REGULATION OF SKELETAL MUSCLE AMP DEAMINASE

Effects of limited proteolysis on the activity of the rabbit enzyme

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1. Introduction

The physiological function of skeletal muscle AMP deaminase is not clearly defined. However, following its characterization as an allosteric regulated enzyme [1,2], the kinetic properties of AMP deaminase have been extensively investigated. This has provided useful information about the factors which affect AMP deaminase activity and which may regulate the relative concentrations of adenine nucleotides 'in vivo'. Nucleoside di- and triphosphates, inorganic phosphate, creatine phosphate and several other organic phosphate compounds, in the concentration range found in muscle, have a profound influence on AMP deaminase activity at substrate concentrations lower than K_m suggesting that they may play a significant regulatory role. It should be emphasized, however, that the effect of all these metabolites is strongly dependant on KCl concentration [3–5]. For example, the effects of ADP and GTP, which, respectively, are the most efficient activator and inhibitor of AMP deaminase, disappear when KCl is raised to > 200 mM [4]. At < 100 mM KCl, < 5 μM ATP inhibits the enzyme, while > 5 μM activates it [4,6]. At > 100 mM KCl, ATP is an inhibitor at all concentrations [3,4]. Attempts to correlate the state of aggregation of AMP deaminase with the kinetic parameters of the enzyme in the presence of nucleotide effectors have up to now been unsuccessful [7]. It is therefore not possible to propose a plausible scheme for the regulation of the enzyme as long as the possible interpretations of the effect of each modifier remain unclear. Here we report the influence of limited proteolysis on the activity of rabbit skeletal muscle AMP deaminase. After incubation with trypsin the enzyme activity is shown no longer dependant on KCl concentration. The effect of proteolysis on the regulatory properties of AMP deaminase enables us to distinguish between the binding sites for GTP and adenine nucleotides.

2. Materials and methods

Rabbit muscle AMP deaminase was purified by cellulose phosphate chromatography essentially as in [8], but eluting the enzyme with 1.0 M KCl after a previous elution with 0.6 M KCl (pH 7.0) which yielded one small peak of lower specific AMP deaminase activity attributable to the isoenzyme present in red muscle [9]. The specific activity of the enzyme was 1200 μmol AMP deaminated.min^{-1}.mg protein^{-1} at 20°C in 50 mM imidazole–HCl (pH 6.5), 60 mM KCl and 2 mM AMP.

Limited proteolysis of AMP deaminase was performed at 20°C in a reaction mixture containing 0.5 mg/ml AMP deaminase, 5 μg/ml trypsin and 1 M KCl adjusted to pH 7.0 with 1 M K_2HPO_4. At intervals samples of the incubation mixture were removed and immediately tested for catalytic activity using a 1000-fold dilution in 50 mM imidazole–HCl (pH 6.5), 0.1 mM AMP and 2 mM or 60 mM KCl. Of the digestion mixture, 50 μl were taken at 0 and 30 min incubation and added to an equal volume of 1 mM phenylmethyl sulfonyl fluoride. The two samples were then denatured in sodium dodecylsulphate and examined by gel electrophoresis as in [10]

in 0 1 M Tris/0 1 M Bicine [*N,N*-bis-(2-hydroxyethyl)-glycine] (pH 8 3) For the molecular weight determination, the following standards were run phosphorylase (94 000), transferrin (77 000), bovine serum albumin (66 500), glutamate dehydrogenase (53 000), ovalbumin (44 000), chymotrypsinogen (25 700) For the kinetic experiments, the trypsin-treated enzyme was prepared by incubating AMP deaminase with trypsin as above for 25 min and terminating the reaction by lowering the temperature of the incubation mixture to 0°C During further 24 h incubation at this temperature the specific activity and regulatory properties of AMP deaminase did not vary The kinetic studies with treated AMP deaminase reported here were accomplished within 1–2 h from the start of the treatment

Aliquots of the incubation were diluted 1000-fold in the assays at 20°C containing 0 2 mM phenylmethyl sulfonyl fluoride to inhibit the small quantity of trypsin present Trypsin and the trypsin inhibitor did not have any effect on initial velocity of native AMP deaminase in the various assay conditions adopted AMP deaminase activity was routinely determined spectrophotometrically as in [11] AMP, ATP, phenylmethyl sulfonyl fluoride, Bicine, trypsin, transferrin, albumin and chymotrypsinogen were purchased from Sigma Chemical Co ADP, GTP, phosphorylase and glutamate dehydrogenase were from Boehringer.

