Adenylate Metabolism in the Heart

REGULATORY PROPERTIES OF RABBIT CARDIAC ADENYLATE DEAMINASE

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The kinetic properties of a 300-fold purified cardiac AMP deaminase were studied and compared with those of the corresponding enzyme from skeletal muscle. The heart enzyme is activated by ATP and less efficiently by ADP, and is inhibited by P_i , phosphocreatine and GTP. ATP, even at micromolar concentrations, is able to abolish the effects of the inhibitors. The affinity of the enzyme for AMP is low in the absence of activators (K_m 3.1 mM), but, in the presence of ATP, becomes as high as that of skeletal-muscle AMP deaminase (K_m 0.4 mM). The maximal activation by ATP is observed at alkaline pH (pH 7.5–8.0). Under the same conditions ATP is maximally inhibitory for skeletal-muscle enzyme. These results suggest that AMP deaminase in the heart is always in the activated state, whereas in skeletal muscle the enzyme is active only during exhaustive contractions.

The breakdown of AMP in muscle can take place by two different pathways, either the direct deamination to IMP or the sequential dephosphorylation and deamination to inosine with the intermediate formation of adenosine (Purzycka, 1962; Burger & Lowenstein, 1967). It is not yet clear what factors determine the rate of degradation of AMP by the two pathways under different physiological conditions. However, it is known that the distribution of AMP-catabolizing enzymes is different in several tissues and is correlated with their different metabolic activities (Arch & Newsholme, 1978; Purzycka & Zydowo, 1960). AMP deaminase is present in higher concentration in phasic white muscles (Raggi *et al.*, 1969; Winder *et al.*, 1974), whereas the enzymes of the alternative pathway are prevalent in red muscles and mainly in the heart (Conway & Cooke, 1939; Bockman, 1977). As far as AMP deaminase is concerned, the enzyme has been shown to exist as different isoenzymes in white and red muscles (Raggi *et al.*, 1975). Only one form is present in the heart, which has the chromatographic properties of the isoenzyme prevalent in red muscle. The white-muscle-specific enzyme has been extensively studied (Smiley & Suelter, 1967; Ronca-Testoni *et al.*, 1970; Coffee & Solano, 1977). Our studies (Ronca-Testoni *et al.*, 1970) on the effects of several metabolites on the K^+ -activated enzyme from five different species showed that AMP deaminase is inhibited by ATP,

P_i and phosphocreatine; ADP is the most efficient metabolite in counteracting the effect of these inhibitors, and an increase in the $[ADP]/[ATP]$ ratio is likely to be the stimulus for the activation of AMP deaminase in the periods of intense muscular activity. In agreement with these suggestions, Coffee & Solano (1977) have shown that rat muscle AMP deaminase is activated by a decrease of the adenylate energy charge. The properties of the cardiac enzyme are less well known, but the available data on the concentrations of nucleotides during several phases of heart activity (and mainly the constancy of IMP concentration) (Rubio *et al.*, 1973) suggest a different regulation pattern for this isoenzyme. It is of special interest to study the regulatory features of this particular adenylate deaminase because this enzyme is located at the metabolic crossroad leading to the production of adenosine. This nucleoside is thought to be an important physiological regulator of coronary blood flow. There is experimental evidence that perfusion of isolated hearts with adenosine or adenosine deaminase inhibitors brings about a fall in resistance of coronary vessels (Katori & Berne, 1966; Cobbin *et al.*, 1974). Furthermore, it is known that during episodes of coronary-artery occlusion adenosine or its catabolic products are released from hypoxic myocardial cells (Berne, 1963; Berne & Rubio, 1974).

In the present paper we examine the kinetic properties of the partially purified rabbit cardiac AMP deaminase. Our results give strong evidence of different regulatory properties of cardiac and skeletal-muscle enzymes.

