Regulatory properties of AMP deaminase isoenzymes from rabbit red muscle

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We examined the kinetic and regulatory properties of the two isoenzymes of red muscle AMP deaminase, forms A and B, corresponding respectively to the single isoenzymes present in the heart and white skeletal muscle. At the optimal pH value, 6.5, both enzymes show hyperbolic substrate-velocity curves and are inhibited by GTP, inducing sigmoid kinetics. An effect similar to that of GTP is exerted on form B by ATP, whereas form A is almost insensitive to this nucleotide. At pH 7.1 both enzymes follow sigmoid kinetics. ATP enhances the sigmoidicity of the substrate-velocity curve of form B, but it stimulates form A, reverting sigmoidal to hyperbolic kinetics shown by the enzyme at optimal pH. At pH 7.1, form A is also less sensitive to the inhibitory action of P_i and GTP. These results suggest that, owing to the presence of form A, AMP deamination occurs in red muscle also at moderate work intensity. A possible role of this process in counteracting the production of adenosine by 5'-nucleotidase is hypothesized.

INTRODUCTION

AMP deaminase (AMP aminohydrolase, EC 3.5.4.6) catalyses the deamination of AMP to generate IMP and ammonia. The physiological function of this enzyme remains unclear. However, in skeletal muscle, where it is present at a much higher concentration than in any other tissue, including cardiac and smooth muscle (Conway & Cooke, 1939), AMP deaminase may be assumed to play a significant role in the regulation of the relative concentrations of adenine nucleotides (Ronca-Testoni *et al.*, 1970; Chapman & Atkinson, 1973; Coffee & Solano, 1977).

The kinetic studies which followed the characterization of skeletal-muscle AMP deaminase as an allosteric regulated enzyme (Smiley & Suelter, 1967) have provided useful information about the nucleotides that may regulate the reaction *in vivo*. For a long time it has been believed that the regulatory properties of skeletal-muscle AMP deaminase were similar to those of the enzyme from other sources, in showing inhibition by guanine nucleotides and activation by adenine nucleotides (Zielke & Suelter, 1971). Our studies on the regulatory properties of skeletal-muscle AMP deaminase from five different species at a KCl concentration approximating to that found in muscle demonstrated a constant inhibitory effect by all nucleoside triphosphates, which was maximal at alkaline pH; since ADP was the most efficient metabolite in counteracting this inhibition, a decrease in the ATP/ADP ratio, together with a decrease in the tissue pH, has been suggested to be the stimulus for the activation of AMP deaminase in periods of intense muscular activity (Ronca-Testoni *et al.*, 1970).

The opposite conclusion has been reached for cardiac AMP deaminase. Indeed, the observation that this enzyme is stimulated by ATP, with a maximal effect at physiological pH, has led to the suggestion that it is always active under normoxic conditions (Barsacchi *et al.*, 1979).

Our previous distribution data showed that white muscles from different species deaminate AMP 4–10 times more effectively than do red muscles (Raggi *et al.*, 1969). More recently, we demonstrated that both

quantitative and qualitative dissimilarities exist between the preparations of AMP deaminase from the different types of striated muscle (Raggi *et al.*, 1975). By chromatography on cellulose phosphate of extracts of rabbit red muscles, two peaks of AMP deaminase activity are obtained (forms A and B), the position of the former corresponding to that of the single form present in the heart, and the second has the same chromatographic properties as the single form of the white muscles. These results have been confirmed by Ogawara *et al.* (1983), who also showed that the activities of forms A and B behaved electrophoretically as well as immunologically similarly to the activities of heart and white muscle enzymes respectively.

The observation that cardiac and skeletal-muscle AMP deaminases possess different regulatory properties stimulated our interest in studying the kinetic properties of the two forms of AMP deaminase that are present in red muscle. The results of the present paper give strong evidence of qualitative differences in the regulatory properties of these two isoenzymes which confirm the hypothesis that the variation in the kinetic properties of AMP deaminases from different types of striated muscle is due to the predominance of one or other of the two isoenzymes.

MATERIALS AND METHODS

Nucleotides and cellulose phosphate were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.); other chemicals were of reagent grade. Red muscle (anconeus, semitendinosus, soleus) dissected from the legs of four rabbits shortly after killing were freed of fat and tendons, pooled, finely minced and homogenized in a Waring Blendor with 5 vol. of ice-cold 90 mM-potassium phosphate (pH 6.5) containing 180 mM-KCl (buffer A). After stirring for 20 min, the homogenate was centrifuged for 20 min at 20000 g. After filtration through cheesecloth, the cellulose phosphate resin equilibrated with buffer A was added to the supernatant and, after standing for 90 min, the slurry was placed on a sintered-glass funnel and washed with

buffer A until the A_{280} of the effluent was lower than 0.1 and then with 5 mM-succinate/50 mM-Tris (pH 6.5) (buffer B) containing 0.45 M-KCl until the A_{280} of the effluent was lower than 0.05. The effluent of these two successive washings contained no AMP deaminase activity.

