Purification and properties of a novel nucleotide-hydrolysing enzyme (5'-nucleotidase) from *Boophilus microplus*

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The tick *Boophilus microplus* contains a nucleoside phosphate-hydrolysing enzyme which, in many respects, resembles the well characterized 5'-nucleotidase from mammalian tissue. The tick enzyme has been purified to homogeneity. It is a membrane-bound glycoprotein with an apparent M_r of 67000 and, although it fails to hydrolyse a range of nucleoside 2'- or 3'-monophosphates, it has broad specificity for the 5' derivatives. Further investigation of the enzyme's substrate specificity, however, shows some important differences from the mammalian nucleotidases. It hydrolyses both bis-*p*-nitrophenyl phosphate and *p*-nitrophenyl phenylphosphonate, typical substrates for phosphodiesterases. However, the tick enzyme is most strikingly different from the mammalian enzymes in that it hydrolyses not only AMP but ADP and ATP as well. Further, the products of the hydrolysis of ATP are adenosine and tripolyphosphate, a reaction which has not been reported previously. The products of ADP hydrolysis are adenosine and pyrophosphate.

INTRODUCTION

The 5'-nucleotidases are a group of enzymes that have proved to have not only considerable intrinsic interest but also much practical usefulness in various areas of biochemical research. They have long been considered one of the best enzyme markers of plasma membranes (Evans, 1978), but they are, in addition, one of the best characterized of the ectoenzymes. In many tissues, 5'-nucleotidase is largely confined to this extracellular location (Luzio *et al.*, 1986).

We are interested in several aspects of the biochemistry of the tick *Boophilus microplus*, a serious problem to cattle production in many tropical and sub-tropical parts of the world. In studies with this parasite, an appropriate enzyme marker for the plasma membrane would be invaluable. The tick does contain an enzyme with many similarities to the mammalian 5'-nucleotidases, so much so that we have referred to it, for the sake of convenience, as a 5'-nucleotidase. Nevertheless, further investigation has revealed an interesting enzyme catalysing a unique series of reactions.

EXPERIMENTAL

Materials

Adenosine deaminase was obtained from Boehringer Mannheim; [γ - 32 P]ATP and [14 C]AMP were from Amersham International, Amersham, Bucks., U.K. Nucleosides, nucleotides, p[CH₂]pA and sodium tripolyphosphate were from Sigma Chemical Co., St. Louis, MO, U.S.A. [β - 32 P]ADP was prepared essentially by the method of Dawson *et al.* (1985), then purified by chromatography, first on a C-18 Novapak (Waters) radial compression column in 0.1 M-phosphate buffer, pH 6.0, and then on a Mono Q column as described below. AMP-Sepharose and Mono Q columns were obtained from Pharmacia. Tick material was prepared as described previously (Willadsen *et al.*, 1988).

Assay of 5'-nucleotidase

The enzyme was routinely assayed in 60 mM-Tris/HCl buffer, pH 7.5, containing 10 mM- β -glycerophosphate (Baillyes *et al.*, 1982), 1 mM-MgCl₂ and 10 μ M-AMP containing [8- 14 C]AMP as a tracer. The release of [14 C]adenosine was measured after 30 min at 37 °C (Avruch & Wallach, 1971; Gentry & Olsson, 1975).

The hydrolysis of AMP, ADP and ATP was measured spectrophotometrically in 0.10 M-Tris/HCl buffer, pH 7.4, containing 50 μ M nucleotide and 0.1 unit of adenosine deaminase/ml. The decrease in absorbance was measured at 263 nm. Results were calculated by using a $\Delta\epsilon$ of 8080 (Cercignani, 1987). The hydrolysis of nitrophenyl esters was assayed at a range of substrate concentrations in the same buffer, the release of *p*-nitrophenolate ion being measured at 400 nm. Enzyme activity was expressed in units and specific activity as units/mg of protein, where one unit of enzyme catalysed the hydrolysis of 1 μ mol of substrate/min. Steady-state kinetic parameters were estimated by the method of Eisenthal & Cornish-Bowden (1974), by using initial rates measured at a minimum of six substrate concentrations.

