Regulation of rat heart cytosol 5'-nucleotidase by adenylate energy charge

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A 5'-nucleotidase (EC 3.1.3.5) was highly purified from the soluble fraction of rat heart. The preparation appeared homogeneous by the criterion of polyacrylamide-gel electrophoresis. The enzyme was activated by ATP and ADP, and inhibited by P_i . When AMP was used as substrate, the velocity/substrate-concentration plot was sigmoidal. ATP or ADP changed the plot to hyperbolic and decreased $s_{0.5}$. P_i increased both the sigmoidicity of the plot and $s_{0.5}$. When IMP was used as substrate, the velocity/substrate plot was hyperbolic. ATP or ADP decreased K_m and increased V . P_i changed the plot to sigmoidal and increased $s_{0.5}$. Within the range of adenylate energy charge observed in surviving mammalian cells (0.7–0.9), the rate of AMP-hydrolysing activity catalysed by the 5'-nucleotidase increased sharply with decreasing energy charge. The highest activity was observed at an energy-charge value of about 0.6. The response was also observed in the presence of P_i . No change in IMP-hydrolysing activity was observed in the physiological range of adenylate energy charge, but in the presence of P_i the activity gradually increased with increasing energy charge. These results suggest the possibility that this enzyme participates in production of adenosine, a vasodilator, during hypoxia and in removal of IMP, which accumulates during the hypoxia, in the heart.

INTRODUCTION

It has been suggested that adenosine participates in the metabolic regulation of various cardiac functions. In addition to modulating impulse formation in the sinusoidal node and His bundle, and impulse propagation through the atrioventricular node (Belardinelli *et al.*, 1983), it has a potent coronary vasodilator effect (reviewed by Berne, 1980).

On the basis of results on isolated cat hearts and intact hearts of open-chest dogs, Berne (1963) proposed that a deficiency of oxygen increases the rate of release of adenosine from the muscle cells of mammalian heart. Adenosine caused relaxation of the smooth muscle of the coronary arterioles, which results in vasodilation and an increased blood flow to the heart muscle. Thus more oxygen is made available for the heart. Evidence provided by many investigators seems to support this theory (reviewed by Arch & Newsholme, 1978).

Adenosine can be formed from AMP by action of 5'-nucleotidase (5'-ribonucleotide phosphohydrolase, EC 3.1.3.5) or alkaline phosphatase (EC 3.1.3.1) and acid phosphatase (EC 3.1.3.2), or from S-adenosylhomocysteine by S-adenosylhomocysteine hydrolase (EC 3.3.1.1). Rubio *et al.* (1973) reported that a decrease in adenine nucleotides during ischaemia in the heart is associated with a marked increase in myocardial content of adenosine, and this has been confirmed by several investigators (Jennings *et al.*, 1981; Swain *et al.*, 1982). These results suggest that the main pathway for production of adenosine in the heart during ischaemia is through dephosphorylation of AMP.

A 5'-nucleotidase associated with a membrane fraction was highly purified from rat heart, and its properties were

extensively investigated (Naito & Lowenstein, 1981). This 5'-nucleotidase activity has been reported to be associated mostly with endothelial and myocardial membranes lining compartments open to the extracellular space (Rubio *et al.*, 1973). Functional studies on this enzyme by Frick & Lowenstein (1976) also suggested that it is an ectoenzyme.

On the basis of results of experiments using isolated guinea-pig heart, Schütz *et al.* (1981) proposed that, in hypoxia in the heart, (a) the ecto-5'-nucleotidase most probably is not involved in the formation of adenosine, (b) release of adenosine from heart requires adenosine to be transported across the membrane, and (c) adenosine is predominantly formed intracellularly by a process involving dephosphorylation of AMP. More recently, similar results have been reported by Meghji *et al.* (1985), using neonatal-rat heart cells in culture.

Searching for the enzymes that participate in intracellular dephosphorylation of AMP, Lowenstein *et al.* (1983) suggested the presence of another 5'-nucleotidase in the cytosolic fraction of rat heart. Independently, the evidence for existence of a cytosol 5'-nucleotidase in chicken heart has been reported more recently (Itoh & Oka, 1985).

To elucidate mechanisms of adenosine formation during hypoxia in mammalian heart, we purified a 5'-nucleotidase from rat heart soluble fraction to apparent homogeneity by the criterion of polyacrylamide-gel electrophoresis, and studied some regulatory properties of the preparation. The results of the present study strongly suggest the possibility of participation of this 5'-nucleotidase in production of adenosine in hypoxia in the heart.