The cake was then transferred to a glass column (bed dimensions 2 cm $\times$ 7 cm) and eluted with 0.6 M-KCl in buffer B. Of the original AMP deaminase activity, 27% emerged as a single peak (form A), with a purification factor of 500. When the A_{280} of the effluent was lower than 0.01, 0.9 M-KCl in buffer B was applied to the cellulose phosphate column. In this way we obtained a second peak of AMP deaminase activity (form B), corresponding to 42% of the original activity and with a purification factor of 700. The specific activity of the two AMP deaminase isoenzymes, devoid of contamination by 5'-nucleotidase, adenylate kinase and adenosine deaminase, was 90 and 100 μ mol of AMP deaminated/min per mg of protein for forms A and B respectively in 50 mM-imidazole/HCl (pH 6.5)/100 mM-KCl (buffer C) containing 2 mM-AMP at 20 °C.

The AMP deaminase activity was determined spectrophotometrically at 265 or 285 nm; $\Delta\epsilon_{\text{nm}}$ values of 8.86 and 0.23 were used at 265 and 285 nm respectively for the calculation of the μ mol of substrate deaminated (Ronca *et al.*, 1968). The spectrophotometric determinations were carried out in a Zeiss PMQ II spectrophotometer equipped with a Zeiss TE-converter and a Varian G-2000 recorder. Protein concentration was determined by the micro-biuret method, with purified skeletal-muscle AMP deaminase as the reference protein.

RESULTS AND DISCUSSION

The aim of the present paper was to re-investigate the catalytic properties of the two isoenzymes present in red muscle and to compare them with those of heart and white-muscle AMP deaminases. By the purification procedure described in the Materials and methods section, we obtained the two isoenzymes with a specific activity about 70 times that of the AMP deaminase prepared from red muscle by Ogasawara *et al.* (1983). The kinetic investigations were performed with the two forms in 50 mM-imidazole/HCl at the required pH, containing 100 mM-KCl. This salt concentration, beside being in the physiological range, had been shown by preliminary experiments to be almost optimal for both isoenzymes assayed at physiological concentration of AMP (0.1 mM). In this discussion we assume the kinetic properties of white-muscle AMP deaminase as being represented by those described in the literature for rabbit skeletal-muscle AMP deaminase. The reason for this assumption arises from the low percentage of red muscle in the hind-leg tissue usually used as source for rabbit skeletal-muscle AMP deaminase; furthermore, kinetic experiments carried out with AMP deaminase extracted from the rabbit white muscle biceps femoris gave results very similar to those obtained with the enzyme prepared from the whole hind leg of the rabbit (A. Raggi, unpublished work).

Effect of pH on the activity

Like the cardiac and white-muscle AMP deaminase, both forms of red-muscle AMP deaminase display maximal activity at pH 6.5. Nevertheless, the concen-

Fig. 1. pH-dependence of the two forms of red muscle AMP deaminase

Activity was assayed in the presence of 0.1 mM-AMP in buffer C (see the Materials and methods section) at the indicated pH values. $\circ$, form A; $\bullet$, form B. The activity at pH 6.5 is taken as 100%.

tration of H^+ influences the activity of the two isoenzymes differently (Fig. 1). Whereas with form A a nearly symmetrical bell-shaped activity-versus-pH curve is obtained, form B shows a more abrupt decrease of activity on the alkaline side of the curve, these two behaviours closely resembling those of cardiac and white-muscle AMP deaminases respectively (Schmidt, 1928; Barsacchi *et al.*, 1979).

At pH 6.5 both red-muscle isoenzymes display almost hyperbolic saturation kinetics, with a Hill coefficient of 1.1 (Fig. 2). K_m values of forms A and B are 0.8 and 0.6 mM respectively, both being slightly higher than the reported value for white-muscle AMP deaminase (0.4 mM-AMP; Zielke & Suelter, 1971). These results show that all forms of skeletal-muscle AMP deaminase possess an affinity for substrate much higher than that of the cardiac enzyme ($K_m = 3\text{--}5$ mM; Barsacchi *et al.*, 1979; Purzycka-Preis *et al.*, 1978).

