The pigeon heart 5'-nucleotidase responsible for ischaemia-induced adenosine formation

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1. A 5'-nucleotidase with a strong preference for AMP over IMP was characterized in homogenates and subcellular fractions of pigeon heart by using concentrations of ATP, ADP and AMP which mimicked those present in the ischaemic tissue. 2. The AMP-5'-nucleotidase had a neutral pH optimum and an apparent K_m in the range 4.6–5.2 mM. It was stimulated by ATP plus ADP, and was inhibited by other nucleoside monophosphates, P_i and *p*-nitrophenyl phosphate, but not by ribose 5-phosphate or β -glycerophosphate. The enzyme was not inhibited by [$\alpha\beta$ -methylene]ADP or by 5'-deoxy-5'-isobutylthioadenosine, an inhibitor of the previously purified IMP-preferring cytosolic 5'-nucleotidase. 3. Subcellular-fractionation studies indicated that the enzyme has access to cytosolic AMP, although it may be associated by weak ionic interactions with an organelle present in the low-speed particulate fraction. 4. A 5'-nucleotidase was detected under similar conditions in homogenates of rat heart. 5. The activity of the pigeon heart AMP-5'-nucleotidase was sufficient to account for previously measured rates of ischaemia-induced adenosine formation. The similar activity in rat heart could, however, account for only part of ischaemia-induced adenosine formation in this tissue.

INTRODUCTION

Ischaemia induces adenosine formation in hearts of many species, including dogs (Rubio *et al.*, 1969), rats (Deuticke & Gerlach, 1966), rabbits (Imai *et al.*, 1964), guinea-pigs (Schrader & Gerlach, 1976), pigeons (Meghji *et al.*, 1988) and man (Edlund *et al.*, 1985). Adenosine formation, which is most rapid in the earliest stages of ATP breakdown (Lomax & Henderson, 1973; Newby & Holmquist, 1981; Worku & Newby, 1983; De Witt *et al.*, 1983; Meghji *et al.*, 1988), may mediate a co-ordinated response aimed at limiting ATP depletion (Newby, 1984). The mechanism by which adenosine formation is accelerated during myocardial ischaemia is, however, not understood.

Excessive formation of adenosine contributes, in large measure, to the loss of total purines which occurs during ischaemia (Achterburg *et al.*, 1985; Meghji *et al.*, 1988). Identification of the enzyme or enzymes responsible for adenosine formation may thus open up the possibility of preventing nucleoside loss pharmacologically, and hence of improving recovery of myocardial function on reperfusion.

The plasma-membrane 5'-nucleotidase has its active site confined to the extra-cytoplasmic face of the plasma membrane (Stanley *et al.*, 1980; Baron *et al.*, 1986), and inhibition of the enzyme does not prevent adenosine formation in rat (Frick & Lowenstein, 1976) or guinea-pig (Schutz *et al.*, 1981) perfused hearts or in rat heart cells in culture (Meghji *et al.*, 1985). Moreover, ischaemia induces rapid formation of adenosine in pigeon hearts, which lack the ecto-enzymes (Meghji *et al.*, 1988).

A cytoplasmic ATP-stimulated 5'-nucleotidase is widespread in rat tissues (Newby *et al.*, 1987) and has been purified from the livers and hearts of both rats and chickens (Naito & Tsushima, 1976; Itoh, 1981; Itoh &

Oka, 1985; Itoh *et al.*, 1986). Except in rat polymorphonuclear leucocytes poisoned with 2-deoxyglucose (Worku & Newby, 1983), the enzyme has not been shown to be sufficiently active to explain observed rates of adenosine formation. Indeed, the strong preference of the enzyme for IMP and its high K_m for AMP in the range 2.5–10 mM argue against this (Meghji *et al.*, 1988).

To search for a 5'-nucleotidase activity in pigeon hearts sufficiently active to account for adenosine formation, an assay system was developed suitable for use with unfractionated homogenates. This strategy aimed to avoid inactivating or discarding any previously unrecognized enzyme during purification and to preserve any protein-protein interactions which might be necessary for expression of activity. The assay was conducted at a pH and with concentrations of ATP, ADP and AMP mimicking those in the cytoplasm of ischaemic hearts.