3 Results

When 0 1 mM AMP is used in the assay mixture, the activity of rabbit skeletal muscle AMP deaminase at 60 mM KCl is ~14-fold higher than that at 3 mM KCl (fig 1) When the enzyme is incubated with trypsin at a ratio of 1/100 (trypsin/AMP deaminase, w/w) as in section 2, a rapid activation of the enzyme at 3 mM KCl is observed (fig 1) Within 20 min the activity reaches a level of 15-fold of the original and remains constant during further incubation The activity at 60 mM KCl is only slightly increased by trypsin so that after 20 min incubation the activities at 3 mM and 60 mM KCl of the treated enzyme do not noticeably differ This observation may be related to the nature of the activation of AMP deaminase by trypsin which is due to the transition of the enzyme to a form exhibiting

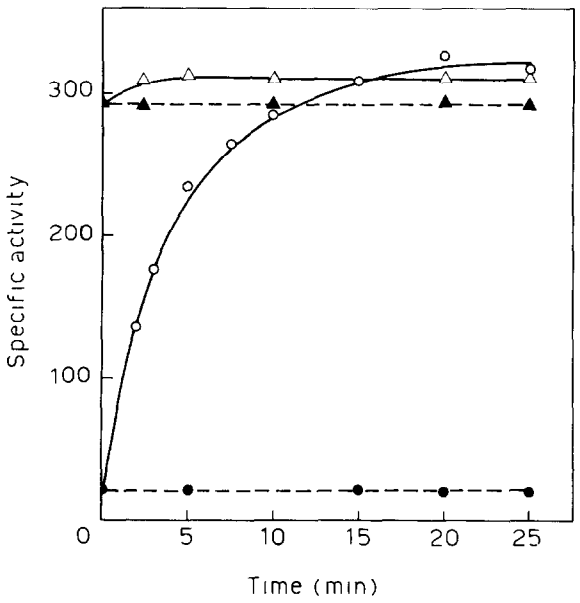

Fig 1 Effect of trypsin on the activity of AMP deaminase The enzyme (0 5 mg/ml) was incubated with 5 μg/ml trypsin for the indicated times and assayed for the catalytic activity at 0 1 mM AMP in the presence of 3 mM (○) or 60 mM (△) KCl The dotted lines show control incubations which did not contain trypsin

hyperbolic kinetics even at 3 mM KCl, where the native enzyme shows a sigmoidal substrate velocity curve (fig 2A) Hill plots of the data (not shown) had slopes of 1 0 and 2 9 (native and digested enzyme, respectively) Since both native and trypsin-treated enzymes follow hyperbolic kinetics at 60 mM KCl, at this salt concentration no clear differences are observed between their activities at low concentrations of substrate (fig 2B) From fig 2 it can also be seen that higher AMP concentrations have a different effect on the activity of the two enzymes At both salt concentrations the V reached by the treated enzyme is ~20% lower than the native enzyme V

The influence of proteolysis on the regulatory properties of AMP deaminase was also investigated The effect of increasing concentration of ADP, ATP and GTP on the activity of both native and trypsin-treated enzymes was studied in the presence of 3 mM KCl At this salt concentration ADP markedly activates the native enzyme while the trypsin-treated enzyme shows an almost complete loss of sensitivity towards this nucleotide (fig 3) It follows that in the