Measurement of protein concentration

Protein concentrations were normally measured with the Pierce BCA protein assay reagent according to the manufacturer's instructions, with bovine serum albumin as a standard. For measurements on inverted gut preparations, a dye-binding method (Bio-Rad) was used. At low protein concentrations, a fluorimetric procedure was used (Willadsen *et al.*, 1988).

Polyacrylamide-gel electrophoresis

Analytical SDS/polyacrylamide-gel electrophoresis was run on gradient gels in the buffer system of Laemmli (1970), and protein was detected with the silver stain of Morrissey (1981). M_r values of reduced proteins were

calculated by comparison with the proteins in the Pharmacia low- M_r standards.

Purification of 5'-nucleotidase

The ticks used in the enzyme isolation were semi-engorged female *Boophilus microplus*, weighing an average of about 25 mg. Membrane material was prepared and stored frozen at -20°C until required (Willadsen *et al.*, 1988). The material precipitated between 600 and 20000 g (Willadsen *et al.*, 1988) was thawed as required and homogenized in 10 mM-Tris/HCl buffer (pH 8.0)/0.15 M-NaCl. The extract was diluted to a protein concentration of 3 mg/ml, cooled to below 7°C , and a cold solution of Triton X-114 (10%, w/v) added in an extract:detergent ratio of 5:1 (v/v). The suspension was stirred for 30 min in an ice bath, then centrifuged at 10000 g for 30 min at 0°C . The supernatant was warmed to $30-35^\circ\text{C}$ for 30 min and centrifuged at low speed to separate the detergent-rich and detergent-poor phases. The detergent-rich phase was redissolved to the original volume of the detergent extract in 0.025 M-Tris/HCl buffer, pH 8.0 at 4°C , then centrifuged (20000 g, 30 min) to remove insoluble material.

The Triton X-114 extract was loaded on a column of DEAE-Sepharose at 4°C . To remove as much Triton X-114 as possible, the column was then washed in sequence with 0.025 M-Tris/HCl buffer, pH 8.0 at 4°C , then in this buffer containing 0.1% Zwittergent 3-14 at 4°C , and finally in Tris/Zwittergent buffer at 20°C . The column was then eluted in 0.025 M-Tris/HCl buffer, pH 8.0, containing 0.1% Zwittergent 3-14 and a linear gradient of 0–0.6 M-NaCl. Fractions containing 5'-nucleotidase activity were pooled.

Pooled fractions from the DEAE-Sepharose column were passed through a column of AMP-Sepharose in a buffer containing 0.025 M-Tris, 2 mM- β -glycerophosphate and 0.1% Zwittergent 3-14, adjusted to pH 7.5–7.6 with HCl. Bound material was eluted with 10 mM-AMP in the same buffer. Fractions containing 5'-nucleotidase activity were pooled. Occasionally, not all of the enzymic activity was bound on a single pass through an AMP-Sepharose column. In this case, the chromatography was repeated and a combined pool of nucleotidase activity made.

These fractions were concentrated on an Amicon Centricon Microconcentrator, then loaded on a Mono Q column attached to a Waters 650 chromatography system and chromatographed in 0.05 M-Tris/HCl buffer (pH 7.5)/0.1% Zwittergent 3-14 with a linear gradient of 0–0.5 M-NaCl in the same buffer. Fractions were analysed by SDS/polyacrylamide-gel electrophoresis and pooled on the basis of purity and nucleotidase activity.

Preparations of inverted gut and assay of nucleotidase

Guts from adult female ticks 4 mm in length were dissected into 10 mM-Tris/HCl buffer (pH 7.6)/0.15 M-NaCl, then sliced longitudinally with a fine needle. The guts then everted spontaneously. They were cut into sections and washed for approx. 1 h in several changes of cold buffer. Portions were homogenized in buffer, and nucleotidase was assayed in the sections of both inverted gut and gut homogenate by the normal method using [^{14}C]AMP, though with the addition of NaCl to give a final ionic strength of 0.155 M. After 30 min, reactions were stopped in all assays by addition of p[CH₂]pA. The assay mixtures containing intact cells were then homogenized; portions were used immediately for

determination of released adenosine and the remainder for protein determinations.

