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Partial purification and properties of an AMP-specific soluble 5′-nucleotidase from pigeon heart

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A soluble 5′-nucleotidase was purified 200-fold from pigeon heart. The enzyme (1) had an apparent molecular mass close to 150 kDa, (2) had a neutral pH optimum and hydrolysed a wide range of nucleoside 5′-monophosphates with a 15-fold preference for AMP over IMP, (3) at near-physiological concentrations of AMP was activated by ADP but not by ATP, (4) was inhibited by high Mg^{2+} concentration and high ionic strength, (5) was weakly inhibited by *p*-nitrophenol phosphate and P_i, and (6) was non-competitively inhibited more potently by 5′-deoxy-5′-isobutylthioinosine than by 5′-deoxy-5′-isobutylthioadenosine, but not by [α,β-methylene]ADP. The data show that the enzyme is distinct from the ecto-5′-nucleotidase and from the previously purified IMP-specific 5′-nucleotidase. They also predict that the enzyme is activated during ATP catabolism and hence will generate a more-than-linear increase in the adenosine-formation rate in response to an increase in cytosolic AMP concentration.

INTRODUCTION

The enzymes involved in cellular ATP degradation may have important homoeostatic roles and are also potential sites for pharmacological intervention to protect organs against nucleotide depletion. Hydrolysis of nucleoside monophosphates is a key step in the degradative pathway, because the product nucleosides can diffuse out of the cell, leading to loss of total purines. One of these nucleosides, namely adenosine, may in addition serve as signal which increases the supply of O_2 and substrates and decreases energy utilization, thereby tending to halt or reverse ATP degradation (Newby, 1984).

The identity of the phosphohydrolase responsible for adenosine formation in mammalian cells undergoing ATP breakdown is still uncertain. In the heart, for example, the activity of a soluble IMP-specific 5′-nucleotidase (Itoh *et al.*, 1986*a*) towards AMP is probably insufficient to account for ischaemia-induced adenosine formation (Meghji *et al.*, 1988*a*). The ecto-5′-nucleotidase appears unable to hydrolyse cytosolic AMP, since it can be inhibited in rat or guinea-pig anoxic perfused hearts (Frick & Lowenstein, 1976; Schutz *et al.*, 1981) or rat and chick cardiac myocytes (Meghji *et al.*, 1985, 1988*b*) without affecting rates of adenosine efflux, in agreement with data from other cell types (Newby *et al.*, 1987). This enzyme may, however, be responsible for hydrolysing extracellular-fluid AMP derived from nucleotides released from endogenous nerve endings, as suggested by the recent work of Imai *et al.* (1989).

In pigeon heart, which lacks the ecto-5′-nucleotidase, an active AMP-specific 5′-nucleotidase has been described (Newby, 1988). The enzyme, which was stimulated by nucleoside di- and tri-phosphates, was shown to be in the cytosolic compartment, possibly in weak association with contractile proteins (Newby, 1988). An apparently similar soluble AMP-specific 5′-nucleotidase was found in rat heart, where it was separable from the IMP-specific enzyme (Truong *et al.*, 1988). This suggests that distinct phosphohydrolases are responsible for the hydrolysis of AMP and the IMP derived from AMP by AMP deaminase, which is known to be active in the hearts of several species (Skladanowski, 1988).

More information is needed about the nature and properties of the AMP-specific 5′-nucleotidase before its role in adenosine formation can be evaluated. The enzyme was therefore partially purified from pigeon heart and its properties were studied.

MATERIALS AND METHODS

Materials

ATP, AMP (disodium salts) and ADP (monopotassium salt) were obtained from Boehringer Corp., Lewes, East Sussex, U.K. ATP and ADP were subjected to chromatography on DEAE-Sephacel in order to remove traces of other nucleotides (Pharmacia Fine Chemicals, 1974). dAMP, 3′,5′-cyclic AMP, IMP, dIMP, GMP, dGMP, CMP, dCMP, UMP, TMP, [α,β-methylene]ADP, 5′-deoxy-5′-isobutylthioadenosine (IBTA), adenosine, ribose 5-phosphate, *p*-nitrophenyl phosphate dithiothreitol and polyethyleneimine (PEI)–cellulose sheets were purchased from Sigma Chemical Co., Poole, Dorset, U.K. 5′-Deoxy-5′-isobutylthioinosine (IBTI) was prepared as described previously (Skladanowski *et al.*, 1989). Phosphocellulose was a product of Whatman, Maidstone, Kent, U.K. [2-^{3}H]AMP (540 GBq/mmol) was obtained from The Radiochemical Centre (Amersham, Bucks., U.K.). [2-^{3}H]IMP (180 GBq/mmol) was prepared from [2-^{3}H]AMP by the method of Worku & Newby (1982). A prepacked size-exclusion-chromatography column (UltroPak TSK G4000 SW; 10 μm particle size; column dimensions 7.5 mm × 300 mm) was obtained from Pharmacia LKB, Central Milton Keynes, Bucks., U.K. $(NH_4)_2SO_4$ was of enzyme grade, low in heavy-metal ions, and was purchased from BDH (Poole, Dorset, U.K.). All other reagents were of the highest grade available and were obtained as described previously (Meghji *et al.*, 1988*a*).