MATERIALS AND METHODS

Materials

ATP, ADP and AMP (disodium salts) were obtained from Boehringer Corp., Lewes, Sussex, U.K. Activated charcoal, [$\alpha\beta$ -methylene]ADP, NADH, NADPH, 4-methylumbelliferyl *N*-acetyl- β -D-glucosaminide and 5'-deoxy-5'-isobutyladenosine were obtained from Sigma, Poole, Dorset, U.K. [α - 32 P]ATP (PB107; 0.37–1.85 TBq/mmol) was obtained from Amersham International, Little Chalfont, Bucks., U.K. Antiserum to purified rat liver ecto-5'-nucleotidase was raised in sheep as described by Stanley *et al.*, (1980). Cytosolic 5'-nucleotidase (specific activity 16–58 μ mol of IMP hydrolysed at 37 °C/min per mg of protein) was purified from rat liver and stored in buffer containing 1 mg of bovine serum

Abbreviations used: 2'-deoxycoformycin, *R*-3-(2-deoxy- β -D-erythro-pentofuranosyl)-3,6,7,8-tetrahydro[4,5-*d*]diazapin-8-ol; 5-iodotubercidin, 4-aminio-5-iodo-7-(β -D-ribofuranosyl)pyrrolo[2,3-*d*]pyrimidine.

albumin/ml as described by Itoh (1981). All other reagents were of the highest grade available and were obtained as described previously (Meghji *et al.*, 1988).

Homogenization of pigeon and rat hearts

Pigeons (*Columba livia*) of either sex were obtained from Abbot Brothers, Thuxton, Norfolk, U.K. Wistar rats were bred in the Animal Unit, University of Wales College of Medicine. Pigeons and rats were killed by decapitation.

Isolated hearts were briefly perfused via the aorta to remove blood with homogenizing buffer, containing 20 mM-sodium dimethylglutarate, pH 6.9, 0.1 mM-dithiothreitol and either 0.14 M-KCl or 0.25 M-sucrose. The ventricles were dissected free from the atria and great vessels, and were then homogenized at 4 °C in 9 vol. of buffer with a Polytronic homogenizer (Northern Media Supply, Hessle, N. Humberside, U.K.). Vigorous homogenization comprised two bursts for 10 s each at setting 2. Gentle homogenization comprised one burst for 5 s at setting 1.5.

Measurement of 5'-nucleotidase activity

Where indicated in the text, homogenates and subcellular fractions were dialysed for 2 h at 4 °C against 100 vol. of homogenizing buffer containing 0.1% (w/v) activated charcoal to remove endogenous nucleotides before measurement of 5'-nucleotidase activity as follows. Fractions (50 μ l) were mixed with 50 μ l of assay cocktail containing (final concns.) 20 mM-sodium dimethylglutarate, pH 6.9, 0.1 mM-dithiothreitol, 100 mM-KCl, 4.8 mM-ATP, 2.8 mM-ADP, 0.66 mM-AMP or 0.66 mM-IMP, 6.3 mM-MgCl₂, 1 μ M-5-iodotubercidin and 1 μ M-2'-deoxycoformycin and incubated at 37 °C for 0, 30 or 60 s. After these times, 0.9 ml of 5% (w/v) trichloroacetic acid was added. The mixture was maintained at 4 °C for 30 min and then centrifuged at 1000 *g* for 10 min. The supernatants were neutralized and filtered, and concentrations of ATP, ADP, AMP and adenosine were determined by h.p.l.c. methods as described previously (Newby *et al.*, 1983). 5'-Nucleotidase activity was calculated as the rate of adenosine formation.

The purified rat liver soluble 5'-nucleotidase was assayed by a modification of the method of Worku & Newby (1982). Incubations were conducted at 37 °C for 0, 10 and 20 min in a total volume of 0.1 ml containing 100 mM-Tes/NaOH, pH 6.5, 50 mM-MgCl₂, 500 mM-NaCl, 3 mM-IMP (plus 2–3 kBq of [2-³H]IMP), 1 mg of bovine serum albumin/ml and 0.025–0.1 μ g of enzyme. Incubations were terminated by boiling for 3 min, and 0.5 ml of water was then added and the mixture was centrifuged at 12000 *g* for 2 min. Portions of supernatant (100 μ l) were assayed in duplicate for P_i concentration (Itaya & Ui, 1966), and a further portion (300 μ l) was analysed for inosine concentration by chromatography on Dowex AG1X2 (Worku & Newby, 1982). Inosine was eluted with 3.7 ml of water, and radioactivity was then determined by scintillation counting.

Ecto-5'-nucleotidase activity was measured in rat hearts by the radioassay method of Avruch & Wallach (1971) as modified by Newby *et al.* (1975).

