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5′-Nucleotidases in rat heart

Evidence for the occurrence of two soluble enzymes with different substrate specificities

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Chromatography of soluble proteins from rat heart on phosphocellulose columns separates two 5′-nucleotidases. The first to emerge from the column shows a preference for AMP over IMP as substrate, whereas the second shows a preference for IMP over AMP. The properties of the IMP-preferring enzyme, including the conditions under which it is eluted from phosphocellulose columns, show it to be the enzyme studied by Itoh, Oka & Ozasa [Biochem. J. (1986) **235**, 847–851]. The kinetic properties of the AMP-preferring enzyme indicate that it is likely to be the enzyme responsible for the production of adenosine under conditions of hypoxia and increased work load, and with metabolic stresses such as a high load of acetate.

INTRODUCTION

Soluble 5′-nucleotidases have been studied in heart of pigeon (Gibson & Drummond, 1972), chicken (Itoh & Oka, 1985), rat (Lowenstein *et al.*, 1983; Itoh *et al.*, 1986) and rabbit (Collinson *et al.*, 1987) and in liver of chicken and rat (Naito & Tsushima, 1976; Itoh, 1981). Soluble enzymes have been purified to homogeneity from liver (Naito & Tsushima, 1976; Itoh, 1981), placenta (Berry *et al.*, 1986) and heart (Itoh *et al.*, 1986). These 5′-nucleotidases are believed to be cytosolic enzymes, although careful fractionation studies to establish this point convincingly do not appear to have been made. The purified soluble enzymes studied by Itoh *et al.* (1986) and Itoh (1981) show a high preference for IMP over AMP; for example, $V_{max.}/K_{app.}$ for AMP divided by $V_{max.}/K_{app.}$ for IMP is 0.07 and 0.02 for the enzymes from rat heart and liver respectively. In the course of purifying a cytosolic 5′-nucleotidase from high-speed supernatant of rabbit heart homogenate, we observed an enzyme that showed a preference for AMP over IMP (Collinson *et al.*, 1987). In the report below, we establish that rat heart cytosol contains two soluble 5′-nucleotidases which can be separated by chromatography on phosphocellulose; by the criterion of $V_{max.}/K_{app.}$, one prefers AMP over IMP, whereas the other prefers IMP over AMP.

MATERIALS AND METHODS

Materials

All reagents were of the highest purity commercially available.

Methods

Rat hearts were removed immediately after the animals were killed and were placed on crushed ice. The hearts were suspended (at 3 ml/g of heart) in an ice-cold solution containing 25% (v/v) glycerol, 0.1 mM-dithiothreitol, 1 mM-EDTA, 0.2 mM-α-toluenesulphonyl fluoride and 50 mM-Hepes/NaOH buffer, pH 7.0 (hereafter termed Solution 1). The mixture was homogenized with a Polytron (Brinkman), with three bursts of 30 s each followed by an interval of 2 min. During this procedure the container was surrounded with crushed ice. Alternatively, rat hearts were purchased frozen and shipped at the temperature of solid CO_2 (Bio-Lab Corp., St. Paul, MN, U.S.A.). The frozen hearts were pulverized in a stainless-steel percussion mortar at the temperature of liquid N_2. The resulting powder was suspended in ice-cold extraction medium and homogenized as described above. All subsequent procedures were carried out at 5 °C. The homogenate was centrifuged at 12000 *g* for 30 min. The resulting supernatant was filtered through gauze cloth and centrifuged at 150000 *g* for 60 min.

$(NH_4)_2SO_4$ precipitation

$(NH_4)_2SO_4$ (258 g/l) was added with continuous stirring to the high-speed supernatant prepared as described above. After the addition was complete, the mixture was left on ice for 2 h before being centrifuged at 12000 *g* for 30 min. The resulting precipitate was dissolved in Solution 1 and dialysed against Solution 1 for 12 h. The mixture was centrifuged at 10000 *g* for 10 min to remove insoluble material, and the supernatant was applied to a phosphocellulose column.