Purification of cytosolic AMP-specific 5′-nucleotidase

Part of the procedure was a modification of that described by Truong *et al.* (1988). Pigeons (*Columbia livia*) were killed by decapitation. Hearts (three or four) were perfused briefly via the aorta to remove blood, each with 20 ml of buffer A, which

Abbreviations used: IBTA, 5′-deoxy-5′-isobutylthioadenosine; IBTI, 5′-deoxy-5′-isobutylthioinosine; PMSF, phenylmethanesulphonyl fluoride; PEI, polyethyleneimine; $s_{0.5}$, concentration giving 50% of maximal activity.

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contained 20 mM-sodium dimethylglutarate, pH 7.0, 0.1 mM-dithiothreitol, 0.14 M-KCl and 0.2 mM-PMSF. The atria, great vessels and fat were removed and the ventricles minced with a scalpel. The ventricular tissue (13–18 g) was homogenized at 4 °C in 7.5 vol. of buffer A with a Polytron homogenizer (Northern Media Supply, Hessle, N. Humberside, U.K.) with two bursts for 30 s each at setting 2. The homogenate was centrifuged at 105000 *g* for 60 min. The resulting supernatant was filtered through cloth gauze, and $(NH_4)_2SO_4$ (258 g/l) was added within 2–3 h with continuous stirring. The mixture was left on ice for 2 h before being centrifuged at 12000 *g* for 30 min. The precipitate was dissolved in a total volume of 3–4 ml of buffer B, which contained 25% (v/v) glycerol, 0.1 mM-dithiothreitol, 1 mM-EDTA, 0.2 mM-PMSF and 0.05 M-Hepes/NaOH, pH 7.0, and was then dialysed overnight against 1 litre of buffer B. The resulting suspension was centrifuged at 12000 *g* for 15 min to remove insoluble material, and the supernatant was applied to a phosphocellulose column (1.5 cm × 6 cm) previously equilibrated with buffer B. The column was washed with 60 ml of buffer B, followed by a linear gradient of 0–0.8 M-NaCl in buffer B. The volumes of each buffer were 25 ml. The column was eluted at 0.25 ml/min and 1 ml fractions were collected. Fractions with the highest specific activity of 5′-nucleotidase were pooled (total vol. 5–6 ml), diluted 5-fold with buffer B and applied to a small phosphocellulose column (1 cm × 3 cm). The column was washed with 20 ml of buffer B, and concentrated active protein was then eluted with 0.8 M-NaCl in buffer B. Alternatively the first phosphocellulose-chromatography pool was concentrated by using Minicon blocks (Amicon, Danvers, MA, U.S.A.). The concentrated enzyme (0.2 ml lots) was subjected to high-efficiency size-exclusion chromatography on an UltroPak TSK G4000 SW column eluted with buffer C, which contained 20% glycerol (v/v), 0.05 M-Hepes/NaOH, pH 7.0, 1 mM-EDTA, 0.1 mM-dithiothreitol, 0.1 M-Na_2SO_4 and 0.05 M-$MgSO_4$. In some preparations 0.5–1.0 mM-ADP or -ATP were included in an attempt to protect the enzyme from denaturation. The column was eluted at a flow rate of 0.4 ml/min, and 0.6 ml fractions were collected. The fractions of highest specific activity were pooled, dialysed if necessary against buffer C to remove added nucleotides, and concentrated in a Minicon block. The enzyme thus obtained was used for the kinetic studies reported here, unless indicated otherwise.

Activities of 5′-nucleotidase and other enzymes

The purified pigeon heart soluble 5′-nucleotidase was assayed by a modification of the method described for purified rat liver soluble 5′-nucleotidase (Newby, 1988). Incubations were conducted at 37 °C from 2–10 min in a total volume of 0.1 ml containing 100 mM-Tes/NaOH, pH 6.5, 10 mM-$MgCl_2$, 30 mM-NaCl, AMP or IMP concentrations in the range 0.1–15 mM (plus 2–3 kBq of [2-^{3}H]AMP or [2-^{3}H]IMP as appropriate), activators or inhibitors as indicated in the text and protein concentrations in the range 4–20 μg/ml. Throughout the purification procedure assays were conducted with 10 mM-AMP plus 0.25 mM-ATP or 1 mM-ADP. The reaction was started by adding different amounts of enzyme protein designed so that the overall substrate conversion did not exceed 25%. Reactions were terminated and the concentrations of radioactive nucleoside were determined as described by Newby (1988). When other unlabelled substrates (at 10 mM) were used, the progress of reaction was measured by P_i liberation alone, by using the method of Itaya & Ui (1966).