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Materials and Methods

Nucleotides and cellulose phosphate were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and rabbit hearts from a local slaughterhouse; cellulose phosphate was activated by successive rinsing with NaOH and HCl and finally equilibrated with 90mM-potassium phosphate (pH 6.5) containing 180mM-KCl (buffer A). Any other reagent employed was of the highest purity available and used without further purification. Rabbit skeletal-muscle AMP deaminase was purified by phosphocellulose chromatography essentially as described by Smiley *et al.* (1967), but by eluting the enzyme with 1.0M-KCl after the column had been washed with 0.6M-KCl; in this way we obtained an enzyme completely devoid of isoenzyme A, which is the prevalent AMP deaminase form in red muscles (Raggi *et al.*, 1975). Protein concentration was determined by the method of Bradford (1976), by using purified skeletal-muscle AMP deaminase as a reference protein.

During the purification procedure the enzyme activity was assayed both by measuring NH_3 formation and spectrophotometrically. Similar results were obtained with both procedures. In the former procedure the enzyme was incubated at 25°C with 3mM-AMP in 0.1M-succinate buffer (pH 6.5) containing 0.15M-KCl. The reaction was stopped by adding trichloroacetic acid (5%, w/v, final concentration) and portions of the protein-free filtrate were analysed for NH_3 by reaction with the Nessler reagent. Details of the spectrophotometric assay, which was accomplished by monitoring the change in A_{265} when the total nucleotide concentration was lower than 0.2mM and in A_{285} for higher concentrations of nucleotides, are found in Ronca *et al.* (1968). The enzyme was purified from hearts frozen

immediately after the animals were killed; after rinsing with 150mM-KCl, the tissue was chopped and homogenized in a Waring Blendor with 3.2vol. of buffer A. After stirring for 20min, the homogenate was centrifuged for 20min at 20000g. After filtration through several layers of cheesecloth, the cellulose phosphate resin was added to the supernatant, and after standing for 30min the slurry was placed on a sintered-glass funnel and washed with 25mM-succinate/280mM-Tris (pH 6.5) (buffer B) containing 0.2M-KCl until the A_{280} of the effluent was lower than 0.01. The cake was then transferred to a glass column (bed dimensions 1.5cm $\times$ 18cm in a typical experiment) and eluted with an 80ml linear gradient of 0.2–2.0M-KCl in buffer B. The enzyme emerged as a single symmetrical peak at 1.0M-KCl. AMP deaminase was thus purified about 300-fold with a total yield of 15%. The specific activity of the purified enzyme, devoid of contamination by 5'-nucleotidase, adenylate kinase and adenosine deaminase, was 1.4 $\mu\text{mol}/\text{min}$ per mg of protein. Higher specific-activity values were obtained as a result of a buffer effect in 50mM-imidazole/HCl (pH 6.5)/10mM-Tris/150mM-KCl (buffer C). In these conditions the specific activities with 2mM-AMP were 2.6 and 3.5 in the absence and in the presence of 1mM-ATP, respectively. This latter incubation medium has been adopted for the kinetic investigations, which have been performed by the spectrophotometric assay using about 10 μg of protein/ml.

Results and Discussion

Effectors of cardiac AMP deaminase

Although the physiological function of skeletal-muscle AMP deaminase is not completely clarified, this protein has been widely studied and well charac-

Fig. 1. Activation of cardiac AMP deaminase by adenine nucleotides
Assays contained buffer C (see the Materials and Methods section), 0.1 mM-AMP and either ADP (●) or ATP (○).

terized as an allosteric enzyme. In the heart a distinct isoenzyme of AMP deaminase is found, which differs from the enzyme of skeletal muscle in its chromatographic properties (Raggi *et al.*, 1975). Our aim was to investigate the catalytic properties of this enzyme and to compare them with those of skeletal-muscle adenylate deaminase. The data have been obtained in the presence of moderately high salt concentration (i.e. 150mM-KCl). Preliminary experiments showed that this concentration, beside being in the physiological range, is optimal for heart AMP deaminase and was used throughout.