Phosphocellulose chromatography

The procedures used were those devised either by us or by Itoh (1981*a*), and were as follows. Procedure 1: the phosphocellulose column (2.6 cm × 12 cm) was equilibrated with Solution 1. The enzyme solution was applied to the column, which was then washed with 210 ml of Solution 1. A linear gradient was then run, from Solution 1 to Solution 1 containing 0.8 M-NaCl over 500 ml. Procedure 2: the phosphocellulose column (1.5 cm × 5 cm) was equilibrated with 10 mM-mercaptoethanol/1 mM-EDTA/50 mM-Tris/HCl buffer, pH 7.4 (hereafter termed Solution 2). The enzyme solution, which had been dialysed against Solution 2, was applied to the column, which was then washed with 35 ml of Solution 2. A linear gradient was then run, from Solution 2 to Solution 2 containing 13 mM-ATP over 60 ml. Procedure 2 closely followed that described previously for heart by Itoh *et al.* (1986) and for liver by Itoh (1981).

Assays

All assays were performed at protein concentrations and over time intervals for which the reaction was linear.

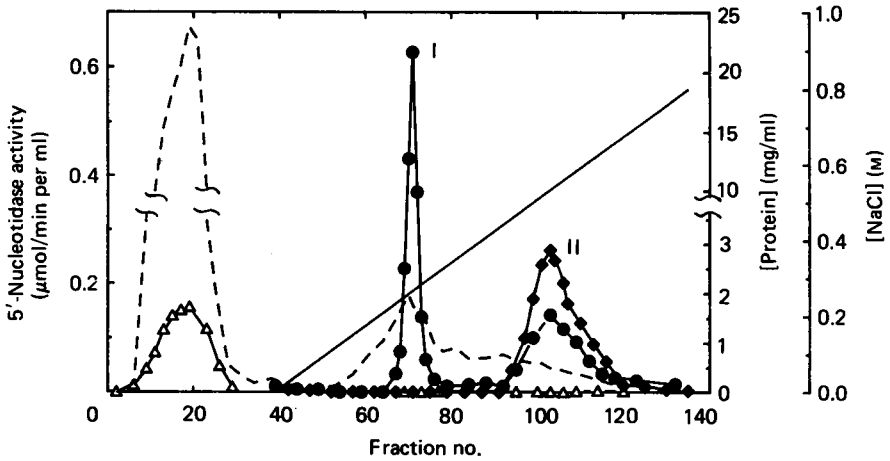

Fig. 1. Elution of soluble 5′-nucleotidases I and II from first phosphocellulose column

Phosphocellulose chromatography was carried out by Procedure 1 as described in the Materials and methods section. Fractions (7.2 ml) were collected and analysed as follows. Assay 1 (●) contained 10 mM-AMP, 1 mM-ATP, 3 mM-$MgCl_2$, and 50 mM-Mops/NaOH buffer, pH 6.5. Assay 2 (◆) contained 10 mM-IMP, 1 mM-ATP, 50 mM-$MgCl_2$, 500 mM-NaCl and 50 mM-imidazole/HCl buffer, pH 6.5. Non-specific phosphatase activity (△) was determined with 3 mM-*p*-nitrophenyl phosphate as substrate in 50 mM-potassium acetate buffer, pH 3.5. The broken line without symbols shows protein concentration. The continuous line without symbols shows the NaCl concentration.

The following assays were employed. Assay 1: the reaction mixture contained 10 mM-AMP, 3 mM-$MgCl_2$ and 50 mM-Mops/NaOH buffer, pH 6.5. Where indicated, the assays also contained 1 mM-ATP. P_i liberated was measured by the method of Sanui (1974). Assay 2: unless otherwise indicated, the reaction mixture contained 10 mM-IMP, 50 mM-$MgCl_2$, 500 mM-NaCl, 0.1% bovine serum albumin and 50 mM-Tris/HCl buffer, pH 6.5. Where indicated, the assay also contained 1 mM-ATP. The reactions were started by adding enzyme and were run at 37 °C, typically for 10 min. The reactions were stopped by adding an equal volume of ice-cold 10% trichloroacetic acid. P_i liberated was measured as above. Assay 3; the reaction mixture contained 50 mM-Mops/NaOH buffer, pH 6.5, 10 mM-$MgCl_2$, 5 mM-ATP and [2-^{3}H]AMP or [2-^{3}H]IMP as indicated, in a final volume of 0.2 ml. The reaction was started by adding enzyme and was run at 37 °C, typically for 3 and 6 min. The reaction was stopped by adding ice-cold 2 mM-EDTA in 50 mM-Tris/HCl buffer, pH 7.0. Radioactive nucleoside formed during the reaction was separated from nucleotide by applying the mixture to a Dowex-1 (Cl^- form) column (1 cm × 8 cm) that had been equilibrated with 50 mM-Tris/HCl buffer, pH 7.0. The column was then washed with 20 ml of the same buffer to elute adenosine or inosine, and the eluate was assayed for radioactivity by liquid-scintillation counting. Protein was determined by the method of Bradford (1976).