Adenylate kinase activity was assayed under the same conditions used for 5′-nucleotidase measurement with 0.1 mM-AMP (plus 2–3 kBq of [2-^{3}H]AMP) and 10 mM-ATP. After terminating the reactions, triplicate 15 μl aliquots were spotted on PEI–cellulose sheets, which were eluted with 0.5 M-formate/Na^+, pH 4.6. ATP and ADP remained close to the origin, whereas AMP had an R_F value of approx. 0.4. The spots containing ADP + ATP were cut from the sheet and radioactivity was determined. The enzyme activity was determined as the rate of conversion of AMP into ATP + ADP.

AMP deaminase was determined by the modified procedure of Skladanowski *et al.* (1979), as described previously (Skladanowski *et al.*, 1989).

One unit of enzyme activity was defined as the amount needed to convert 1 μmol of substrate per min at 37 °C.

Protein was measured as described by Bradford (1976), with BSA as standard.

Estimation of M_r

M_r was estimated by Ultrogel AcA34 column chromatography. The column (45 cm × 2 cm) was eluted with Buffer B at a flow rate of 0.2 ml/min, and 2 ml fractions were collected. The column was calibrated with ferritin (450 kDa), β-glucuronidase (290 kDa), aldolase (158 kDa), hexokinase (100 kDa) and Black Albumin (68 kDa). The elution positions of the standard proteins were assessed either by protein estimation with the procedure of Bradford (1976), or by absorbance at 280 nm or 624 nm in the case of black albumin. The elution of the enzyme was detected by measurement of activity as described above.

Calculations

The calculation of kinetic constants was performed using a program designed for IBM-compatible computers (Marszalek *et al.*, 1989). Initial estimates for kinetic parameters were computed from the data (0.1–0.9 $V_{max.}$) by using a linear regression of the transformed Hill equation, where the $V_{max.}$ entry was the experimental maximum velocity. The final fit of the experimental data to the Hill equation was performed by adopting an iterative non-linear least-squares method with a modified Marquardt (1967) algorithm. S.D. values were derived from the diagonal elements of a co-variance matrix and represent the goodness-of-fit.

RESULTS

Purification of the enzyme

The AMP-specific soluble 5′-nucleotidase was purified by a combination of $(NH_4)_2SO_4$ precipitation, chromatography on phosphocellulose and high-performance gel filtration, as summarized in Table 1. The initial activity of the soluble 5′-nucleotidase was equivalent to 3.7 ± 1.0 units/g of fresh tissue in three experiments (cf. Newby, 1988). A 2-fold increase of total 5′-nucleotidase activity was observed after $(NH_4)_2SO_4$ precipitation, presumably due to removal of factor which interfered with the assay or to the presence of an unidentified endogeneous inhibitor. Subsequent removal of proteins which precipitated at low ionic strength led to a further purification. Phosphocellulose chromatography led to elution of a single peak of 5′-nucleotidase, the ratio of activity of which, measured with AMP and IMP as substrates, was invariant (results not shown). The enzyme which was eluted at approx. 0.5 M-NaCl was purified 4-fold (Table 1). High-performance gel filtration yielded a further 5-fold purification, largely attributable to the removal of proteins of lower molecular mass. An overall purification of more than 200-fold was achieved relative to the original high-speed supernatant. The yield of activity could be increased by including ADP or ATP in the buffer during gel filtration. Their subsequent removal by dialysis caused partial inactivation of the enzyme, however. The enzyme resisted further attempts at purification on ADP–agarose or Affi-gel Blue affinity chromatography.

Table 1. Purification of AMP-specific soluble 5′-nucleotidase from 16 g of pigeon ventricle

The results are representative of three similar experiments.

Step	Volume (ml)	Protein (mg/ml)	Total protein (mg)	Activity (units/ml)	Specific activity (units/mg)	Total activity (units)	Purification (fold)	Yield (%)
Supernatant	116.6	2.95	343.97	0.49	0.17	57.08	1	100
$(NH_4)_2SO_4$ precipitate	5.00	17.91	89.55	24.56	1.37	122.8	8	215
Dialysed $(NH_4)_2SO_4$ precipitate	3.4	16.24	55.22	27.36	1.68	93.02	10	163
Phosphocellulose-concentrated pool	0.83	5.5	4.56	38.9	7.34	32.3	43	56
UltroPak TSK G4000 SW column	0.45	0.27	0.12	9.67	35.83	4.35	211	8

The major activities which would complicate kinetic studies of the enzyme were removed: AMP deaminase by phosphocellulose chromatography, and adenylate kinase during size-exclusion chromatography (results not shown).

The M_r estimated for the enzyme by gel-exclusion chromatography (Fig. 1) was 150000±5000 (mean±range of two estimations).

Substrate specificity of 5′-nucleotidase

The relative rates of dephosphorylation of different nucleotides and other phosphoesters are presented in Table 2. The preferred substrate for the purified enzyme was 5′-AMP, but the enzyme hydrolysed other purine and pyrimidine nucleoside monophosphates. Of these, IMP was among the poorest. Very low rates of dephosphorylation were observed with β-glycerophosphate, ribose 5-phosphate and *p*-nitrophenyl phosphate. The phosphodiester, 3′,5′-cyclic AMP, was not hydrolysed significantly.