Subcellular distributions of 5'-nucleotidase and β -N-acetylglucosaminidase (EC 3.2.1.30)

Pigeon ventricles were homogenized gently (see above), and portions of homogenates were then centrifuged at

20000 *g* for 20 min at 4 °C. The first supernatants and homogenates were then dialysed as described above. The first pellets were rehomogenized in fresh buffer and portions were re-centrifuged, after which the second supernatants were removed while the second pellets were rehomogenized. To release latent enzyme activities, a portion of each fraction was frozen four times over an acetone/solid-CO₂ mixture, followed by thawing over water at 23 °C. Portions of the first pellet which had been frozen and thawed were re-centrifuged, and the second supernatants containing released activities were removed, while the second pellets were rehomogenized in fresh buffer. Samples of each fraction were assayed for 5'-nucleotidase and β -N-acetylglucosaminidase by the method of Barrett & Heath (1977).

Subcellular distributions of 5'-nucleotidase and marker enzymes

Pigeon ventricles were homogenized vigorously in buffer containing 0.25 M-sucrose, and homogenates were then centrifuged at 300 *g* for 5 min at 4 °C. The first supernatants were removed, and the first (nuclear) pellets were resuspended to the original homogenate volume. The first supernatants were further centrifuged at 8000 *g* for 20 min at 4 °C and the second supernatants were removed. The second (mitochondrial) pellets were resuspended to the volume of the first supernatants. The second supernatants were re-centrifuged at 100000 *g* for 60 min at 4 °C, and the third supernatants (soluble fraction) were removed. The third (microsomal) pellets were resuspended to two-thirteenths of the volumes of the second supernatants.

Fractions were assayed for 5'-nucleotidase, for adenylate cyclase (EC 4.6.1.1) with 20 mM-NaF as activator as described by Salomon *et al.* (1974), for lactate dehydrogenase (EC 1.1.1.27) as described by Keiding *et al.* (1974), and for succinate-cytochrome *c* reductase (EC 1.3.99.1) and NADPH-cytochrome *c* reductase (EC 1.6.2.4) as described by Mahler (1955). All assays were linear with time and protein concentration over the ranges used. Protein concentration was measured by the method of Bradford (1976), with bovine serum albumin as standard.

RESULTS

5'-Nucleotidase activity in homogenates of pigeon ventricle

Homogenates of pigeon ventricle incubated at pH 6.9 with concentrations of ATP, ADP and AMP which mimicked those present in the ischaemic tissue (Meghji *et al.*, 1988) catalysed linear rates of adenosine formation (results not shown) amounting to 4.4 ± 0.3 (31) μ mol/min per g wet wt. The 5'-nucleotidase activity in the homogenate had a single pH optimum close to pH 7.0 (Fig. 1). Given the complexity of the assay system and the likely presence of ATPases and adenylate kinase in the homogenate, the apparent pH optimum might have arisen from pH-dependent changes in substrate (AMP) or activator (ATP and ADP) concentrations. These were therefore measured at the end of the 30 s incubations. The final concentrations of AMP ranged from 0.75 mM at pH 5.0 to 1.1 mM at pH 9.0 (Fig. 1), and those of ATP ranged from 4.6 to 4.1 mM and those of ADP from 2.5 to 2.7 mM respectively (Fig. 1). Thus the variations in these

concentrations were insufficient to explain the pronounced pH optimum.

After vigorous homogenization of pigeon hearts in KCl-containing buffer, most of the AMP-5'-nucleotidase

activity was found in the soluble fraction (Table 1). A washed particulate fraction contained only $12 \pm 1\%$ of the homogenate activity (Table 1).

The AMP-5'-nucleotidase activity was further charac-

Fig. 1. pH-dependence of pigeon heart 5'-nucleotidase

Pigeon hearts were homogenized vigorously in an unbuffered solution of 0.14 M-KCl/0.1 mM-dithiothreitol, and homogenates were then dialysed for 2 h at 4 °C against 100 vol. of this solution supplemented with 0.1% (w/v) activated charcoal. 5'-Nucleotidase was assayed in duplicate as described in the Materials and methods section, except that the buffer solution consisted of (final concns.) 20 mM-dimethylglutarate, 20 mM-Tris and 10 mM-β-glycerophosphate adjusted to the pH values shown with either HCl or NaOH. After incubation for 30 s at 37 °C, concentrations of ATP (○), ADP (□) and AMP (△) and the rate of adenosine formation (●) were measured by h.p.l.c. Values are means and ranges from two separate experiments.