Some remarkable observations have been obtained during the study of the effects of adenine nucleotides on enzyme activity. Although ATP inhibits and ADP does not modify the activity of the skeletal-muscle enzyme at low substrate concentrations (Ronca-Testoni *et al.*, 1970; Coffee & Solano, 1977), both nucleotides are activators of cardiac AMP deaminase (Figs. 1a and 1b). In the micromolar concentration range ATP has a powerful activating effect which is much greater than that produced by the same concentrations of ADP (Fig. 1a). However, ADP and ATP concentrations in the millimolar range (Fig. 1b) activate to about the same extent. The discrepancy between these observations on the activation by adenine nucleotides depends on the nature of the saturation curve, which is sigmoid for ADP and hyperbolic for ATP.

In order to determine the effects of ATP on the kinetic parameters of cardiac AMP deaminase, substrate-saturation curves have been examined in the absence and in the presence of these activators, both at 1mM concentration (Fig. 2). Under our experimental conditions, heart AMP deaminase

displays almost hyperbolic saturation kinetics. Because of the limits of the spectrophotometric assay, which allows determination of enzyme activity only in the presence of total nucleotide concentration lower than 3mM, measurement of initial velocity at saturating AMP concentration was performed by the NH_3 assay. For this purpose the enzyme was diluted in buffer C containing 10mM-AMP and NH_3 was determined as described in the Materials and Methods section. The specific-activity values obtained were 3.3 without effectors and 3.4 and 3.5 in the presence of 1mM-ADP and -ATP, respectively. These observations indicate an identical V_{max} value for the enzyme both in the presence and in the absence of activators. Subsequent analysis of the data of Fig. 2 by the Lineweaver-Burk procedure shows that the affinity of cardiac AMP deaminase for AMP is much lower than that of the skeletal-muscle enzyme; K_m is 3.1mM, whereas the reported value for skeletal-muscle AMP deaminase is lower (0.4–0.7mM-AMP; Smiley & Suelter, 1967; Ronca-Testoni *et al.*, 1970; Coffee & Solano, 1977). The affinity of the cardiac-muscle enzyme for AMP is increased strikingly by 1mM-ADP or -ATP, when the K_m for AMP is decreased to 1.6 and 0.4mM, respectively. Both these effectors behave as pure modifiers of K_m , since V_{max} remains unchanged. It is noteworthy that the affinity of skeletal-muscle AMP deaminase for AMP is not altered by addition of ADP and is decreased about 2-fold by ATP (Ronca-Testoni *et al.*, 1970; Coffee & Solano, 1977).

By analogy with skeletal-muscle AMP deaminase, the cardiac enzyme is inhibited by P_i , phospho-

Fig. 2. Effect of ADP and ATP on substrate-saturation curve of cardiac AMP deaminase

The reaction mixtures contained buffer C and the reported AMP concentrations either in the absence of effectors (Δ), or in the presence of 1mM-ADP ($\bullet$) or 1mM-ATP ($\circ$).

Fig. 3. Effect of some inhibitors on cardiac AMP deaminase activity

The reaction mixtures contained buffer C, 0.1mM-AMP and GTP (Δ), P_i ($\bullet$) or phosphocreatine ($\circ$).

creatine, GTP and several other organic phosphate compounds (among them 2,3-diphosphoglyceric acid, glucose 6-phosphate, fructose 6-phosphate and fructose 1,6-bisphosphate). In Fig. 3 the data relevant to these inhibitors are presented: of the compounds tested, GTP is the most effective, inhibiting even at micromolar concentrations. Also P_i and phosphocreatine in the millimolar range inhibit the enzyme. Under our conditions, P_i is a more effective inhibitor than phosphocreatine. Similar data were obtained from analogous experiments with rabbit skeletal-muscle AMP deaminase at a concentration giving the same catalytic activity. Interesting differences were observed between the AMP deaminases from the two sources when the action of adenine nucleotides was investigated in the presence of P_i and GTP. The results of this study on the cardiac enzyme are reported in Table 1, which shows that the effects of 0.5 mM- P_i and 5 μ M-GTP are only partially relieved by 20 or 50 μ M-ADP, whereas with the addition of ATP at the same concentrations a full recovery of enzyme activity is obtained. This behaviour is significantly different from that of skeletal-muscle AMP deaminase (Ronca-Testoni *et al.*, 1970), in which ADP, but not ATP, abolished the inhibitions by GTP and P_i .