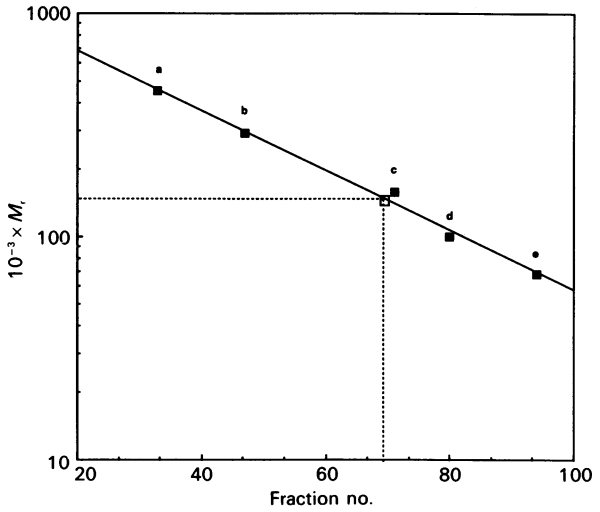

Fig. 1. Gel filtration of pigeon heart 5′-nucleotidase on Ultrogel AcA34

Enzyme purified to the stage of phosphocellulose chromatography (9.7 mg of protein in 0.5 ml of Buffer B) or a mixture of standard proteins: ferritin (M_r 450000, a), β-glucuronidase (290000, b), aldolase (158000, c), hexokinase (100000, d) and black albumin (68000, e), in a total amount of 25 mg, were applied to a column (45 cm × 2 cm) of Ultrogel AcA34. The elution of the various proteins was detected as described in the Materials and methods section.

Table 2. Substrate specificity and inhibition of pigeon heart 5′-nucleotidase

The enzyme was incubated in the presence of 1 mM-ADP with the substrates shown at 10 mM final concentration. Inhibition experiments were performed with 10 mM-AMP (plus 2–3 kBq of [2-^{3}H]AMP) plus 1 mM-ADP and 10 mM-inhibitor unless otherwise stated. Values represent the means (±S.E.M.) for three separate experiments.

Substrate or inhibitor	Relative rate of dephosphorylation (% of AMP-ase)	Inhibition of AMP-ase activity (%)
AMP	100	0
dAMP	66.0 (7.0)	66.1 (2.0)
CMP	61.7 (5.4)	55.4 (2.7)
dIMP	60.9 (3.2)	
dGMP	49.9 (1.2)	
UMP	49.5 (6.5)	74.5 (1.5)
GMP	33.5 (1.4)	42.7 (3.4)
IMP	33.1 (1.2)	36.0 (4.8)
TMP	15.2 (5.2)	
dCMP	12.1 (3.2)	
Ribose 5-phosphate	6.9 (2.7)	14.2 (5.0)
β-Glycerophosphate	5.8 (2.6)	7.7 (6.0)
3′,5′-Cyclic AMP	2.0 (1.1)	
p-Nitrophenyl phosphate	0.9 (0.6)	49.9 (2.9)
[αβ-methylene]ADP (2.5 mM)		−1.8 (1.8)
P_i (20 mM)		31.7 (12.0)
IBTA (3 mM)		21.1 (1.3)
IBTI (7 mM)		54.8 (3.7)

Kinetic properties of pigeon heart 5′-nucleotidase

The purified enzyme had a pH optimum close to pH 7.0 in the absence of effectors (Fig. 2). The pronounced decrease of activity at more acidic pH values was less when ATP, and especially when ADP, was present (Fig. 2). All subsequent kinetic and regulatory studies were performed at pH 6.5 so as to allow comparison with the previously described properties of IMP-specific 5′-nucleotidase (Itoh, 1981*a*).

The AMP- and IMP-saturation curves for pigeon heart 5′-nucleotidase in the presence of 1 mM-ADP are presented in Fig. 3. The kinetic parameters estimated from the AMP curve were: $V_{max.} = 29.5 \pm 1.1$ μmol/min per mg of protein, concentration giving 50% of maximal activity ($s_{0.5}$) = 4.7±0.3 mM and h (Hill coefficient) = 1.3±0.04. The values estimated for IMP as a substrate were: $V_{max.} = 3.3 \pm 0.2$ units/mg of protein, $s_{0.5} = 7.7 \pm 0.8$ mM and $h = 1.8 \pm 0.2$. The slight sigmoidicity