Fig. 2. Concentration-dependence of pigeon heart 5'-nucleotidase

The soluble fraction of pigeon ventricle was prepared after vigorous homogenization in buffer containing 0.14 M-KCl. Incubations contained 10 mM-sodium β-glycerophosphate, pH 6.9, 1 μM-iodotubercidin, 1 μM-2'-deoxycytosine, 4.8 mM-ATP, AMP (0.2–5 mM as shown), ADP concentrations (0.35–7.74 mM) calculated to be in equilibrium with adenylate kinase ($K_{eq} = 0.4$) and $MgCl_2$ (5.22–8.81 mM) calculated to give a free Mg^{2+} concentration of 1 mM (Veloso *et al.*, 1973). Incubations were conducted for 0.5–10 min with enzyme appropriately diluted to give, at most, 5% conversion of substrate. AMP concentrations measured at the end of the incubations were not different from starting concentrations by more than 20% (results not shown). Values are means of duplicate observations in two separate experiments.

Table 1. Specificity and solubility of pigeon heart 5'-nucleotidase

Pigeon hearts were homogenized vigorously (see the Materials and methods section) in KCl-containing buffer, and portions of homogenates were then centrifuged at 100 000 g for 60 min at 4 °C. The first supernatants were removed and the pellets re-homogenized in the original volume of buffer and re-centrifuged. The second supernatants were removed, and the second pellets (washed particulate fractions) were re-homogenized in the original volume of buffer. Homogenates and first supernatants were dialysed to remove endogenous nucleotides, and then 5'-nucleotidase activity was measured with AMP or IMP as substrate in duplicate. Values are means $\pm$ S.E.M. ($n = 6$).

Fraction	Substrate	5'-Nucleotidase activity	
		(nmol/min per g wet wt.)	(% of homogenate AMP-5'-nucleotidase)
Homogenate	IMP	270 $\pm$ 10	6.6 $\pm$ 0.8
	AMP	4300 $\pm$ 400	100
First supernatant	AMP	4200 $\pm$ 200	102 $\pm$ 12
Second supernatant	AMP	350 $\pm$ 20	9 $\pm$ 1
Washed pellet	AMP	500 $\pm$ 80	12 $\pm$ 1
Total recovery	AMP	5000 $\pm$ 500	122 $\pm$ 13

Table 2. Activators and inhibitors of pigeon heart 5'-nucleotidase

Pigeon hearts (two series of six) were homogenized in KCl-containing buffer, and dialysed 100 000 *g* supernatant fractions were prepared. 5'-Nucleotidase activity was measured in duplicate with 0.66 mM-AMP as substrate and (except where indicated) in the presence of 4.8 mM-ATP and 2.6 mM-ADP. When AMP deaminase was added, its activity was calculated to deaminate 0.5 μ mol of AMP/min at saturating concentration. 5'-Nucleotidase activity was related to that of a sample from the same heart incubated without omissions or additions. Values are means $\pm$ S.E.M. for the numbers of hearts in parentheses.

Expt. no.	Omission or addition	Activity (% of control)
1	– AMP + AMP deaminase	18 $\pm$ 8 (4)
	– ATP – ADP	27 $\pm$ 9 (5)
	+ 0.5 mM-[$\alpha\beta$ -methylene]ADP + 10 mM- β -glycerophosphate	99 $\pm$ 4 (6)
	+ 10 mM-P _i	88 $\pm$ 3 (6)
	+ 20 mM-P _i	68 $\pm$ 7 (5)
	+ 10 mM- <i>p</i> -nitrophenyl phosphate	21 $\pm$ 2 (5)
	+ 3 mM-5'-deoxy-5'-isobutylthioadenosine	89 $\pm$ 9 (4)
	+ 0.1 mM-IMP	92 $\pm$ 8 (4)
	+ 1 mM-IMP	90 $\pm$ 8 (4)
2	+ 10 mM-IMP	47 $\pm$ 8 (6)
	+ 10 mM-GMP	22 $\pm$ 3 (6)
	+ 10 mM-GMP	19 $\pm$ 3 (6)
	+ 10 mM-UMP	16 $\pm$ 2 (6)
	+ 10 mM-ribose 5-phosphate	99 $\pm$ 13 (6)

Table 3. Subcellular distribution in sucrose-containing buffer of 5'-nucleotidase, β -N-acetylglucosaminidase and lactate dehydrogenase