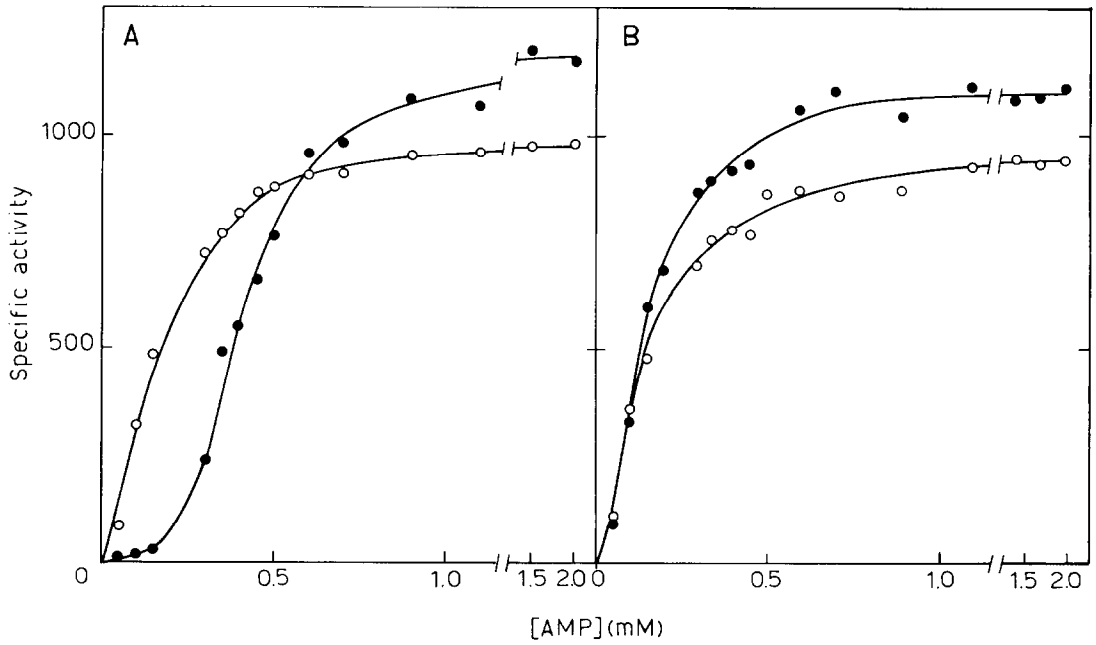

Fig.2. Substrate versus velocity curves of native and trypsin-treated AMP deaminases. The reaction mixture contained 50 mM imidazole–HCl (pH 6.5), the reported AMP concentrations and 3 mM (A) or 60 mM (B) KCl. (•) Native enzyme; (○) trypsin-treated enzyme.

presence of ADP at > 20 μM the specific activity of the native and the treated enzyme are almost the same even at this low KCl concentration. After the limited proteolysis of the enzyme the effect of ATP on AMP deaminase is also markedly modified. As shown by fig.4, ATP exerts a biphasic effect on the native enzyme at 3 mM KCl; ⩽ 5 μM ATP acts as an inhibitor, while ⩾ 5 μM ATP activates the enzyme. The treated enzyme is no longer inhibited by low concentrations of ATP, while the activation by higher concentrations of this nucleotide is markedly reduced. The inhibitory effect of GTP on AMP deaminase appears to be unmodified after proteolysis (fig.5). Inhibition of ~85% is obtained when both native and treated enzymes are assayed in the presence of increasing concentrations of GTP.

Additional experiments (not shown) were performed at 60 mM KCl, where ADP exerts no activation effect on the native enzyme but removes the inhibitory effect by ATP. At this salt concentration the trypsin-treated enzyme revealed a complete loss of sensitivity towards both ADP and ATP. In the

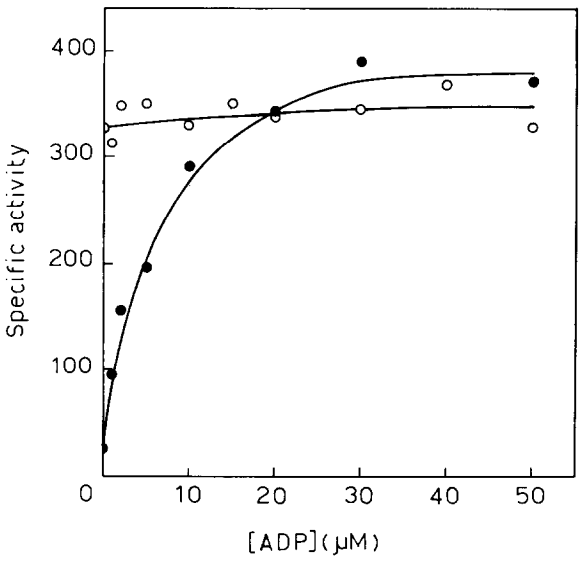

Fig.3. Effect of ADP on the activity of native and trypsin-treated AMP deaminases. The reaction mixture contained 50 mM imidazole–HCl (pH 6.5), 3 mM KCl and 0.1 mM AMP. (•) Native enzyme; (○) trypsin-treated enzyme.