The effect of P_i is to convert the cardiac enzyme into a form which shows a sigmoidal saturation curve, as shown in Fig. 4. The main effect of addition of ATP to the enzyme inhibited by P_i is to reconvert it into a form which follows hyperbolic kinetics.

Effect of pH and Mg^{2+} ions on the activity and speculations on the physiological role of cardiac AMP deaminase

The experiments show that cardiac and skeletal-muscle AMP deaminases can be differentiated on the basis of their kinetic behaviour. To compare further the properties of the two enzymes, we have analysed the effect of pH on their activation state. The reason for this investigation arises from the important role that pH is likely to play in the regulation of skeletal-muscle AMP deaminase. This enzyme is characterized by an acidic optimal pH

value (pH 6.5) and is more sensitive towards inhibitors at pH values above neutrality (Ronca-Testoni *et al.*, 1970). Cardiac AMP deaminase, when assayed at substrate concentrations lower than K_m , displays maximal activity at pH 6.5, without any major difference from that of the skeletal-muscle enzyme except for a broader bell-shaped pH-activity profile (Fig. 5). The optimal pH is nevertheless influenced

Fig. 4. Effect of ATP on the substrate-saturation curve of cardiac AMP deaminase inhibited by P_i . The reaction mixtures contained buffer C, the reported AMP concentrations and either 0.5 mM- P_i (●) or 0.5 mM- P_i plus 50 μ M-ATP (○).

Fig. 5. Effect of pH on cardiac AMP deaminase activity. Assays were performed at 0.1 mM-AMP in the absence of effectors (●) or in the presence of 50 μ M-ATP (□), 50 μ M-ADP (Δ) or 0.5 mM- P_i (○). Substrate and effectors were dissolved in 150 mM-KCl/10 mM-Tris/50 mM-imidazole (adjusted with 5 M-HCl to such a pH that addition of enzyme in buffer C led to the pH values indicated). The assumed 100% enzyme activity is the value obtained at pH 6.5 in the absence of effectors.

Table 1. Effects of ADP and ATP on the inhibition of cardiac AMP deaminase by GTP and P_i

Assays were performed in the presence of 0.1 mM-AMP in buffer C (see the Materials and Methods section). An arbitrary value of 100 was assigned for the enzyme assayed in the absence of effectors.

Additions	No inhibitor	5 μ M-GTP	0.5 mM- P_i
None	100	26	56
ADP (20 μ M)	135	46	57
ADP (50 μ M)	145	53	61
ATP (20 μ M)	190	110	78
ATP (50 μ M)	260	160	95

by the addition of effectors. Although $50\mu\text{M}$ -ADP has no effect on the curve, the addition of $50\mu\text{M}$ -ATP or of 0.5mM - P_i shifts the optimal pH to 7.0. With ATP, this effect results from the higher activating power of this nucleotide at alkaline pH, and for P_i from the lack of inhibition in this pH range. The different behaviour of skeletal and cardiac AMP deaminase is exemplified in Fig. 6, where the sensitivity of both enzymes to the effectors (ATP, ADP and P_i) is analysed as a function of pH in the range 5.6–8.0. Although skeletal AMP deaminase is more influenced by the inhibitors at pH values higher than neutrality, in the same pH range the cardiac enzyme is less sensitive to the inhibitory action of P_i and more to the activation by ATP and ADP. The most apparent difference is observed with ATP. In order to ascertain the possibility that regulation of cardiac AMP deaminase by nucleotides may be Mg^{2+} -dependent, we studied the effect of increasing MgCl_2 concentration in the absence and presence of effectors. Mg^{2+} slightly activated the enzyme at 0.1mM -AMP in the absence of effectors, but made it insensitive towards low concentration of adenine nucleotides ($50\mu\text{M}$) (Table 2) or towards 5– $50\mu\text{M}$ -GTP (results

Fig. 6. Sensitivity of cardiac and skeletal-muscle AMP deaminase to the effectors at different pH values