Pigeon hearts were homogenized gently in buffer containing 0.25 M-sucrose and were then centrifuged at 20 000 *g* for 20 min. The pellet from this centrifugation was re-homogenized in fresh buffer and re-centrifuged. In four experiments, part of the first pellet was frozen and thawed four times before centrifugation. Enzyme activities were measured in portions of each fraction that received no further treatment and in fractions that were frozen and thawed four times. Activities were expressed as a percentage of total activity in the frozen-thawed homogenate, which was 3.8 $\pm$ 0.3 (10) nmol/min per *g* wet wt. for β -N-acetylglucosaminidase, 5.2 $\pm$ 0.5 (6) μ mol/min per *g* wet wt. for 5'-nucleotidase and 41 $\pm$ 2 (8) μ mol/min per *g* wet wt. for lactate dehydrogenase. Values are means $\pm$ S.E.M. for the numbers of hearts shown in parentheses.

Fraction	Recovery of activity (%)				
	β -N-Acetylglucosaminidase		5'-Nucleotidase		Lactate dehydrogenase
	Frozen-thawed	Untreated	Frozen-thawed	Untreated	Frozen-thawed
Homogenate	100	65 $\pm$ 3 (10)	100	105 $\pm$ 5 (6)	100
First supernatant	45 $\pm$ 2 (10)	47 $\pm$ 2 (10)	13 $\pm$ 2 (5)	14 $\pm$ 2 (6)	100 $\pm$ 2 (8)
Second supernatant	11 $\pm$ 1 (8)	11 $\pm$ 1 (8)	5 $\pm$ 2 (4)	7 $\pm$ 2 (4)	3.2 $\pm$ 0.2 (6)
Washed pellet	60 $\pm$ 2 (10)	31 $\pm$ 1 (10)	60 $\pm$ 7 (6)	64 $\pm$ 7 (6)	3.8 $\pm$ 0.3 (8)
Second supernatant after freeze-thaw	–	54 $\pm$ 4 (4)	–	14 $\pm$ 3 (4)	–
Washed pellet after freeze-thaw	–	19 $\pm$ 1 (4)	–	35 $\pm$ 7 (4)	–

terized with the soluble fraction, since the ATP, ADP and AMP concentrations at the end of the incubations did not differ from the starting concentrations by more than 20% (results not shown). The apparent K_m for AMP of the activity was 4.6 and 5.2 mM in two experiments (Fig. 2). The activity was greatly decreased when AMP was omitted and AMP deaminase was added to metabolize AMP formed from the adenylate kinase reaction (Table 2). The activity was also decreased if ATP and ADP were omitted, but was not inhibited by addition of [$\alpha\beta$ -methylene]ADP (Table 2). The activity was not inhibited by addition of β -glycerophosphate or ribose 5-phosphate, but was inhibited by all of the nucleoside 5'-monophosphates tested, with the order of

potency GMP = CMP = UMP > IMP (Table 2). The activity was weakly inhibited by P_i and more potently by *p*-nitrophenyl phosphate.

Effect of 5'-deoxy-5'-isobutylthioadenosine

Inhibitors of the previously purified soluble IMP-5'-nucleotidase have not been described previously, although inosine has been shown to bind to the transition state of the enzyme from rat liver, heart and polymorphonuclear leucocytes (Worku & Newby, 1982, 1983), suggesting that nucleosides with a blocked 5'-position might act as non-competitive inhibitors. Various 5'-substituted adenosine and inosine analogues were tested as inhibitors, and 5'-deoxy-5'-isobutylthioadeno-

Table 4. Subcellular distribution in KCl-containing buffer of 5'-nucleotidase and β -N-acetylglucosaminidase

Pigeon hearts were homogenized gently in buffer containing 0.14 M-KCl and were then centrifuged at 20000 *g* for 20 min. The pellet was re-homogenized in fresh buffer and re-centrifuged. Portions of some fractions were frozen and thawed four times. β -N-Acetylglucosaminidase activity was expressed as a percentage of that [3.8 ± 0.1 (6) nmol/min per g wet wt.] present in the frozen-thawed homogenate. 5'-Nucleotidase activity was expressed as a percentage of that (4.0 ± 0.4 μ mol/min per g wet wt.) in the untreated homogenate. Values are means $\pm$ S.E.M. for six hearts.