H.p.l.c. assay of nucleotide hydrolysis

Substrates (50 μM) were incubated in 0.05 M-Tris/HCl buffer/2 mM-MgCl₂, pH 7.5 at 25°C . A portion of enzyme was added and the reaction followed by repeated sampling of the mixture and separation of substrate and products by reverse-phase chromatography. Separations were on a Waters Novapak radial pack C-18 cartridge in 0.1 M-phosphate buffer, pH 6.0, containing variable amounts of methanol. By adjusting the methanol concentration and concentration range, it was possible to design a series of isocratic or linear gradient systems which allowed the separation of all the nucleosides and nucleotides of interest within less than 12 min. Reactions were therefore monitored by sampling at 12 min intervals. Substrates and products were quantified by integrating the A_{260} by using a Shimadzu C-R3A recording integrator, and a minimum of six estimates was used to calculate rates.

Inhibition of the enzyme with 5'-p-fluorosulphonylbenzoyladenine

The enzyme was incubated at 25°C in 0.05 M-Hepes buffer, pH 7.4, containing bovine serum albumin (1 mg/ml) and 2% (v/v) dimethylformamide, in the presence of 100 μM -5'-p-fluorosulphonylbenzoyladenine. Samples were removed periodically and assayed spectrophotometrically against AMP, ADP or ATP.

Hydrolysis of ^{32}P -labelled ATP and ADP

The hydrolysis mixture contained 1 ml of 50 μM -[γ - ^{32}P]ATP (sp. activity 6.6×10^7 c.p.m./ μmol) in 0.05 M-Hepes buffer, pH 7.4. Incubations were performed in the absence of Mg^{2+} , at 37°C for 1 h after addition of 1 munit of enzyme. Samples were withdrawn for chromatography before the addition of enzyme and immediately after the incubation. Hydrolysis of [β - ^{32}P]ADP (sp. radioactivity 1.3×10^7 c.p.m./ μmol) was performed in a similar manner. In this case, 4 munits of enzyme was added and the incubation was for 75 min.

Samples were chromatographed on a Mono Q column in a Waters 650 chromatography system. The buffer was 0.02 M-Hepes/5 μM -EDTA adjusted to pH 7.4 with KOH. Nucleotides and phosphates were separated with a linear gradient of 0–0.25 M-KCl in the buffer over 48 min at a flow rate of 1 ml/min; 0.5 ml fractions were collected and counted for radioactivity.

The elution times of ortho-, pyro- and triphosphates were estimated from standards injected separately, after determination of orthophosphate (Forbush, 1983), for the two latter compounds after hydrolysis with 1 M-HCl at 95°C for 30 min. Elution times of nucleotides were measured from the A_{260} , by using a Shimadzu C-R3A integrator and, for accurate comparison with the times for the phosphates, adjusted by 0.15 min, the time delay between the detector and eluate collection.

RESULTS

Measurement of nucleotidase activity

The activity of the enzyme was measured routinely by the release of [^{14}C]adenosine from [^{14}C]AMP as described in the Experimental section. The release of product by

both purified enzyme and crude membrane preparations was linear with time to at least 60 min at 37 °C. Rates were linear to at least 50 % hydrolysis of the substrate. The rate of hydrolysis was proportional to the amount of purified or semi-purified enzyme added within the usual working range of the assay, although assays with crude extracts showed some deviation from linearity. It was routinely found during enzyme purification that the total amount of enzyme activity being measured increased during the initial steps, and so there may be some inhibition of the enzyme or interference with the assay in crude extracts. Since the tick extracts contain large quantities of both alkaline and acid phosphatase, β -glycerophosphate was included in the assay to prevent non-specific hydrolysis of the AMP substrate. It was found that the hydrolysis of the substrate by either crude extracts or purified enzyme was inhibited completely by the inclusion of 20 μ M-p[CH₂]pA, an inhibitor of 5'-nucleotidase (Luzio *et al.*, 1986; Zachowski *et al.*, 1981). This suggests that the assay is accurately measuring 5'-nucleotidase activity and is unaffected by the presence of phosphatases. Subsequent experiments showed that the β -glycerophosphate caused partial inhibition of the enzyme at both 25° and 37 °C, but this did not affect the use of the assay to monitor the purification of the enzyme.