Non-specific phosphatase was determined by using 3 mM-*p*-nitrophenyl phosphate and 50 mM-potassium acetate buffer, pH 3.5. The reaction was started by adding enzyme, was run at 37 °C for 10 min, and was stopped by adding 2 vol. of 1 M-NaOH. The colour corresponding to *p*-nitrophenylate was read at 400 nm.

RESULTS

When the dialysed $(NH_4)_2SO_4$ fraction of rat heart cytosol was subjected to phosphocellulose chromatography by Procedure 1, as described in the Materials and methods section, two peaks of activity were observed. The first contained an enzyme which, under the conditions of the assays, hydrolysed AMP much faster than IMP, whereas the second contained an enzyme which showed preference for IMP over AMP (Fig. 1). This finding raised the question whether one of these peaks contained the enzyme previously described by Itoh *et al.* (1986). Fractions containing the first peak of activity were pooled and concentrated by vacuum dialysis against Solution 2. Fractions containing the second peak were treated similarly. Each pooled peak was then subjected to chromatography on a second phosphocellulose

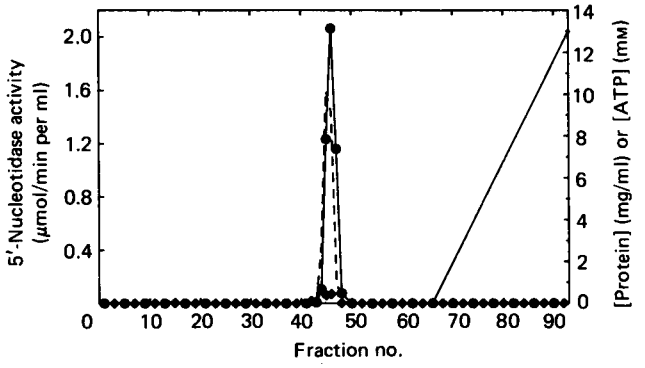

Fig. 2. Elution of 5′-nucleotidase I from second phosphocellulose column

Phosphocellulose chromatography was carried out by Procedure 2 as described in the Materials and methods section. The peak fractions containing 5′-nucleotidase I activity (Fig. 1) were pooled, dialysed against buffer 2, and 8.9 units of activity were applied to the second phosphocellulose column; 2.6 ml fractions were collected. Activity was determined by assays 1 (●) and 2 (◆) as described in Fig. 1. Recovery of activity after column chromatography was 92%. The continuous straight line without symbols shows the concentration of ATP. The broken line without symbols shows protein concentration.