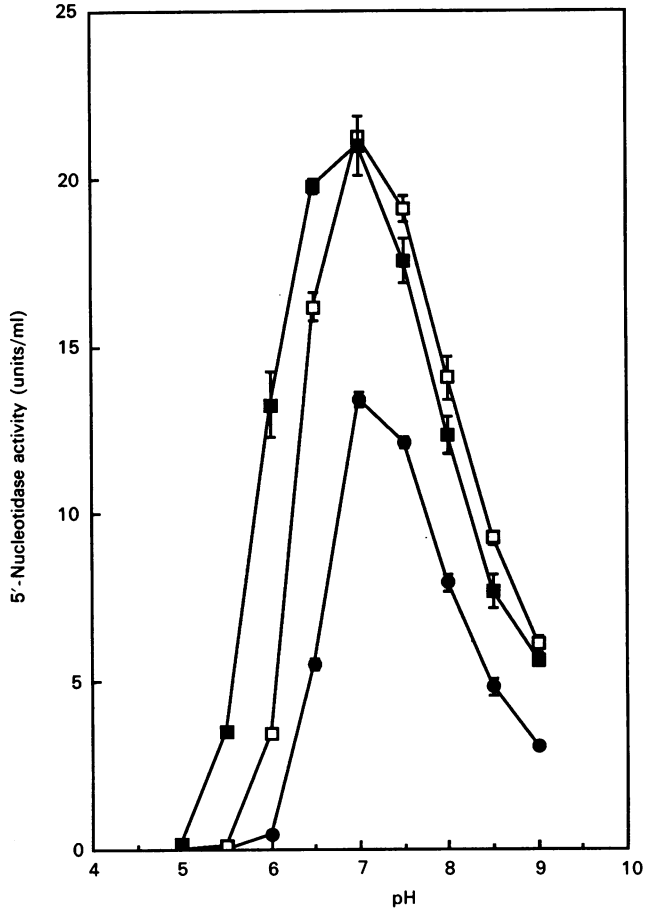

Fig. 2. pH–activity relationship for pigeon heart 5′-nucleotidase

Enzyme (2 μl containing 1.34 μg) was added to an assay mixture containing 40 mM-Mes, 30 mM-Tes and 30 mM-Tris (adjusted to different pH values with HCl or NaOH solutions), 30 mM-NaCl, 20 mM-$MgCl_2$ and 10 mM-AMP (plus 2–3 kBq of [2-^{3}H]AMP) alone (●) or with additions of 10 mM-ATP (□) or 1.5 mM-ADP (■). Reactions were terminated and the activity was measured as described in the Materials and methods section. Each point represents the mean (±S.E.M.) of three incubations.

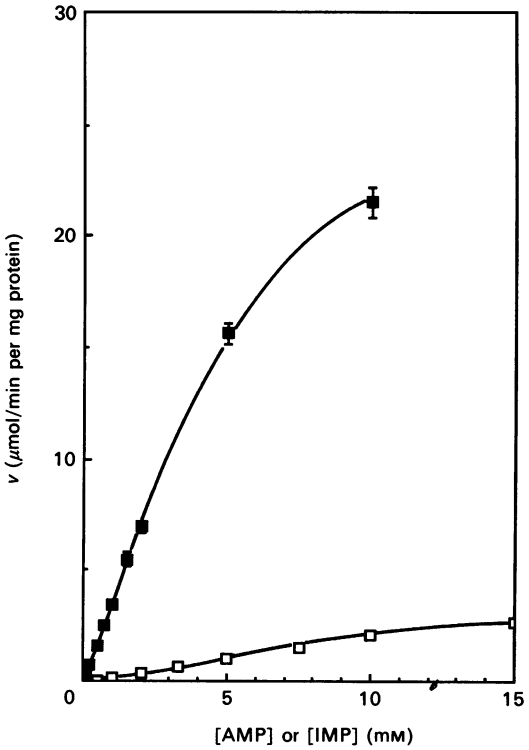

Fig. 3. AMP- and IMP-saturation curves of pigeon heart 5′-nucleotidase

Enzyme (2.5 μl containing 1.7 μg) was added to a reaction mixture containing 0.1 M-Tes/NaOH, pH 6.5, 30 mM-NaCl, 10 mM-$MgCl_2$, different concentrations of AMP with 2–3 kBq of [2-^{3}H]AMP (■) or IMP with 2–3 kBq of [2-^{3}H]IMP (□). The reaction was monitored for 15 min. Termination of reaction and measuring the activity were performed as described in the Materials and methods section. Each point represent the mean (±S.E.M.) for triplicate incubations. The curves are representatives of three experiments with different enzyme preparations.

of the AMP-dependence did not disappear, even at higher concentrations of activators (results not shown).

Activators and inhibitors of 5′-nucleotidase

Mg^{2+} was required for activity of the enzyme, but concentrations above 10 mM were inhibitory (Fig. 4). The enzyme activity with 50 mM-Mg^{2+} was only 29% ($n = 2$) of that at 10 mM-Mg^{2+}. Ionic strength also had biphasic effect. The activity increased slightly from 0 to about 30 mM-NaCl, declined at higher concentrations, and was only 10% ($n = 2$) of the maximum at 500 mM-NaCl (Fig. 4).