Fraction	Recovery of activity (%)		
	β -N-Acetylglucosaminidase		5'-Nucleotidase
	Frozen-thawed	Untreated	Untreated
Homogenate	100	77 ± 1	100
First supernatant	—	52 ± 1	52 ± 9
Second supernatant	—	17 ± 1	16 ± 2
Washed pellet	51 ± 3	34 ± 2	9 ± 1

Table 5. Subcellular distribution in sucrose-containing buffer of 5'-nucleotidase and other marker enzymes

Pigeon hearts were homogenized in buffer containing 0.25 M-sucrose and were then fractionated as described in the Materials and methods section. The total recovered activity of each enzyme in the four fractions was divided by the total recovered protein to give for each heart a value of 1 for relative specific activity. The average absolute values of this parameter are shown in the right-hand column. The specific activities in individual fractions were then expressed relative to this. Hence a value greater than 1 implies enrichment, and a value less than 1 implies depletion of the enzyme in that fraction. Values are means $\pm$ S.E.M. ($n = 6$).

Enzyme	Fraction . . .	Relative specific activity				Total recovered activity/total recovered protein (nmol/min per mg)
		Nuclear	Mitochondrial	Microsomal	Soluble	
5'-Nucleotidase		0.87 ± 0.03	0.42 ± 0.05	0.6 ± 0.2	3.0 ± 0.3	31 ± 2
Adenylate cyclase		0.76 ± 0.07	2.0 ± 0.2	5.6 ± 0.8	0	0.07 ± 0.02
Succinate-cytochrome <i>c</i> reductase		0.68 ± 0.04	3.5 ± 0.3	0.55 ± 0.05	0.05 ± 0.02	50 ± 5
NADPH-cytochrome <i>c</i> reductase		0.66 ± 0.05	3.1 ± 0.5	1.45 ± 0.05	0.9 ± 0.1	5.6 ± 0.4
Lactate dehydrogenase		0.33 ± 0.07	0.16 ± 0.04	0.54 ± 0.09	5.7 ± 0.6	440 ± 40

sine was found to be the most potent. Three different preparations of purified rat liver cytosolic 5'-nucleotidase were reversibly inhibited by 66, 69, and 75 % by 3 mM-5'-deoxy-5'-isobutylthioadenosine, and by 76, 82 and 85 % by 4 mM. Production of both nucleoside and P_i was inhibited to the same extent, and the inhibition was as great in the presence of 5 mM-ATP (results not shown). In contrast, 3 mM-5'-deoxy-5'-isobutylthioadenosine had no significant effect on the AMP-5'-nucleotidase of pigeon hearts (Table 2).

Subcellular fractionation

In view of the previous reports of a lysosomal-matrix 5'-nucleotidase (Arsenis & Touster, 1968; Collinson *et al.*, 1987), the subcellular distribution of the AMP-5'-nucleotidase activity was compared with that of β -N-acetylglucosaminidase. Gentle homogenization in buffer containing 0.25 M-sucrose and centrifugation at moderate *g* forces preserved latent β -N-acetylglucosaminidase activity in both homogenates and washed particulate fractions (Table 3). Of the homogenate β -N-acetylglucosaminidase activity, 60 % was recovered in the washed particulate fraction together with 60 % of the 5'-nucleotidase activity and 4 % of lactate dehydrogenase (Table 3). There was no latent 5'-nucleotidase activity,

however, in any fraction (Table 3), and, unlike β -N-acetylglucosaminidase, the 5'-nucleotidase activity remained mainly particulate after freezing and thawing (Table 3).

When fractionation was conducted in buffer containing 0.14 mM-KCl, 50 % of homogenate β -N-acetylglucosaminidase was recovered in the washed particulate fraction together with only 9 % of the 5'-nucleotidase activity (Table 4) and 2.7 ± 0.3 (6) % of the homogenate lactate dehydrogenase. In KCl-containing buffer, 68 % of the homogenate 5'-nucleotidase activity was recovered in the two soluble fractions, compared with only 19 % in sucrose-containing buffer (cf. Tables 3 and 4), despite similar homogenate and total recovered activities (see the legends to Tables 3 and 4).