Assays were performed at 0.1mM -AMP in buffers prepared as described in the legend of Fig. 5, with the addition of $50\mu\text{M}$ -ATP ($\square$, $\blacksquare$), $50\mu\text{M}$ -ADP ($\triangle$, $\blacktriangle$) or 0.5mM - P_i ($\circ$, $\bullet$). Open symbols refer to cardiac enzyme and filled symbols to skeletal-muscle AMP deaminase. The ordinate shows the ratio of the experimental results in the presence of the effectors to that in their absence at each pH value considered. For this investigation, skeletal-muscle AMP deaminase was diluted in buffer C to a final concentration of approx. $0.2\mu\text{g/ml}$, giving the same catalytic activity as the heart enzyme to avoid interference owing to different protein concentration (Hemphill *et al.*, 1971).

not shown). Evidence of a differential behaviour of the enzyme towards adenine nucleotides is obtained when the effect of 1mM -ADP and -ATP is investigated in the presence of Mg^{2+} . The data of Table 2 show that activation by ATP is enhanced by the bivalent cation (maximally by 4mM - Mg^{2+}), whereas that by ADP is progressively lowered until it is abolished by 20mM - Mg^{2+} .

From the above results of the investigation on the regulatory properties of cardiac AMP deaminase and on the action of pH on the activity we can conclude that *in vivo* under normoxic conditions the enzyme is likely to be constantly in its activated state. This is the consequence of its stimulation by ATP, which is also able to abolish the effects of the inhibitors. Similar conclusions were reached by Burger & Lowenstein (1967), who studied the catabolism of AMP by heart homogenates. According to their results, AMP is degraded with production of IMP only if 2mM -ATP is added to the extracts. The opposite conclusion has been reached for skeletal-muscle AMP deaminase by taking account of its regulatory properties. During moderate contractile activity the enzyme is inhibited by ATP and is activated only during the performance of exhaustive work, when the increase of ADP concentration and the decrease of the tissue pH cancel the action of the inhibitors (Ronca-Testoni *et al.*, 1970). It should be emphasized that, as is reported here for the activatory effect of ATP on cardiac AMP deaminase, the inhibition exerted by this nucleotide on the skeletal enzyme is also strengthened by addition of physiological concentrations of Mg^{2+} (Ronca *et al.*, 1968) to reinforce the functional importance of these modulating mechanisms.

An activatory effect by ATP, similar to that described here for heart AMP deaminase, has been reported for the enzyme isolated from other tissues; in those cases, however, this kinetic property seems to have less physiological significance because of the prevalence of the effect of other modifiers. GTP completely overcomes the activation by ATP of the

Table 2. Effect of Mg^{2+} on sensitivity of AMP deaminase towards activators

Experimental conditions are described in the legend to Table 1.

Additions	Relative activity in the presence of indicated Mg^{2+} concn.			
	0	4mM	10mM	20mM
None	100	103	128	128
ADP ($50\mu\text{M}$)	155	113	125	118
ADP (1mM)	448	341	244	98
ATP ($50\mu\text{M}$)	300	95	95	79
ATP (1mM)	526	731	680	604

enzyme from bovine brain (Setlow *et al.*, 1966). Van den Berghe *et al.* (1977) have reported that, at the concentrations of metabolites physiologically present in liver, the activation by ATP is suppressed by the concomitant action of P_i and GTP. Similarly Lian & Harkness (1974) have demonstrated that in erythrocytes AMP deaminase is present at about one-quarter of its potential activity because of the inhibition exerted by 2,3-diphosphoglyceric acid on the ATP-activated enzyme.

The conviction that cardiac AMP deaminase is tonically active *in vivo* is supported by the determinations of the adenine nucleotide content of the heart reported by Rubio *et al.* (1973). These researchers reported that under normal conditions clamp-frozen heart extracts contain low concentrations of IMP (about 0.1 mM) and that the concentration of this nucleotide is not substantially altered even in the hypoxic heart, in which a large depletion of the total adenine nucleotide concentration takes place. The observed accumulation of inosine and hypoxanthine under ischaemic conditions indicates the activation of the adenosine pathway without modification of the AMP flow through the direct deaminating pathway.