The concentration-dependencies of the effects of ATP and ADP on purified pigeon heart 5′-nucleotidase at two substrate concentrations are presented in Fig. 5. ATP and ADP were equally effective activators of the enzyme at 10 mM-AMP concentration. Only ADP was effective, however, at a lower, close-to-physiological substrate concentration of 0.1 mM (Fig. 5); 50% of maximal activity was achieved with 0.05 mM-ADP under these conditions.

Other nucleoside monophosphates inhibited dephosphorylation of AMP to extents (%) that were approximately proportional to their rates of dephosphorylation when used as substrates: UMP > dAMP > CMP > GMP > IMP (Table 2). Inhibition by 10 mM-β-glycerophosphate and 10 mM-ribose 5-phosphate was less than 10%, whereas 10 mM-p-nitrophenyl phosphate, despite being an equally poor substrate, inhibited by 50% (Table 2). The activity of 5′-nucleotidase was inhibited approx. 30% by 20 mM-P_i (Table 2). IBTA and IBTI, the non-competitive inhibitors of the IMP-specific cytoplasmic 5′-nucleotidases from rat liver, heart and polymorphonuclear leucocytes (Skladanowski *et al.*, 1989), also non-competitively inhibited the pigeon heart AMP-specific enzyme (Table 2 and Fig. 6). The K_i value for IBTI was about 5 mM, whereas that for IBTA was > 20 mM.

The potent inhibitor of ecto-5′-nucleotidase, [α,β-methylene]ADP (Burger & Lowenstein, 1975), did not inhibit purified cytosolic 5′-nucleotidase, either in the presence (Table 2) or in the absence (result not shown) of ADP.

DISCUSSION

An IMP-specific cytosolic 5′-nucleotidase with similar properties has been purified from a variety of mammalian and avian sources, including the heart (Itoh & Oka, 1985; Itoh *et al.* 1986*a*; Truong *et al.*, 1988), liver (Itoh *et al.*, 1978; Itoh, 1981*a*; Van Den Berghe *et al.*, 1977), erythrocytes (Bontemps *et al.*, 1988) and placenta (Spychala *et al.*, 1988). The enzyme is activated by Mg^{2+}, high ionic strength and by ATP more potently than by ADP. The enzyme dephosphorylates a number of purine and pyrimidine 5′-monophosphates with an approx. 30-fold preference for IMP over AMP. The K_m values of the fully activated enzyme for IMP and AMP are approx. 0.2 and 4–8 mM

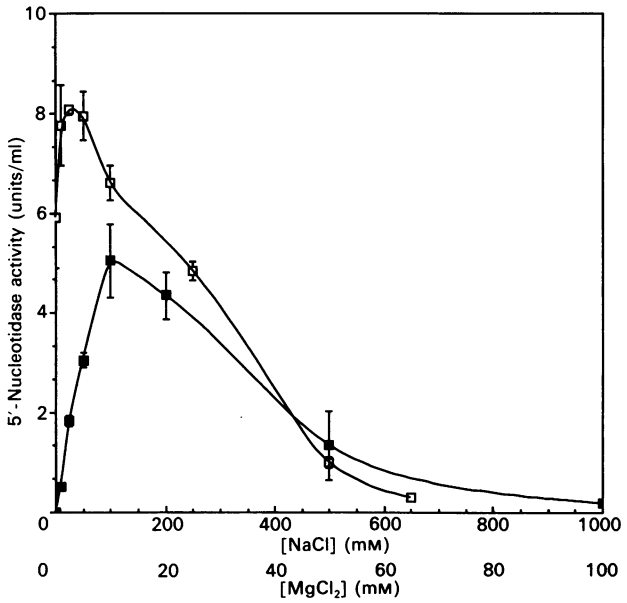

Fig. 4. NaCl- and Mg^{2+}-dependence of the activity of pigeon heart 5′-nucleotidase

Enzyme (5 μl containing 1.7 μg) was added to a reaction medium containing 0.1 M-Tes/NaOH, pH 6.5, 5 mM-AMP (plus 2–3 kBq of [2-^{3}H]AMP), 5 mM-ATP and either different concentrations of NaCl with 10 mM-$MgCl_2$ (□) or different concentrations of $MgCl_2$ with 500 mM-NaCl (■). Reactions were terminated after 15 min and the activity measured as described in the Materials and methods section. Each point represents the mean (±S.E.M.) for three incubations.

respectively, with little difference in $V_{max.}$ (Itoh *et al.*, 1986*b*). The high K_m for AMP, competition between IMP and AMP as substrates, and the rather potent inhibition of the enzyme by P_i (Itoh *et al.*, 1986*a*), suggest that the enzyme is principally responsible for dephosphorylation of IMP rather than adenosine formation during ATP catabolism (Van Den Berghe *et al.*, 1977).