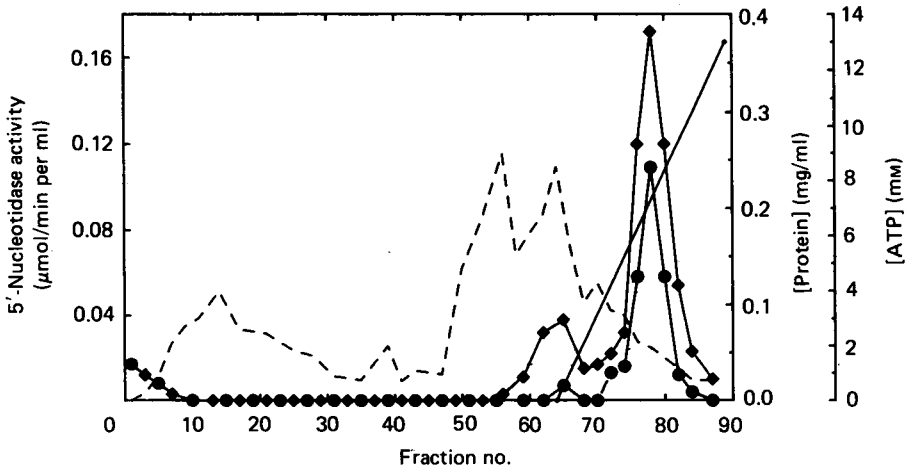

Fig. 3. Elution of 5′-nucleotidase II from second phosphocellulose column

Phosphocellulose chromatography was carried out by Procedure 2 as described in the Materials and methods section. The peak fractions containing 5′-nucleotidase II activity (Fig. 1) were pooled, dialysed against buffer 2, and 3.0 units of activity were applied to the second phosphocellulose column; 2.6 ml fractions were collected. Activity was determined by assay 1 (●) and assay 2 (◆) as described in Fig. 1. Recovery of activity after column chromatography was 93%. The continuous straight line without symbols shows the concentration of ATP. The broken line without symbols shows protein concentration.

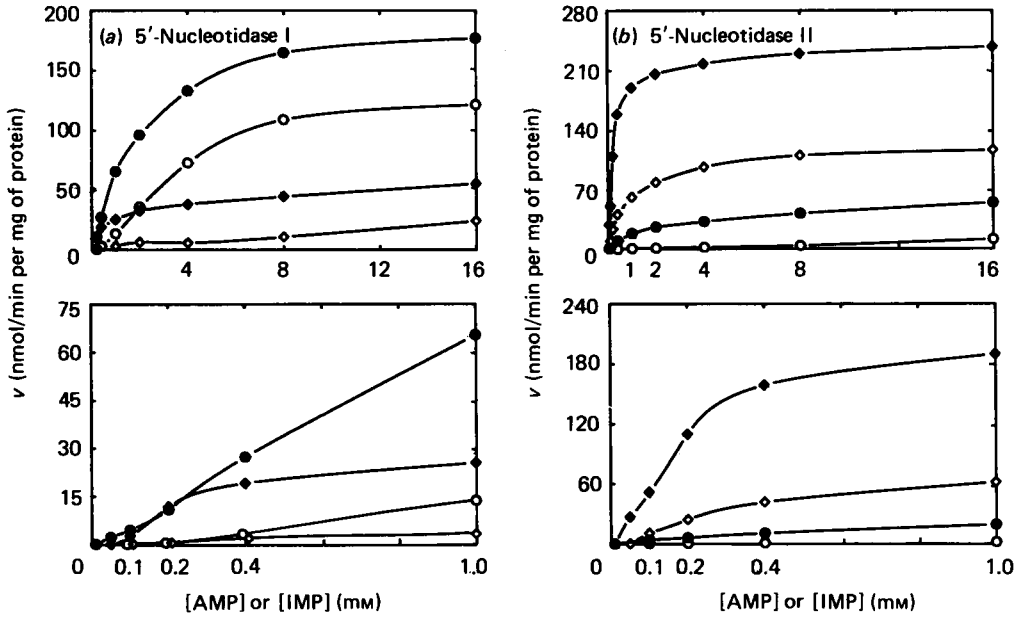

Fig. 4. Effect of ATP on activity of 5′-nucleotidases I (*a*) and II (*b*)

5′-Nucleotidase activity was determined by assay 3 as described in the Materials and methods section. The reaction mixture contained either (○, ●) AMP or (◇, ◆) IMP, 10 mM-$MgCl_2$, 50 mM-Mops/NaOH buffer, pH 6.5, and 5′-nucleotidases I or II (110 μg of protein) from the first phosphocellulose column. The reactions were run in the presence (●, ◆) or absence (○, ◇) of 5 mM-ATP. Two ranges of substrate concentration, 0–1 and 0–16 mM, are shown for each enzyme.

column by Procedure 2, which is based on the procedure used by Itoh (1981) and Itoh *et al.* (1986). The first peak of activity obtained by Procedure 1 was only weakly retained when applied to the column run by Procedure 2 (Fig. 2). The second peak of activity obtained by Procedure 1 was strongly retained when applied to the column run by Procedure 2 (Fig. 3), and was eluted as a minor peak followed by a major peak of activity. The major peak was eluted at a position identical with that described for the isolation of the enzyme by Itoh *et al.* (1986). We did not observe the minor peak in two separate preparations out of three.