In the kinetic studies of the purified enzyme, three types of activity assay were used: a radiometric assay similar to that described above, though without the addition of β -glycerophosphate, a spectrophotometric assay measuring the release of adenosine with adenosine deaminase in a coupled assay, and an assay using separation of substrate and products of hydrolysis on reverse-phase h.p.l.c. The validity of the three methods was checked by measuring the activity of a purified enzyme preparation in the three systems in 0.1 M-Tris/HCl buffer, pH 7.5 at 25 °C, at saturating substrate concentration but in the absence of Mg²⁺ ions and β -glycerophosphate. The radiometric assay gave an enzyme concentration of 0.135 unit/ml, the spectrophotometric assay 0.136 unit/ml and the h.p.l.c. method 0.138 unit/ml. The three methods are thus consistent.

Purification and some properties of the enzyme

The results of a typical enzyme purification are shown in Table 1. Three consecutive purifications of the enzyme gave overall recoveries and specific activities of, respectively, 25 % and 51 units/mg, 53 % and 30 units/mg, and 21 % and 39 units/mg. Part of the variation in specific activity may be due to difficulty in accurately measuring the low protein concentration of the purified enzyme. The final product, as judged by SDS/

Fig. 1. SDS/polyacrylamide-gel electrophoresis of purified 5'-nucleotidase

Samples are (left to right) 0.7 μ g of one preparation and 0.3 and 0.5 μ g of a second.

polyacrylamide-gel electrophoresis, had only minor, if any, contaminants (Fig. 1). Two preparations are shown in Fig. 1. The apparent M_r of the protein in the presence of mercaptoethanol was 67000. The purified protein was found to bind completely to lentil lectin-Sepharose and to be eluted with 50 mg of methyl α -D-mannopyranoside/ml (results not shown).

The effects of a range of compounds on the enzyme activity were investigated by incubating purified nucleotidase in 0.1 M-Hepes buffer, pH 7.4, for 10 min at 37 °C with these compounds, then adding substrate and assaying the enzyme for 30 min as usual. The results shown in Table 2 are the means of two experiments. The largest difference between any two determinations was 3 %. The enzyme is inhibited weakly by anions, whether chloride, sulphate or phosphate, and strongly by both EDTA and concanavalin A. The inhibitory effects of EDTA and concanavalin A have been found with other nucleotidases (Luzio *et al.*, 1986).

The pH optimum of the enzyme was measured against [¹⁴C]AMP as substrate at 20 and 50 μ M concentrations in 1 mM-MgCl₂ and in a series of 0.1 M-Mes and -Hepes buffers at pH values from 5.8 to 8.5. Mes buffers were used at pH 5.8, 6.3 and 6.8, and Hepes buffers at pH 7.4, 7.9 and 8.5. β -Glycerophosphate was not added. The

Table 1. Purification of 5'-nucleotidase from *Boophilus microplus*

Fraction	Protein (mg)	Activity (units)	Specific activity (units/mg)	Yield (%)	Purification factor
Membrane suspension	7640	6.63	0.00087	100	1.0
Triton extract	7920	6.00	0.00076	91	0.9
Detergent-rich phase	1660	5.17	0.0031	78	3.6
DEAE-Sepharose pool	193	3.20	0.017	48	19.0
AMP-Sepharose pool	0.32	1.73	5.33	26	6140
Mono Q pool	0.035	1.37	38.8	21	44600

Table 2. Effects of potential inhibitors on nucleotidase activity

Compound	Final concn.	Residual activity (%)
NaCl	20 mM	71
KCl	20 mM	71
Sodium phosphate	20 mM	20
Na ₂ SO ₄	20 mM	63
EDTA	1.0 mM	5
<i>p</i> -Hydroxymercuribenzoate	1.0 mM	26
Levamisole	2.0 mM	99
Concanavalin A	1.0 mg/ml	1
Concanavalin A	0.1 mg/ml	8

procedure was otherwise as described for the standard assay. The activity of the enzyme was the same within experimental error at both substrate concentrations, hence maximum velocity was being measured. There was a broad pH optimum between pH 6.3 and 7.4. In the absence of substrate, the enzyme was stable for 30 min throughout the pH range investigated.