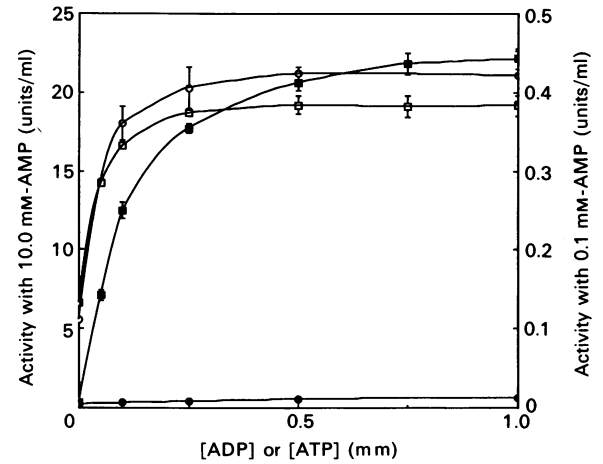

Fig. 5. Activation of pigeon heart 5′-nucleotidase by ADP and ATP

Enzyme (2 μl containing 3.3 μg) was added to a reaction medium containing 0.1 M-Tes/NaOH, pH 6.5, 10 mM-$MgCl_2$, 30 mM-NaCl, alternatively 0.1 mM-AMP (plus 2–3 kBq of [2-^{3}H]AMP) (closed symbols) or 10 mM-AMP (plus 2–3 kBq of [2-^{3}H]AMP) (open symbols) and different concentrations of ADP (squares) or ATP (circles). Reactions were terminated after 10 min and the activity was measured as described in the Materials and methods section. Each point represents the mean (±S.E.M.) for three incubations. The curves are representatives of one from three similar experiments.

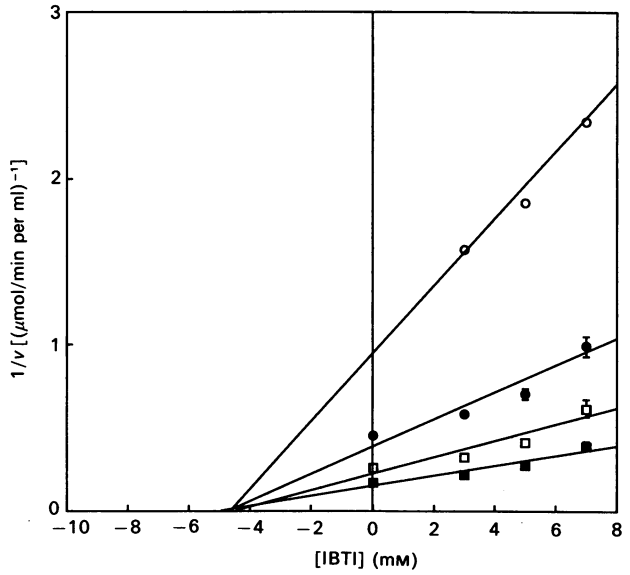

Fig. 6. Inhibition of pigeon heart cytosolic 5′-nucleotidase by IBTI

Dixon (1953) plots relating to the velocities of reaction, measured as the amount of [2-^{3}H]adenosine liberated from [2-^{3}H]AMP during 15 min incubation at 1 mM- (○), 2.5 mM- (●), 5 mM- (□) and 10 mM- (■) AMP in the presence of 0.25 mM-ATP. Values represent the means±S.E.M. for triplicate observations. Curves are representatives of one of two similar experiments.

Nonetheless, the positive dependence of adenosine formation on a fall in energy charge (Itoh, 1981*b*; Itoh *et al.*, 1986*a*) suggests that the enzyme may have some role in adenosine formation. Consistent with this, in 2-deoxyglucose-poisoned rat polymorphonuclear leucocytes the observed rates of adenosine formation correspond closely with the predicted activity of the purified enzyme (Worku & Newby, 1983) and are decreased by inhibition of the enzyme in intact cells with IBTA or IBTI (Skladanowski *et al.*, 1989). It is doubtful, however, whether the activity and regulatory properties of this enzyme can account for adenosine formation rates in the hypoxic (Schutz *et al.*, 1981) or ischaemic (Meghji *et al.* 1988*a*) heart or in the ischaemic brain (Worku, 1984).

Another cytoplasmic 5′-nucleotidase has been detected in impure preparations of pigeon (Gibson & Drummond, 1972; Newby, 1988), rabbit (Collinson *et al.*, 1987) and rat (Truong *et al.*, 1988) heart. An observed preference for AMP over IMP (Newby, 1988; Truong *et al.*, 1988), its less potent inhibition by IBTA (Newby, 1988) and its different chromatographic properties on phosphocellulose (Truong *et al.*, 1988) suggested that this AMP-specific 5′-nucleotidase may be distinct from the previously purified IMP-specific enzyme. The present study provides definitive evidence for difference between the two enzymes when preparations of the pigeon enzyme having specific activities similar to those of the most highly-purified preparations of the IMP-specific enzyme (Itoh *et al.*, 1986*a*) were used. The pigeon heart enzyme was inhibited by high concentrations of Mg^{2+} and high ionic strength. At near physiological concentrations of AMP, the enzyme was activated potently by ADP, but not by ATP. In partially purified preparations we observed an apparent inhibition of the enzyme activity by ATP, especially when using low concentrations (0.1 mM) of AMP (results not shown). This proved to result from removal of substrate AMP by contaminating adenylate kinase. Such contamination may therefore also explain the inhibition by ATP that has previously been described for the pigeon (Gibson & Drummond, 1972) and rabbit (Collinson *et al.*, 1987) enzymes. The pigeon heart enzyme