Another phosphocellulose column with identical dimensions was used to analyse rat heart cytosol for the elution position(s) of 5′-nucleotidase under conditions identical with those described by Itoh (1981) and Itoh *et al.* (1986). A single peak of activity was observed under these conditions, which was eluted at a position identical with that described by Itoh *et al.* (1986) (results not shown). We conclude that the second peak of activity

Table 1. Comparison of rat heart cytosolic 5′-nucleotidases I and II

The data are derived from the results shown in Fig. 4. When present, ATP was at 5 mM. $K_{app.}$ is defined as the substrate concentration at which the reaction velocity is 0.5 $V_{max.}$.

	Type I		Type II	
	+ATP	−ATP	+ATP	−ATP
AMP				
$K_{app.}$ (mM)	1.2	3.3	1.8	6
$V_{max.}$ (nmol/min per mg of protein)	202	128	57.3	13.6
$V_{max.}/K_{app.}$	168	39	32	2.3
IMP				
$K_{app.}$ (mM)	0.8	10	0.2	0.8
$V_{max.}$ (nmol/min per mg of protein)	47.3	24.5	245	121
$V_{max.}/K_{app.}$	59	2.4	1225	151
$\frac{V_{max.} \text{ for AMP}}{V_{max.} \text{ for IMP}}$	4.3	5.2	0.23	0.11
$\frac{V_{max.}/K_{app.} \text{ for AMP}}{V_{max.}/K_{app.} \text{ for IMP}}$	2.8	16	0.026	0.015

observed by our Procedure 1 is probably identical with the enzyme described by Itoh *et al.* (1986), and that the first peak of activity observed by us represents a new form of 5′-nucleotidase which favours AMP as a substrate over IMP. Below, we refer to the enzymes in the first and second peaks as 5′-nucleotidases I and II respectively. 5′-Nucleotidase I is relatively unstable in the absence of glycerol. Neither glycerol nor other stabilizing agents were added by Itoh *et al.* (1986), and this may explain why they did not observe the type I enzyme. Moreover, after applying their extracts to the phosphocellulose column, Itoh *et al.* (1986) washed the column with 0.2 M-NaCl. Under these conditions 5′-nucleotidase I is eluted soon after the break-through volume.

The kinetics of both types of enzyme were compared with AMP and IMP as substrates, in the presence and absence of 5 mM-ATP. 5′-Nucleotidase I had a higher $V_{max.}$ with AMP than with IMP (Fig. 4). At low substrate concentrations the enzyme showed sigmoidal saturation behaviour. The affinity of 5′-nucleotidase I for AMP is somewhat higher than that for IMP, in both the presence and the absence of ATP. 5′-Nucleotidase II had a higher $V_{max.}$ with IMP than with AMP, in both the absence and the presence of 5 mM-ATP. At low substrate concentrations, the enzyme showed sigmoidal saturation behaviour with IMP, but not with AMP. The affinity of the enzyme for IMP was substantially higher than that for AMP. The kinetic differences between the two 5′-nucleotidases are summarized in Table 1. By the criterion of ($V_{max.}/K_{app.}$ for AMP)/($V_{max.}/K_{app.}$ for IMP), 5′-nucleotidase I clearly favours AMP as substrate over IMP, in both the presence and the absence of 5 mM-ATP. On the other hand, 5′-nucleotidase II clearly favours IMP as substrate over AMP, in both the presence and the absence of 5 mM-ATP.

DISCUSSION

Initiation of hypoxia, anoxia and increased work load leads to an increase in the AMP concentration in the heart. Physiological concentrations of AMP in rat heart are in the range 0.1–0.5 mM (Williamson, 1965; Frick & Lowenstein, 1976). The AMP content of heart also rises in response to the infusion of acetate (Williamson, 1965; Liang & Lowenstein, 1978), a situation of practical interest in the case of kidney dialysis, for which acetate is currently used as the major anion in the dialysing fluid. In aerobic perfused heart, the IMP content is much lower than the AMP content. Both AMP and IMP increase upon initiation of anoxia, but the AMP content remains more than double the IMP content (Frick & Lowenstein, 1976; Swain *et al.*, 1982). AMP and IMP of cultured heart cells also increase in response to 2-deoxyglucose; in this case, the IMP content exceeds the AMP content (Meghji *et al.*, 1985). IMP is removed rapidly upon reoxygenation of heart myocytes (Geisbuhler *et al.*, 1984).