Kinetic studies by Ogasawara *et al.* (1983) showed that the K_m values for AMP of forms A and B were very similar to those of heart and white-muscle enzymes respectively. However, the K_m of the white-muscle enzyme (7.7 mM) was much higher than that of the heart enzyme (0.6 mM), this clearly being in contrast with the data in the literature (Zielke & Suelter, 1971; Raggi *et al.*, 1975; Barsacchi *et al.*, 1979). This discrepancy can be resolved with the particular assay conditions, 20 mM-maleate buffer (pH 6.9), used by Ogasawara *et al.* (1983). In fact, it has been shown that the activity of skeletal-muscle AMP deaminase is strongly influenced by anions and decreases with increasing numbers of negative charges of the anions (Ronca *et al.*, 1972).

The substrate-saturation plots of forms A and B at pH 7.1 are almost superimposable (Fig. 2), with a Hill coefficient of 1.6. This indicates that the inhibition caused by alkalization at low substrate concentration induces sigmoid kinetics in red-muscle AMP deaminases as well as in the enzyme from rat skeletal muscle (Ranieri-Raggi *et al.*, 1980) and rabbit white muscle (A. Raggi, unpublished work).

Fig. 2. Substrate-saturation curves of AMP deaminase isoenzymes at pH 6.5 and 7.1

Assays contained buffer C and the reported AMP concentrations at pH 6.5 (○, ●) or 7.1 (□, ■). ○, □, form A; ●, ■, form B.

Influence of effectors

At the optimal pH value of 6.5 (Fig. 3a), both red-muscle isoenzymes are almost insensitive towards ADP and display an inhibitory effect by GTP similar to that reported for white muscle as well as for cardiac AMP deaminase (Ronca-Testoni *et al.*, 1970; Tomozawa & Wolfenden, 1970; Barsacchi *et al.*, 1979). The two forms of AMP deaminase can be differentiated on the basis of their behaviour towards ATP, since form A is not affected by this nucleotide, whereas form B shows an inhibition by ATP which is partially diminished as the concentration of ATP is increased above 10 μ M. This biphasic behaviour with respect to ATP was observed by several authors with rabbit as well as with rat skeletal-muscle AMP deaminase (Tomozawa & Wolfenden, 1970; Ronca *et al.*, 1972; Wheeler & Lowenstein, 1979; Raggi & Ranieri-Raggi, 1979; Ranieri-Raggi &

Fig. 4. Effect of P_i on AMP deaminase isoenzymes

The reaction mixtures contained the reported P_i concentrations, 0.1 mM-AMP and buffer C at pH 6.5 (○, ●) or 7.1 (△, ▲). ○, △, form A; ●, ▲, form B.

Raggi, 1979). It has been shown that the extent of stimulation observed in the second phase depends on the K^+ concentration in the assay mixture. At low K^+ concentrations a stimulation above the zero-ATP value follows the inhibition observed in the first phase. At K^+ concentrations of the order of those required for complete activation of the enzyme, ATP inhibits all the skeletal AMP deaminases to a limited extent (Ronca-Testoni *et al.*, 1970; Ronca *et al.*, 1972). We have observed the same behaviour with form B of red muscle AMP deaminase, 60% inhibition being seen above 1 mM-ATP (results not shown).

Fig. 3. Effect of some nucleotides on AMP deaminase isoenzymes

The reaction mixtures contained buffer C at pH 6.5 (a) or 7.1 (b), 0.1 mM-AMP and the reported concentrations of ADP (○, ●), ATP (□, ■) or GTP (△, ▲). ○, □, △, form A; ●, ■, ▲, form B.

Table 1. Effect of pH and some modifiers on the kinetics of red muscle AMP deaminases

Experimental conditions are described in the legend to Fig. 2.

Additions	pH . . .	Hill coefficient of the substrate-saturation curve			
		Form A		Form B	
		6.5	7.1	6.5	7.1
None		1.1	1.6	1.1	1.6
ATP (50 μ M)		1.1	1.1	1.6	1.8
GTP (50 μ M)		2.0	2.1	2.2	2.8
P _i (2 mM)		1.3	1.6	1.6	1.8

To compare the regulatory properties of the two isoenzymes further, we have studied the influence of the nucleoside triphosphates at pH 7.1 (Fig. 3b). In this condition, ATP activates form A, whereas the inhibitory effect of GTP on the same enzyme is slightly decreased compared with that at pH 6.5. By analogy with white muscle AMP deaminase (Barsacchi *et al.*, 1979), alkalization causes an increase of the inhibition of form B by both nucleoside triphosphates.