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MATERIALS AND METHODS

Chemicals

AMP, ADP, ATP and IMP were products of Yamasa Shoyu Co. (Cyoshi, Japan). Concentrations of all nucleotide solutions were determined spectrophotometrically. Phosphocreatine was a product of Sigma Chemical Co. (St. Louis, MO, U.S.A.). $[8\text{-}^{14}\text{C}]\text{IMP}$ (60 Ci/mol) and $[8\text{-}^{14}\text{C}]\text{AMP}$ (59 Ci/mol) were purchased from Amersham International (Amersham, Bucks., U.K.). All other chemicals were of reagent grade or of the highest quality available.

Assay of 5'-nucleotidase

5'-Nucleotidase was assayed by determination of $[8\text{-}^{14}\text{C}]\text{inosine}$ released from $[8\text{-}^{14}\text{C}]\text{IMP}$ or of $[8\text{-}^{14}\text{C}]\text{-adenosine}$ from $[8\text{-}^{14}\text{C}]\text{AMP}$ as described previously (Itoh, 1981a). The reaction mixture for kinetic experiments contained 100 mM-imidazole/HCl (pH 6.5), 50 mM- MgCl_2 , 0.1% bovine serum albumin, an appropriate amount of enzyme preparation and various amounts of a substrate and effectors.

Determination of protein

This was done by the method of Bradford (1976), with crystalline bovine serum albumin as a standard.

Estimation of kinetic parameters

When the velocity/substrate-concentration plot was hyperbolic, V and K_m values were estimated by the direct linear plot described by Eisenthal & Cornish-Bowden (1974). When the plot of the reaction was sigmoidal, V , $s_{0.5}$ and h were determined by the method of Atkinson (1977).

Purification of 5'-nucleotidase from rat heart

Rat heart cytosol 5'-nucleotidase was prepared by essentially the same method as used for purification of rat liver enzyme (Itoh, 1981a). The procedure consists of five steps: extraction, first and second phosphocellulose column chromatography, fractionation with $(\text{NH}_4)_2\text{SO}_4$ and precipitation at low ionic strength. The rat heart enzyme was precipitated between 0 and 40% saturation of $(\text{NH}_4)_2\text{SO}_4$. The fraction was dialysed against 50 mM-Tris/HCl (pH 6.5) for 7 h; the precipitate, containing impurities, was removed by centrifugation, and the supernatant was used for the following experiments.

Preparation of reaction mixtures of various adenylate energy charges

Reaction mixtures of various adenylate energy charges, $([\text{ATP}] + 0.5[\text{ADP}])/([\text{ATP}] + [\text{ADP}] + [\text{AMP}])$ (Atkinson, 1968), were prepared by the method of Chapman & Atkinson (1973). The relative amounts of ATP, ADP and $[^{14}\text{C}]\text{AMP}$ at different energy charges were calculated from a value of 0.8 for the equilibrium constant of the adenylate kinase reaction. The total Mg^{2+} concentration corresponding to 1 mM free Mg^{2+} at various adenylate energy-charge values was calculated as described by Chapman *et al.* (1976).

Polyacrylamide-gel electrophoresis

This was done by the method of Davis (1964) in 7.5%-acrylamide gels. To identify the position of 5'-nucleotidase, the gels were incubated in a reaction

mixture containing 100 mM-Tris/maleate buffer (pH 6.5), 50 mM- MgCl_2 , 3 mM-IMP and 2 mM- $\text{Pb}(\text{NO}_3)_2$ at 37 °C until a white precipitate of lead phosphate appeared on the gels. Polyacrylamide-gel electrophoresis in the presence of 0.1% SDS was done by the method of Laemmli (1970) in 10%-acrylamide gels.

RESULTS

Purity of the enzyme preparation

Rat heart 5'-nucleotidase was purified about 3000-fold with 3% recovery and a specific activity of the final preparation of 28.5 μmol of inosine formed from IMP/min per mg of protein. On SDS/polyacrylamide-gel electrophoresis, the purified enzyme preparation gave one Coomassie-Blue-staining band, corresponding to M_r of 58000.

On polyacrylamide-gel electrophoresis without SDS at pH 8.9, the preparation gave one Coomassie-Blue-staining band, the position of which corresponded to the 5'-nucleotidase activity detected with IMP as a substrate (Fig. 1).

Effects of various compounds on the 5'-nucleotidase activity

When 4 mM-AMP was used as the substrate, ATP markedly stimulated the enzyme. The AMP-hydrolysing activity was markedly inhibited by P_i . Phosphocreatine and IMP also inhibited the enzyme, but were much less effective than P_i (Fig. 2a).

When 0.5 mM-IMP was used as the substrate, the effects of ATP, P_i and phosphocreatine were similar to those with AMP as substrate. AMP at relatively low concentrations stimulated the IMP-hydrolysing activity, but at higher concentrations it was inhibitory (Fig. 2b).