Nucleotidase as an ectoenzyme

Evidence suggestive of ectopic localization of some of the enzyme was provided by the demonstration that a proportion of total cellular nucleotidase activity was measurable in an intact cell preparation. The gut was chosen for this, as it is a large organ that can be dissected and inverted so that a maximum number of cells are accessible. The specific activity of nucleotidase in intact cells was 35 ± 18 μ units/mg (mean $\pm$ S.D. for 16 determinations), whereas in homogenates of comparable segments of gut it was 280 ± 120 μ units/mg (mean $\pm$ S.D. for 7 determinations). This suggests that no more than 13 % of the total nucleotidase in such cell preparations is readily accessible to substrate.

The enzyme activity measured in intact cells was not due to leakage of a cytoplasmic form of nucleotidase from damaged cells, since the enzyme was almost entirely membrane-bound in this tissue. In three separate homogenates of washed gut, the maximum amount of soluble enzyme was 2.7 % of the total activity. However, the best evidence that some of the nucleotidase is an ectoenzyme is its demonstration on the surface of gut cells by histological methods (D. H. Kemp & J. M. Gough, personal communication).

Specificity of the enzyme for nucleoside monophosphates

Substrate specificity was initially investigated by measuring the rates of hydrolysis of a series of nucleoside 2', 3'- and 5'-monophosphates by using the h.p.l.c. method described in the Experimental section. The compounds were incubated at 50 μ M concentration in 0.10 M-Tris/HCl buffer, pH 7.5 at 25 °C. The rate of the enzymic reaction was routinely measured by both the loss of substrate and the appearance of the nucleoside product, both being quantified after separation by reverse-phase chromatography. The rates were then averaged from the two rate estimates, although they were, as expected, the same within experimental error. For example, for the series of nucleoside 5'-monophosphates, the maximum difference between the two

estimates for any hydrolysis was 4 %. The rates are listed in Table 3 and are expressed relative to the rate of hydrolysis of 5'-AMP under the same conditions.

Hydrolysis of simple phosphate esters and inhibition by p[CH₂]₁₇pA

The hydrolyses of *p*-nitrophenyl phosphate, bis-*p*-nitrophenyl phosphate and *p*-nitrophenyl phenylphosphonate were measured spectrophotometrically in Tris buffer without Mg²⁺. There was no detectable hydrolysis of 2 mM-*p*-nitrophenyl phosphate; thus it is hydrolysed at less than 0.5 % of the rate of AMP. Both of the other esters were substrates, and their hydrolysis appeared to follow simple Michaelis-Menten kinetics. The results are given in Table 4. p[CH₂]₁₇pA (17 nm) gave mixed inhibition of the enzyme, with bis-*p*-nitrophenyl phosphate as substrate (Table 4).

Characterization of ADPase and ATPase activities of the purified 5'-nucleotidase

Unexpectedly, it was found that both ADP and ATP were hydrolysed by the enzyme and that the loss of either substrate was accompanied by the simultaneous production of an equivalent amount of adenosine (Fig. 2). There was no detectable production of intermediates such as ADP or AMP from ATP hydrolysis. Under these circumstances, it was feasible to measure the rates of hydrolysis of AMP, ADP and ATP spectrophotometrically in a coupled enzyme assay utilizing adenosine deaminase. These assays, performed as described in the Experimental section with excess adenosine deaminase,

Table 3. Relative rates of hydrolysis of nucleotide derivatives

Derivative	Rate
5'-AMP	1.00
2'-AMP	< 0.01
3'-AMP	< 0.01
5'-CMP	10.6
2' + 3'-CMP	< 0.01
5'-GMP	2.71
2' + 3'-GMP	< 0.01
5'-UMP	11.0
2' + 3'-UMP	< 0.01
5'-ADP	0.18
5'-ATP	0.62

Table 4. Steady-state kinetics for hydrolysis of various substrates

Substrate	<i>K_m</i> (μ M)	<i>V</i> (μ mol/min per mg)
AMP	0.3	9.4
ADP	0.09	2.0
ATP	0.1	6.2
Bis- <i>p</i> -nitrophenyl phosphate	280	1.80
Bis- <i>p</i> -nitrophenyl phosphate + 17 nm-p[CH ₂] ₁₇ pA	810	1.18
<i>p</i> -Nitrophenyl phenylphosphonate	870	3.1

Fig. 2. Hydrolysis of ADP and ATP to adenosine

The Figure shows the time course for the loss of ATP (●) or ADP (□), the production of adenosine (○) and the sum of the two (■) throughout hydrolysis.

gave linear rates which were proportional to the amount of nucleotidase added.