hydrolysed a broad range of nucleoside 5′-monophosphates, but had a 15-fold preference for AMP over IMP on the basis of values of $V_{max.}/K_m$. Somewhat higher activities of the enzyme towards other 5′-nucleotides than found in previous studies (Gibson & Drummond, 1972; Collinson *et al.*, 1987) may be explained by our use of a preparation fully activated with ADP. The pigeon heart enzyme was apparently much less potently inhibited by P_i than was the IMP-specific 5′-nucleotidase (Itoh *et al.*, 1986*a*). The inhibition of the pigeon heart enzyme by *p*-nitrophenyl phosphate (cf. Newby, 1988) was confirmed, although the compound was shown here to be a poor substrate for the enzyme. Weaker inhibition by IBTA of the pigeon heart as compared with the IMP-specific enzyme was also confirmed (cf. Newby, 1988). We report here that inhibition of the two enzymes by IBTI was equipotent (cf. Skladanowski *et al.*, 1989). The inhibition of both AMP- and IMP-specific enzymes by IBTI, which is believed to bind to a phosphoenzyme intermediate of the IMP-specific enzyme, suggests that the AMP-specific enzyme shares this reaction pathway.

The M_r of pigeon heart 5′-nucleotidase appeared to be less than the reported values of 200000 for chicken (Naito & Tsushima, 1976) and 182000–225000 for rat liver (Itoh, 1982) IMP-specific 5′-nucleotidases. The possible structural differences between the AMP- and IMP-specific 5′-nucleotidases, suggested by this preliminary data, merit further investigation.

The existence of a soluble, low-K_m 5′-nucleotidases has been proposed in the heart (Schutz *et al.*, 1981) and other tissues (Fritzon *et al.*, 1986; Madrid-Marina & Fox, 1986; Le Hir & Dubach, 1988). It is possible that these represent fragments of the ectoenzyme which are liberated from the membrane during homogenization (Fritzon *et al.*, 1986), possibly as a result of phospholipase activation (Stochaj *et al.*, 1989). The inhibition of these enzymes by nucleotide di- and tri-phosphates, including [$\alpha\beta$-methylene]ADP and by anti-(ecto-5′-nucleotidase) antibodies is diagnostic of their identity with the ectoenzyme, as has been shown in rat and guinea-pig heart (Schutz *et al.*, 1981; Meghji *et al.*, 1988*a*). The IMP-specific soluble 5′-nucleotidase has been distinguished from the membrane ecto-5′-nucleotidase by its higher K_m, activation, rather than inhibition, by ATP, and by its resistance to inhibition by [$\alpha\beta$-methylene]ADP and anti-(ecto-5′-nucleotidase) antibodies (Worku & Newby, 1983). As shown here, the pigeon heart 5′-nucleotidase fulfils similar criteria and, in particular, is unaffected by [$\alpha\beta$-methylene]ADP.

The K_m values for AMP of the fully activated AMP- and IMP-specific 5′-nucleotidases are similar and are far above the likely free concentrations of AMP in the cytoplasm (Bünger & Soboll, 1986; Haedrick & Willis, 1989). Both enzymes may then respond at least linearly to an increase in AMP concentration. The potent activatory effect of ADP, much weaker competitive inhibition by IMP, and weaker inhibition by P_i, suggest, moreover, that the AMP-specific enzyme is likely to be more than linearly activated during ATP catabolism. The participation of the AMP-specific enzyme in adenosine formation therefore appears plausible. The total activity of the enzyme found in pigeon ventricle, and the concentrations of ATP (4.8 mM), ADP (2.8 mM), AMP (0.66 mM) and P_i (20–30 mM) after 2 min of ischaemia (Meghji *et al.*, 1988*a*) predict a rate of adenosine formation of 0.5 μmol/min per g wet wt., slightly greater than the observed rate of 0.41 ± 0.4 μmol/min per g wet wt. (Meghji *et al.*, 1988*a*).

AMP- and IMP-specific 5′-nucleotidase appear to occur together in rat ventricle (Newby, 1988; Truong *et al.*, 1988). We were, however, unable to detect the IMP-specific enzyme in pigeon heart by phosphocellulose chromatography. Neither did we detect a biphasic dependence of enzyme activity on IMP concentration in our purified enzyme preparation, which would have revealed the presence of contaminating IMP-specific enzyme. Conversely, only the IMP-specific enzyme was found in rat polymorphonuclear leucocytes. The hypothesis that the AMP-specific enzyme occurs selectively in tissues active in adenosine formation is therefore worth further evaluation.

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