Velocity/substrate-concentration plots of the rat heart 5'-nucleotidase are shown in Fig. 3. When AMP was used as substrate, the plot was sigmoidal. ATP and ADP were activators of the enzyme, and changed the plot to hyperbolic and decreased $s_{0.5}$. P_i was an inhibitor of the

Fig. 1. Polyacrylamide-gel electrophoresis of the purified rat heart 5'-nucleotidase

Electrophoresis of 1 μg of the 5'-nucleotidase preparation was done on a SDS/10%-polyacrylamide slab gel, which was then stained with Coomassie Blue (a). Electrophoresis of 8 μg of the 5'-nucleotidase preparation was done on 7.5%-polyacrylamide disc gels without SDS at pH 8.9. Protein was stained with Coomassie Blue (b), or activity was detected by incubating the gel with IMP and $\text{Pb}(\text{NO}_3)_2$ (c).

Fig. 2. Effects of various compounds on (a) AMP- or (b) IMP-hydrolysing activity of the rat heart 5'-nucleotidase

The reaction mixture contained 100 mM-imidazole/HCl (pH 6.5), 50 mM-MgCl₂, 0.1% bovine serum albumin, 4 mM-AMP as substrate and ATP (□), IMP (○), phosphocreatine (●) or P_i (△) at the concentrations indicated (a). Similar experiments were done with 0.5 mM-IMP as substrate, with ATP (□), AMP (○), phosphocreatine (●) or P_i (△) at the concentrations indicated (b). The ratio of reaction velocities in the presence of an effector (*v*) to those in its absence (*v*₀) is indicated on the ordinate.

Fig. 3. Effects of ATP, ADP and P_i on the 5'-nucleotidase activity as a function of AMP (a) or IMP (b) concentration

The reaction mixture contained 100 mM-imidazole/HCl (pH 6.5), 50 mM-MgCl₂, 0.1% bovine serum albumin, various concentrations of AMP (a) or IMP (b) as substrate, and effectors as indicated: ○, no addition; □, 5 mM-ATP; ●, 5 mM-ADP; △, 1 mM-P_i.

enzyme, and increased both the sigmoidicity of the curve and *s*_{0.5}.

When IMP was used as substrate, the plot was hyperbolic. ATP and ADP were activators of the enzyme, and decreased *K*_m and increased *V*. P_i was an inhibitor of the enzyme, and changed the plot to sigmoidal and increased *K*_m.

Changes in some kinetic parameters of the enzyme, with AMP or IMP as substrate, by various effectors are summarized in Table 1.

Response of the cytosol 5'-nucleotidase activity to variations in adenylate energy charge

The response of the AMP-hydrolysing activity of rat heart 5'-nucleotidase to variations in adenylate energy

charge is shown in Fig. 4(a). The total adenine nucleotide concentration and free Mg²⁺ concentration were held at 4 mM and 1 mM respectively in this experiment. The activity was low at the normal physiological energy-charge value (0.9), and was markedly stimulated when the energy-charge value decreased within the physiological range of surviving cells (0.7–0.9). The maximum activity was obtained at an energy-charge value of about 0.6.

The AMP-hydrolysing activity was strongly suppressed in the presence of 5 mM-P_i, but the response to the changes in adenylate energy charge was still maintained. The effect of changes in P_i concentration on AMP-hydrolysing activity at energy-charge values of 0.6 and 0.9 is shown in Fig. 4(b).

The response of IMP-hydrolysing activity of the

Table 1. Effect of ATP, ADP and P_i on kinetic parameters of the rat heart 5'-nucleotidase

Conditions for the assay were the same as in Fig. 3. *V*, *s*_{0.5} and *h* were estimated as described in the text. The *V* with no addition with IMP as substrate was taken as 1.0.

Substrate	Effector	<i>V</i>	<i>s</i> _{0.5}	<i>h</i>
IMP	None	1.0	0.9	1.0
	5 mM-ATP	2.8	0.1	1.0
	5 mM-ADP	3.4	0.3	1.0
	1 mM-P _i	1.0	3.2	1.3
AMP	None	3.4	25	1.5
	5 mM-ATP	3.4	4.5	1.0
	5 mM-ADP	3.4	9.8	1.0
	1 mM-P _i	3.4	40	2.5

enzyme to variations in adenylate energy charge is shown in Fig. 5. The total adenine nucleotide concentration and free Mg²⁺ concentration were also held at 4 mM and 1 mM respectively, and 0.5 mM-IMP was used as substrate. Almost no response of IMP-hydrolysing activity to variations in adenylate energy charge within the physiological range was observed in the absence of P_i. In the presence of 5 mM-P_i, the activity gradually increased with increasing adenylate energy charge.