Two reasons could be envisaged for the fact that the 5'-mono-, di- and tri-phosphates of adenosine were hydrolysed: either the reaction was due to a single enzyme with broad substrate specificity, or it was due to the presence of contaminants in the purified enzyme. As evidence for the former possibility, it was shown that the three activities were catalysed by very similar, if not identical, enzymes. The AMPase, ADPase and ATPase activities all bound to lentil lectin-Sepharose and were eluted by methyl mannopyranoside (50 mg/ml); the ratios of the three activities remained the same before and after the affinity chromatography. None of the three activities required free Mg^{2+} . Spectrophotometric assay of the hydrolysis of AMP, ADP and ATP showed that the ratios of activity in the presence of 2 mM- $MgCl_2$ to that in its absence were 1.02, 1.04 and 0.81 respectively.

To obtain convincing evidence that a single enzyme hydrolysed all three nucleotides, the enzyme was incubated at 25 °C with 100 μ M-5'-*p*-fluorosulphonylbenzoyl-adenosine, and samples were withdrawn periodically and assayed against AMP, ADP or ATP. The loss of activity towards all substrates was first order to greater than 90 % inhibition. First-order rate constants

Fig. 3. Products of the hydrolysis of ATP

(a) Chromatography of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ before hydrolysis. (b) Chromatography of the products after 60 min hydrolysis. Elution times of standards determined separately are indicated by the points of the arrows. Abbreviation: PPP_i , linear triphosphate.

for inhibition were measured from a minimum of five data points, and the results of four separate experiments for each substrate were averaged. For the AMPase activity, the first-order rate constant for loss of activity had a mean $\pm$ S.E.M. of $0.20 \pm 0.02 \text{ min}^{-1}$; for the ADPase it was $0.19 \pm 0.02 \text{ min}^{-1}$ and for the ATPase $0.22 \pm 0.01 \text{ min}^{-1}$. These values are the same, within experimental error. In the absence of 5'-*p*-fluorosulphonylbenzoyl-adenosine, the enzyme was stable.

Since it seems extremely unlikely that three different enzymes would be inhibited by this compound at the same rate, it is concluded that all three adenosine phosphates are hydrolysed by the same enzyme.

Mechanism of ATP and ADP hydrolysis

In general terms, two mechanisms of hydrolysis of ATP and ADP can be envisaged: either there is sequential removal of phosphate residues to produce, finally, adenosine, or there is a single cleavage to release adenosine and triphosphate or pyrophosphate respectively. The fact that, during the hydrolysis of ATP and ADP as monitored by h.p.l.c., the only u.v.-absorbing compounds seen were the substrate and adenosine suggested that the second mechanism is correct. However, there is the possibility that the kinetics

Fig. 4. Products of the hydrolysis of ADP

(a) Chromatography of [β -³²P]ADP before hydrolysis. (b) Chromatography of the products after 70 min hydrolysis. Elution times of standards determined separately are indicated by the points of the arrows.

of the hydrolysis are such that intermediates produced during the sequential removal of orthophosphates from, for example, ATP, would be in very low concentration.

The kinetics of the hydrolysis of AMP, ADP and ATP were therefore measured spectrophotometrically as described in the Experimental section. The concentration-dependence followed simple Michaelis-Menten kinetics, and the results are listed in Table 4. Adenosine deaminase activity was not rate-limiting at any substrate concentration. The estimates of K_m are approximate, since the minimum substrate concentrations that could be used experimentally were in excess of K_m . Nevertheless, the values for the steady-state kinetic parameters are inconsistent, for example, with a sequential hydrolysis of ATP to ADP, AMP and adenosine.

Direct evidence that the hydrolyses of both ADP and ATP occurred by cleavage at the phosphate residues α to the 5' carbon atom of the ribose residue was obtained by enzymically hydrolysing both [γ -³²P]ATP and [β -³²P]ADP and separating the products by ion-exchange h.p.l.c. The results are shown in Figs. 3 and 4. The radioactive product of ATP hydrolysis had a chromatographic mobility identical with that of tripolyphosphate and clearly different from that of any of the other compounds which might be expected: ATP, ADP, AMP, pyrophosphate or orthophosphate. In an analogous way, β -³²P]ADP gave pyrophosphate as a labelled product.

