrated.

p-Nitrophenylphosphatase (alkaline phosphatase)

As it seemed possible that the ATPase activity of the preparation might be due to other non-specific enzymes, the *p*-nitrophenylphosphatase activity of the preparation was determined. From Table II it may be seen that the ATPase-stimulative anions bicarbonate, borate and selenite have no stimulative effect on the *p*-nitrophenylphosphatase although to some extent the enzyme is inhibited by these anions as compared with halogen ions. Selenite is furthermore the most potent inhibiting ion in the series. Thiocyanate however, which markedly inhibits the ATPase activity, has no pronounced effect on the *p*-nitrophenylphosphatase.

DISCUSSION

The results show that the ATPase from *H. cecropia* midgut resembles an ATPase isolated from frog gastric mucosa by KASBEKAR AND DURBIN[5,6].

The ATPases from both tissues have a high pH optimum, 8.7 for the midgut enzyme and 8.3 for the gastric enzyme.

The graphs of the bicarbonate stimulation of both enzymes have almost identical profiles. The stimulation of the midgut ATPase by selenite and borate, two chemically widely different compounds, has a profile similar to that of bicarbonate. This may be due to a catalytic effect which these inorganic oxygen acids exert on certain chemical reactions as for example the reversible hydration of CO_2 (ref. 7), and possibly on a step in the ATPase-catalyzed sequence.

Thiocyanate inhibits both the midgut and the gastric ATPase. The gastric acid production is inhibited by thiocyanate, and this may be explained as an effect on the ATPase. The effect of thiocyanate on potassium transport or H^+ gradient across the *H. cecropia* midgut has not been investigated.

The inhibition of the midgut ATPase by imidazole may be related to the results of ALONSO, RYNES AND HARRIS[8], who found that 10 mM imidazole depressed the acid secretion of frog gastric mucosa even more than thiocyanate in the same concentration.

The present ATPase from *H. cecropia* midgut is probably mainly mitochondrial, but all tested fractions of the homogenate were stimulated by bicarbonate. The frog gastric mucosa ATPase was a microsomal fraction containing little or no evidence of intact mitochondria, but activity was found in nuclear and mitochondrial fractions as well, and all fractions were stimulated by bicarbonate and inhibited by thiocyanate[6].

The gastric mucosa and the *H. cecropia* midgut both maintain a great difference in pH between lumen and blood (in gastric mucosa the H^+ gradient is in the opposite direction to that of the *H. cecropia* midgut) and like gastric mucosa the *H. cecropia* midgut contains carbonic anhydrase (B. O. TURBECK, unpublished observations).

The epithelium of the *H. cecropia* midgut transports potassium actively against an electrical gradient of more than 100 mV from haemolymph to lumen. Potassium carries about 90 % of the current generated by the midgut when the potential is short circuited[1].

Bioelectrical experiments have shown that the presence of the carbonic anhydrase inhibitor ethoxolamide (cardrase) at 1 mM in the media inhibits the short circuit current of the *H. cecropia* midgut about 36 % (ref. 2).

The carbonic anhydrase of the midgut epithelium may, like that of gastric mucosa, be involved in the maintenance of the pH gradient across the epithelium. The effect of ethoxolamide on the potassium transport may therefore suggest a connexion between the maintenance of concentration differences of both K^+ and H^+ between lumen and haemolymph. The concentration of inorganic anions in haemolymph is low and one of the likely anions to accompany potassium to maintain electroneutrality during its excretion is bicarbonate (or carbonate). The carbonic anhydrase-dependent reaction in the midgut epithelium may be the formation of bicarbonate ions as it is in gastric mucosa. This may explain why the potassium transport of the *H. cecropia* midgut is inhibited by ethoxolamide.

The potassium transport of the midgut is independent of sodium in the bathing

media and is not inhibited by ouabain[2] consistent with the finding that the ATPase of the tested fractions of the tissue showed neither Na^+–K^+ stimulation nor ouabain sensitivity under the conditions described. The gastric ATPase too showed lack of these properties.

Rubidium is also transported actively, even when all potassium is substituted by rubidium. Lithium and sodium cannot substitute for potassium[9]. This may be correlated to the fact that potassium and rubidium are better stimulators of the midgut ATPase than are sodium and lithium, and more so in a range corresponding to the intracellular potassium concentration which is 70 mM (ref. 10). On the other hand it has been shown that liver mitochondrial ATPase is stimulated by alkali metal ions increasing in the order: Li^+, Na^+, K^+, Rb^+, and Cs^+, which is the order of decreasing hydrated ionic radii[11].

The present rather crude ATPase preparation probably has a great number of enzymatic activities, few of which have yet been investigated. Its ability to split *p*-nitrophenylphosphate represents the alkaline phosphatase activity, but both the pH optimum and the anion sensitivity of this enzyme is found to be different from that of the ATPase.

The frog gastric mucosa ATPase which has many points in common with the midgut ATPase is believed to participate directly in the sequence of reactions leading to acid secretion[5,6]. It therefore seems possible that the *H. cecropia* midgut ATPase is involved in the maintenance of the H^+ gradient across the intestinal epithelium.

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REFERENCES

1 W. R. HARVEY AND S. NEDERGAARD, *Proc. Natl. Acad. Sci. U.S.*, 51 (1964) 757.
2 J. A. HASKELL, R. D. CLEMONS AND W. R. HARVEY, *J. Cellular Comp. Physiol.*, 65 (1965) 45.
3 B. VESTERGAARD-BOGIND, *Scand. J. Clin. Lab. Invest.*, 16 (1964) 457.
4 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
5 D. K. KASBEKAR AND R. P. DURBIN, *Biochim. Biophys. Acta*, 105 (1965) 472.
6 R. P. DURBIN AND D. K. KASBEKAR, *Federation Proc.*, 24 (1965) 1377.
7 F. J. W. ROUGHTON AND V. H. BOOTH, *Biochem. J.*, 32 (1938) 2049.
8 D. ALONSO, R. RYNES AND J. B. HARRIS, *Am. J. Physiol.*, 208 (1965) 1183.
9 S. NEDERGAARD AND W. R. HARVEY, *J. Exptl. Biol.*, 48 (1968) 13.
10 W. R. HARVEY AND K. ZERAHN, *J. Exptl. Biol.*, in the press.
11 R. CEREIJO-SANTALO, *Can. J. Biochem.*, 46 (1967) 55.