Pigeon hearts were vigorously homogenized in buffer containing 0.25 M-sucrose and fractionated into nuclear, mitochondrial, microsomal and soluble fractions. Under these conditions, 28 ± 6 % of the homogenate 5'-nucleotidase activity was found in the soluble fraction. After allowance for contamination by soluble fraction, 33 ± 3 % of the homogenate activity was present in the nuclear fraction, 3.7 ± 0.5 % in the mitochondrial fraction and 0.5 ± 0.1 % in the microsomal fraction. As shown in Table 5, only the soluble fraction was enriched in

Table 6. 5'-Nucleotidase in rat heart homogenates

Homogenates of rat hearts were prepared by gentle homogenization in buffer containing 0.25 M-sucrose. Homogenates were then dialysed with or without the addition of sheep anti-(rat liver ecto-5'-nucleotidase) serum at a final dilution of 1:100 as indicated. Samples were assayed for ecto-5'-nucleotidase activity with 0.2 mM-AMP as substrate and in the absence of ATP and ADP. The ATP+ADP-activated 5'-nucleotidase was assayed under the same conditions as those used for pigeon heart. Values are means $\pm$ S.E.M. ($n = 6$).

Assay condition	Enzyme activity (nmol/min per g wet wt.)		
	+ 10 mM- β -glycerophosphate	+ 20 mM- β -glycerophosphate	+ 20 mM- β -glycerophosphate + 0.5 mM-[$\alpha\beta$ -methylene]ADP
Ecto-5'-nucleotidase	2700 $\pm$ 100	2700 $\pm$ 100	110 $\pm$ 10
Ecto-5'-nucleotidase + antiserum	600 $\pm$ 100	620 $\pm$ 50	50 $\pm$ 20
ATP + ADP-activated 5'-nucleotidase + antiserum	170 $\pm$ 10	160 $\pm$ 20	150 $\pm$ 10

5'-nucleotidase activity, but the enrichment was less than that of lactate dehydrogenase. Conversely, the depletion of 5'-nucleotidase activity from the nuclear fraction was less than for lactate dehydrogenase. 5'-Nucleotidase activity was depleted in the mitochondrial fraction, despite enrichment of succinate-cytochrome *c* reductase and NADPH-cytochrome *c* reductase, and from the microsomal fraction, despite enrichment of NADPH-cytochrome *c* reductase and adenylate cyclase.

5'-Nucleotidase activities in rat ventricle homogenates

Ecto-5'-nucleotidase activity was detected in rat ventricle homogenates and was characteristically inhibited by [$\alpha\beta$ -methylene]ADP and anti-(rat liver ecto-5'-nucleotidase) serum (Table 6). In the presence of antiserum and 10 mM- β -glycerophosphate and under the conditions used for pigeon hearts, an AMP-5'-nucleotidase activity was detected which was not inhibited by further addition of β -glycerophosphate or [$\alpha\beta$ -methylene]ADP (Table 6). The AMP-5'-nucleotidase was inhibited by 50 $\pm$ 11 % (6) by 10 mM-*p*-nitrophenyl phosphate, but by only 6 $\pm$ 7 % (6) by 5'-deoxy-5'-isobutylthioadenosine.

DISCUSSION

Knowledge of the kinetic properties of purified 5'-nucleotidases is not helpful in testing their role in adenosine formation, unless they can be shown to have access to cytosolic AMP and to be sufficiently active under the conditions present during ATP catabolism. For these reasons, we chose to test for the presence of AMP-5'-nucleotidase in unfractionated homogenates. Pigeon hearts were chosen since they contain a very low activity (0.016 $\pm$ 0.04 μ mol/min per g wet wt.) of the ecto-5'-nucleotidase (Meghji *et al.*, 1988). Concentrations of ATP, ADP and AMP were chosen so as to mimic average tissue concentrations between 0 and 2 min of normothermic ischaemia (Meghji *et al.*, 1988). Since there are no published measurements of intracellular pH in ischaemic pigeon hearts, the value of 6.9 found for rat hearts after 1 min of ischaemia (Garlick *et al.*, 1979) was employed. Nucleotide concentrations were approximately in equilibrium with the adenylate kinase reaction, and thus varied little on incubation with the soluble fraction of hearts. The presence of active ATPases in

the homogenates and particulate fractions (results not shown) limited the time course over which nucleotide concentrations remained constant. AMP deaminase did not deplete AMP concentrations, nor could it contribute to formation of adenosine, which was clearly separated from IMP and inosine by the h.p.l.c. method used (Newby, 1980). Fractions were diluted minimally during enzyme assays in the hope of preserving any important protein-protein interactions.