The different influence of pH on the two forms of red muscle AMP deaminase is further clarified by Fig. 4, where the influence of P_i at pH 6.5 and 7.1 is presented. The inhibitory action of P_i on the two isoenzymes is almost the same at pH 6.5, and that on form B is slightly increased when the pH is shifted to 7.1. At this pH value the behaviour of form A is significantly different, since it shows a lack of inhibition by P_i, by analogy with what was observed with cardiac AMP deaminase (Barsacchi *et al.*, 1979).

Effect of pH on the kinetics of AMP deaminase in the presence of the effectors

In order to determine the effects of the modifiers on the kinetic parameters of the two isoenzymes, substrate-saturation curves have been obtained in the presence of each effector at pH 6.5 and 7.1. The results are presented in Table 1, which reports the Hill coefficients obtained from the curves. As far as form B is concerned, nucleoside triphosphates as well as P_i cause the hyperbolic substrate-velocity curve obtained at pH 6.5 in the absence of effectors to revert to sigmoidal curves, with Hill coefficients (*h*) ranging from 1.6 (in the presence of either P_i or ATP) to 2.2 (in the presence of GTP). The modifiers exert on the same enzyme a similar action at pH 7.1, where they increase the sigmoidicity of the substrate-saturation curve.

The results obtained with form A give evidence of a differential behaviour towards nucleoside triphosphates, since at either pH 6.5 or pH 7.1 the effect of GTP on enzyme kinetics is quite similar to that observed with form B, inducing sigmoid kinetics at pH 6.5 and enhancing the positive co-operativity observed at pH 7.1, whereas in the presence of ATP the enzyme follows hyperbolic kinetics at pH 6.5 as well as at pH 7.1 (*h* = 1.1), with *K_m* for AMP decreased to 0.3 mM. P_i

inhibits form A at pH 6.5 by decreasing the affinity of the enzyme for AMP (*K_m* = 1.0 mM) with only a slight alteration of the sigmoidicity (*h* = 1.3). At pH 7.1 the substrate-saturation curves of form A in the presence or absence of P_i were almost superimposable (results not shown).

The data of the present paper are essentially of enzymological value. Nevertheless, a potential physiological meaning of the results can be obtained, on the basis of two considerations. First, since the activity of both forms of skeletal-muscle AMP deaminase increases with decreasing pH and, as is well known, the pH of muscle decreases during contraction, the rate of deamination in red muscle should increase during exercise. Second, at alkaline pH, although the action of the inhibitors on form B is enhanced, form A is less sensitive to the action of the inhibitors and is stimulated by ATP, these effects being similar to those observed with the corresponding single forms present in white muscle and in the heart. This observation deserves consideration in the light of the report that in red muscle the accumulation of IMP, which was coincident with the decrease of ATP, was observed only during tetanic stimulation as fast-twitch fibres began to be recruited (Meyer *et al.*, 1980). Although the AMP deamination occurring in these circumstances may be ascribed to the activity of form B of AMP deaminase, the presence in red muscle of an ATP-activated AMP deaminase, as shown by the data of the present paper, could permit, even during work of low intensity (when predominately slow-twitch fibres are used), an AMP deamination with which the AMP regeneration catalysed by the other two enzymes of the purine nucleotide cycle, namely adenylosuccinate synthetase and adenylosuccinase, could keep pace (Lowenstein, 1972).

Regarding the physiological role in red muscle of the purine nucleotide cycle, one possibility is that, similarly to what has been suggested for cardiac muscle (Barsacchi *et al.*, 1979), it may function to decrease the rate of adenosine production under normoxic conditions by subtracting AMP from 5'-nucleotidase. In fact, it has been reported that in cat soleus muscle adenosine production is stimulated only in the presence of some degree of hypoxia, a positive correlation being found between adenosine and lactate formation (Bockman & McKenzie, 1979). It is then possible that in red muscle adenosine exerts its role as mediator of hyperaemia (Bockman *et al.*, 1976) when the ATP concentration falls and stimulation of form A of AMP deaminase is consequently released. On the other hand, the activation of form B of AMP deaminase occurring under these conditions operates to keep adenosine at low concentrations. Considering that red muscles are primarily slow, but contain a small proportion of fast-twitch fibres (Peter *et al.*, 1972), it appears that further studies to establish the ratio of the two forms of AMP deaminase in each fibre type may provide an index of the rate of adenosine production under conditions that cause the muscle to fatigue.

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