Kinetic comparisons of 5′-nucleotidases I and II (Table 1) indicate that 5′-nucleotidase I is likely to be the enzyme responsible for the production of adenosine from AMP. For example, comparison of ($V_{max.}/K_{app.}$ for AMP)/($V_{max.}/K_{app.}$ for IMP) shows that in the presence of 5 mM-ATP these ratios are 2.8 for 5′-nucleotidase I and 0.026 for 5′-nucleotidase II. In the absence of ATP, these ratios are 16 and 0.015 respectively. Furthermore, Itoh *et al.* (1986) showed that 5′-nucleotidase II from rat heart is inhibited by over 90% by 5 mM-P_i, but we find that 5′-nucleotidase I is inhibited less than 10% by 5 mM-P_i (A. R. Collinson & J. M. Lowenstein, unpublished work). The P_i concentration rises during ATP and phosphocreatine depletion, such as occurs during hypoxia (for discussion see Collinson *et al.*, 1987). Since adenosine production increases under these conditions, the strong inhibition of 5′-nucleotidase II by physiological concentrations of P_i has been difficult to reconcile with its possible role in adenosine production. These considerations make it unlikely that the enzyme purified and studied by Itoh *et al.* (1986) is responsible in a major way for the production of the adenosine under these conditions. This raises the question of the function of 5′-nucleotidase II. Heart contains much lower activity of

adenylate deaminase than does skeletal muscle (Lowenstein, 1972); however, this enzyme is presumably responsible for most of the IMP produced by the heart. 5′-Nucleotidases I and II and adenylate deaminase are activated to different extents by ATP and ADP (A. R. Collinson & J. M. Lowenstein, unpublished work). The rate of adenosine production depends on the available concentration of AMP and on competition for AMP by 5′-nucleotidase I and adenylate deaminase. The outcome of such competition will depend on the differences in the regulation of these enzymes.

Lysosomes also contain soluble 5′-nucleotide-hydrolysing activity (Collinson *et al.*, 1987). The specificity and regulatory properties of this activity are quite different from those of the two soluble enzymes described above. For instance, AMP hydrolysis is inhibited competitively by both ADP and ATP, with K_i values of 79 and 90 μM respectively.

The rate of nucleoside plus hypoxanthine production by rat heart perfused under aerobic and anaerobic conditions has been reported to be 0.068 and 3.54 μmol/10 min per g dry wt. respectively (Frick & Lowenstein, 1976). The wet-weight/dry-weight ratio of perfused heart is about 5. Hence the rate is $3.54/(5 \times 10) = 0.071$ μmol/min per g fresh wt. Fully activated 5′-nucleotidase I is present in rat heart with an activity of 300 nmol of AMP hydrolysed/min per g fresh wt., assuming 100% recovery in the peak obtained by phosphocellulose chromatography. This is a minimum rate, because the recovery of activity is almost certainly less than 100%. The AMP content of rat heart perfused aerobically is 0.60 ± 0.04 μmol/g dry wt. (Frick & Lowenstein, 1976), which is equivalent to 0.12 μmol/g fresh wt., or about 0.24 mM-AMP, assuming that 50% of the wet weight is intracellular water. The corresponding concentration for anaerobic heart is 1.4 mM-AMP. According to Fig. 4, at 0.24 mM- and 1.4 mM-AMP fully activated 5′-nucleotidase I exhibits 8.1 and 46%, respectively, of $V_{max.}$ or 24 and 138 nmol of AMP hydrolysed/min per g fresh wt. respectively. This compares with a rate of nucleoside plus hypoxanthine production of 71 nmol/min per g fresh wt. Thus the amount of 5′-nucleotidase I recovered from rat heart is sufficient to account for the rate of nucleoside plus hypoxanthine production observed to occur under anaerobic conditions. Much remains to be learned about the regulatory behavior of the enzyme *in vitro* before we can consider its regulation *in vivo*.

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