With 0.66 mM-AMP, a mean homogenate AMP-5'-nucleotidase activity of 4.4 μ mol/min per g wet wt. was obtained. Given a K_m value for AMP of 4.9 mM, this implies a V_{max} of the order of 40 μ mol/min per g wet wt., which compares with the value of 19 μ mol/min per g wet wt. reported by Gibson & Drummond (1972). The 5'-nucleotidase activity was more than sufficient to account for the initial rate of adenosine formation (0.41 $\pm$ 0.04 μ mol/min per g wet wt.) in ischaemic pigeon ventricles (Meghji *et al.*, 1988). The discrepancy may arise because the free concentration of AMP is lower than total tissue concentration (Nishiki *et al.*, 1978; Bungler & Soboll, 1986), although at 2 min of ischaemia and beyond estimates of free AMP concentration from the creatine kinase and adenylate kinase equilibria become highly inaccurate, as phosphocreatine becomes exhausted (Meghji *et al.*, 1988). The presence of IMP (0.1–0.2 mM) and P_i (20–30 mM) would also decrease the 5'-nucleotidase activity by approx. 50 %. The activity of the enzyme decreased sharply with pH values below 7.0, suggesting that lowered intracellular pH as well as depletion of ATP may contribute to the decreased adenosine formation rates observed after prolonged periods of ischaemia (Meghji *et al.*, 1988).

The enzyme responsible for adenosine formation in pigeon heart homogenates appeared to be a 5'-nucleotidase, since it was not inhibited by ribose 5-phosphate or β -glycerophosphate but was inhibited by nucleoside 5'-monophosphates with some specificity for the base moiety. The enzyme showed a 15-fold selectivity for adenosine formation from AMP over inosine formation from IMP, confirming that it showed base-specificity. The 5'-nucleotidase was activated by ATP and ADP, distinguishing it from the ecto-enzyme, which is inhibited by di- and tri-nucleotides, including [$\alpha\beta$ -methylene]ADP (Burger & Lowenstein, 1975).

The pigeon homogenate enzyme is similar to the

previously purified cytosolic 5'-nucleotidase in being activated by ATP plus ADP, in being inhibited by supra-millimolar concentrations of IMP and P_i , in having a neutral pH optimum and in having a K_m for AMP in the millimolar range (Naito & Tsushima, 1976; Van den Berghe *et al.*, 1977; Itoh, 1981; Itoh & Oka, 1985; Itoh *et al.*, 1986). The enzymes are, however, clearly distinguishable, since the enzyme previously purified from both avian and mammalian heart shows an approx. 30-fold substrate preference for IMP over AMP (Itoh & Oka, 1985; Itoh *et al.*, 1986). In addition, a newly discovered inhibitor of the purified IMP-5'-nucleotidase, 5'-deoxy-5'-isobutylthioadenosine, does not inhibit the pigeon heart AMP-5'-nucleotidase. The different substrate preferences of the two enzymes suggest that each may be responsible, physiologically, for hydrolysis of their preferred nucleoside monophosphates.

The subcellular-distribution studies of the pigeon heart AMP-5'-nucleotidase indicate that it has access to cytosolic AMP. Although a large proportion of the enzyme was associated with the particulate fraction, on homogenization in sucrose-containing buffer it did not show latency, nor was it solubilized by freezing and thawing. It could, in contrast, be solubilized by homogenization in KCl-containing buffer, suggesting that it was eluted thereby from weak ionic interaction with a subcellular organelle. The 5'-nucleotidase activity did not co-purify with markers for plasma membranes or endoplasmic reticulum, but appeared associated with the low-speed particulate fraction containing the nuclei and myofibrils (Swynghedauw *et al.*, 1973). The possibility of an association with the contractile proteins should be investigated further, since it might facilitate in signalling the imbalance between ATP utilization for contraction and its availability to the myofibrillar ATPase.

The studies with homogenates of pigeon ventricle demonstrate, for the first time, that a myocardial 5'-nucleotidase with access to cytoplasmic AMP is sufficiently active to account for ischaemia-induced adenosine formation. Homogenates of rat hearts contained an AMP-5'-nucleotidase with similar characteristics to the pigeon enzyme. The activity was, however, only 0.15–0.17 $\mu\text{mol}/\text{min}$ per g wet wt., which is less than previously measured rates of ischaemia-induced adenosine formation ($0.47 \pm 0.06 \mu\text{mol}/\text{min}$ per g wet wt.; Meghji *et al.*, 1988). This suggests either that other phosphatases contribute to adenosine formation in rat heart or that the full activity of the AMP-5'-nucleotidase is not preserved during extraction and assay. Selective inhibitors of the pigeon heart AMP-5'-nucleotidase are needed to test the role of similar enzymes in adenosine formation in the rat heart and other tissues.

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