DISCUSSION

To characterize the enzyme which participates in formation of adenosine, a powerful vasodilator, from AMP in cardiac tissue, we purified a 5'-nucleotidase from the soluble fraction of rat heart. In contrast with the membrane-associated ecto-5'-nucleotidase of the heart, which is strongly inhibited by ATP or ADP (Sullivan & Alpers, 1971), the purified 5'-nucleotidase by us was effectively activated by these compounds. The kinetic

Fig. 5. Response of IMP-hydrolysing activity of the rat heart 5'-nucleotidase to variations in energy charge

The reaction mixture contained 100 mM-imidazole/HCl (pH 6.5), 100 mM-KCl, 0.1% bovine serum albumin, 1 mM free Mg²⁺, 0.5 mM-IMP, ATP, ADP and AMP at a total concentration of 4 mM at the energy-charge values indicated on the abscissa, with (Δ) or without (○) 5 mM-P_i.

properties of the enzyme suggest that the 5'-nucleotidase is similar to the cytosol 5'-nucleotidases previously described in the liver of various species (Itoh *et al.*, 1967, 1968) and chicken heart (Itoh & Oka, 1985).

Fig. 4. Response of AMP-hydrolysing activity of the rat heart 5'-nucleotidase to variations in the adenylate energy charge (a) and effect of changes in P_i concentration on the activity at high or low adenylate energy-charge value (b)

The reaction mixture contained 100 mM-imidazole/HCl (pH 6.5), 100 mM-KCl, 0.1% bovine serum albumin, 1 mM free Mg²⁺, and ATP, ADP and [¹⁴C]AMP at a total concentration of 4 mM at the energy-charge values indicated on the abscissa with (Δ) or without (○) 5 mM-P_i (a). Effect of changes in P_i concentration on AMP-hydrolysing activity at energy-charge values of 0.6 (○) and 0.9 (Δ) is also shown (b).

Results of experiments *in vitro* revealed that AMP-hydrolysing activity of the cytosol 5'-nucleotidase from rat heart increased sharply with decreasing adenylate energy charge within the physiological range, as has been reported for the cytosol 5'-nucleotidase of the liver (Itoh, 1981b). This response was observed in the reaction conditions with physiological concentrations of total adenine nucleotides, free Mg^{2+} and P_i . It is believed that the intracellular concentration of P_i , an inhibitor of this enzyme, increases in the heart during hypoxic conditions, and this has an adverse effect on the stimulation of AMP-hydrolysing activity by the decrease in adenylate energy charge. However, under our experimental conditions, AMP-hydrolysing activity at an energy charge of 0.6 in the presence of 10 mM- P_i was still higher than that at an energy charge of 0.9 in the presence of 4 mM- P_i .

These results imply that a change in AMP concentration is the primary determinant of the rate of intracellular degradation of AMP by this enzyme, since a decrease in adenylate energy charge within the physiological range is accompanied by a marked increase in AMP concentration and a much smaller decrease in the sum of the concentrations of ATP and ADP, which are activators of this enzyme (Atkinson, 1968). This is consistent with the finding by Liang & Lowenstein (1978) that the adenosine content of the myocardium and the hypoxanthine concentration in coronary blood flow in the anaesthetized dog increased under conditions of AMP accumulation induced by infusion of acetate.

A similar response to changes in adenylate energy charge of AMP deaminase from mammalian liver has been reported (Chapman & Atkinson, 1973). This implies that two enzymes which participate in the first steps of AMP catabolism, dephosphorylation and deamination, in the liver share common regulatory properties. But in the heart, a relatively small amount of IMP accumulation is observed during hypoxia (Swain *et al.*, 1982), and AMP deaminase of the heart has been reported to show virtually no variation in activity with the energy-charge value between zero and 0.9 (Solano & Coffee, 1978). This means that in the heart, dephosphorylation of AMP, which is presumed to be the main pathway of its catabolism, is effectively regulated by changes in adenylate energy charge, resulting in adenosine production in hypoxic conditions.

Accumulation of IMP in rat heart myocytes in anaerobiosis and its rapid removal during reoxygenation has been reported (Geisbuhler *et al.*, 1984). This removal of IMP induced by reoxygenation could be facilitated, at least in part, by the increase in IMP-hydrolysing activity with increasing energy charge in the presence of P_i .

Various results of experiments which have been reported by many investigators on purine nucleotide catabolism, especially adenosine formation from adenine nucleotides, in the heart can easily be explained by considering the participation of the 5'-nucleotidase described in the present paper.

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