The hypothesis of a tonic activation of AMP deaminase in the heart is in agreement with the literature, but the only paper available on the effects of adenine nucleotides on a partially purified preparation of heart enzyme is the paper by Chung & Bridger (1976), who reported a greater stimulation by ADP than by ATP. The discrepancy between their and our results cannot be resolved, because experimental details were not given.

Because of its importance as the direct source of adenosine and the possible role of adenosine in the regulation of coronary blood flow, AMP catabolism in the heart must be the object of further research. The most important topic in this respect is probably the activity of 5'-nucleotidase and the regulation of this enzyme in its intact membrane environment. It suffices here to remember that production of adenosine is triggered by relieving the inhibition of 5'-nucleotidase by ADP and ATP (Sullivan & Alpers, 1971). It is of special interest that the regulatory properties of this enzyme are opposite to those of AMP deaminase. This may represent another case of reinforced metabolic control.

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References

- Arch, J. R. S. & Newsholme, E. A. (1978) *Biochem. J.* **174**, 965-977
- Berne, R. M. (1963) *Am. J. Physiol.* **204**, 317-322
- Berne, R. M. & Rubio, R. (1974) *Circ. Res.* **34/35**, Suppl. 3, 109-118
- Bockman, E. L. (1977) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **36**, 792
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248-254
- Burger, R. & Lowenstein, J. M. (1967) *J. Biol. Chem.* **242**, 5281-5288
- Chung, L. & Bridger, W. A. (1976) *FEBS Lett.* **64**, 338-341
- Cobbin, L. B., Einstein, R. & Maguire, M. H. (1974) *Br. J. Pharmacol.* **50**, 25-33
- Coffee, C. J. & Solano, C. (1977) *J. Biol. Chem.* **252**, 1606-1612
- Conway, E. J. & Cooke, R. (1939) *Biochem. J.* **33**, 479-492
- Hemphill, R. M., Zielke, C. L. & Suelter, C. H. (1971) *J. Biol. Chem.* **246**, 7237-7240
- Katori, M. & Berne, R. M. (1966) *Circ. Res.* **19**, 420-425
- Lian, C. & Harkness, D. R. (1974) *Biochim. Biophys. Acta* **341**, 27-40
- Purzycka, J. (1962) *Acta Biochim. Pol.* **9**, 83-93
- Purzycka, J. & Zydowo, M. (1960) *Bull. Acad. Pol. Sci. Ser. Sci. Biol.* **8**, 483-484
- Raggi, A., Ronca-Testoni, S. & Ronca, G. (1969) *Biochim. Biophys. Acta* **178**, 619-622
- Raggi, A., Bergamini, C. & Ronca, G. (1975) *FEBS Lett.* **58**, 19-23
- Ronca, G., Raggi, A. & Ronca-Testoni, S. (1968) *Biochim. Biophys. Acta* **167**, 626-629
- Ronca-Testoni, S., Raggi, A. & Ronca, G. (1970) *Biochim. Biophys. Acta* **198**, 101-112
- Rubio, R., Berne, R. M. & Dobson, J. G., Jr. (1973) *Am. J. Physiol.* **225**, 938-953
- Setlow, B., Burger, R. & Lowenstein, J. M. (1966) *J. Biol. Chem.* **241**, 1244-1245
- Smiley, K. L., Jr. & Suelter, C. H. (1967) *J. Biol. Chem.* **242**, 1980-1981
- Smiley, K. L., Jr., Berry, A. J. & Suelter, C. H. (1967) *J. Biol. Chem.* **242**, 2502-2506
- Sullivan, J. M. & Alpers, J. B. (1971) *J. Biol. Chem.* **246**, 3057-3063
- Van den Berghe, G., Bronfman, M., Vanneste, R. & Hers, H. G. (1977) *Biochem. J.* **162**, 601-609
- Winder, W. W., Terjung, R. L., Baldwin, K. M. & Holloszy, J. O. (1974) *Am. J. Physiol.* **227**, 1411-1414