DISCUSSION

The tick enzyme described above has many features in common with mammalian 5'-nucleotidases and it has,

for convenience, been referred to as a nucleotidase throughout this paper. It is a membrane-bound enzyme and a glycoprotein: in common with other nucleotidases (Luzio *et al.*, 1986), it binds to lentil lectin and is inhibited by concanavalin A. The M_r of 67000 estimated by SDS/polyacrylamide-gel electrophoresis is close to that of the rat liver enzyme, 70000 (Luzio *et al.*, 1986). It is inhibited by EDTA. It is a minor cell component: for example, it is less than 1 part in 100000 of the protein in a washed tick gut.

In the absence of good histological evidence, it is difficult to prove that the enzyme is an ectoenzyme, particularly since the activity must be measured in complex tissues rather than cell suspensions. In everted tick gut, intact tissue shows only about 13% of the activity of gut homogenates. This value is low, but comparable with the estimates for rat fibroblasts, where the enzyme has been used as a probe for pinocytosis (Widnell *et al.*, 1986). In this respect, it may be relevant that tick digest cells carry out very active endocytosis as a prelude to digestion (Agbede & Kemp, 1985).

The substrate specificity as examined in this paper again shows many similarities to that of the mammalian nucleotidases, but also some fascinating differences. The enzyme, like the mammalian nucleotidases, has broad specificity for the nucleoside 5'-monophosphates, but fails to hydrolyse either the 2' or 3' derivatives. It is clearly not a simple phosphatase: the fact that it hydrolyses AMP in the presence of a 1000-fold excess of β -glycerophosphate, the pH optimum of around pH 7, the specificity seen in the hydrolysis of nucleoside monophosphates, the failure to hydrolyse a phosphatase substrate such as *p*-nitrophenyl phosphate and the lack of inhibition by levamisole, are all evidence for this.

A further strong similarity which the tick enzyme shares with the mammalian nucleotidases is its inhibition by very low concentrations of $p[CH_2]_pA$ (Worku & Newby, 1983; Naito & Lowenstein, 1985; Pearson, 1986).

Nevertheless, the enzyme clearly has much broader specificity than the normal 5'-nucleotidases. It hydrolyses both ADP and ATP as well as AMP, unlike the mammalian enzymes, for which ATP and ADP are merely good inhibitors (Naito & Lowenstein, 1985; Luzio *et al.*, 1986). That the three activities are due to a single enzyme is shown by a number of facts, most convincingly by the identity of rates of inhibition of all three activities by fluorosulphonylbenzoyladenine.

The way in which a single enzyme could catalyse the hydrolysis of AMP, ADP and ATP became clear once the mechanism of hydrolysis was identified. The absence of detectable amounts of intermediates in the hydrolysis of ADP and ATP to the final product, adenosine, coupled with the steady-state kinetics for the three adenosine phosphates suggested that all the hydrolytic reactions occur by a cleavage at the phosphate α to the 5' carbon of the ribose. From the enzyme's point of view, then, the length of the phosphate chain would then be of relatively little importance. Direct proof of this was obtained by showing that ATP was hydrolysed to produce tripolyphosphate and ADP to produce pyrophosphate. With such a mechanism, it was not surprising to find that the enzyme also hydrolysed such phosphodiesterase substrates as bis-*p*-nitrophenyl phosphate and *p*-nitrophenyl phenylphosphonate (Kelly *et al.*, 1975).

Tripolyphosphate is a product of at least two other

enzymic reactions (Markham *et al.*, 1987; Ohta & Beck, 1976), but the simple hydrolysis of ATP to adenosine and triphosphosphate, as reported here, is a unique biochemical reaction. What might be the role of this enzyme in the organism? Considering its broad specificity for 5'-phosphate esters of nucleosides, it is difficult to imagine that the enzyme's function is anything other than the hydrolysis of such compounds to release the free nucleoside. Why the tick should require this activity is unknown, but it is tempting to speculate that it serves in a single protein the functions of the multiple ecto-AMP-, -ADP- and -ATP-ases of some mammalian cells (Pearson, 1986; Slakey *et al.*, 1986).

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