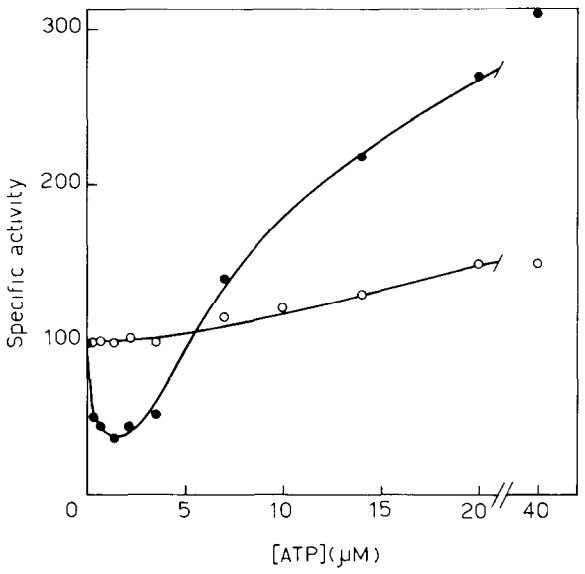

Fig 4 Activity of native and trypsin-treated AMP deaminases as a function of ATP concentration The reaction mixture contained 50 mM imidazole–HCl (pH 6 5), 3 mM KCl and 0 1 mM AMP (•) Native enzyme, (○) trypsin-treated enzyme

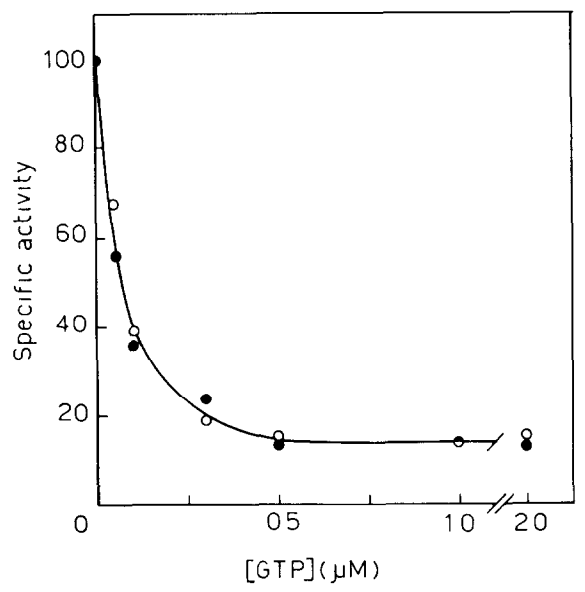

Fig 5 Activity of native and trypsin-treated AMP deaminases as a function of GTP concentration The reaction mixture contained 50 mM imidazole–HCl (pH 6 5), 3 mM KCl and 0 1 mM AMP (•) Native enzyme, (○) trypsin-treated enzyme

same conditions the inhibitory properties of GTP with the digested enzyme were quite similar to those shown with the native enzyme As a first attempt to elucidate the molecular basis of the observed activation of AMP deaminase by trypsin, we compared the gel electrophoretic pattern of AMP deaminase before and after the limited tryptic digestion Rabbit skeletal muscle AMP deaminase is considered to contain 4 identical subunits with molecular weight ranging from 69 000–79 000 [7,12] In our hands, gel electrophoresis of the native enzyme in the presence of sodium dodecyl sulphate (fig 6) gave rise to a major protein band of mol wt 79 000 ± 2000, termed α, and a minor band of mol wt ~73 000, termed α The last component only occurred in trace amounts, but it was always present in all enzyme preparations After 30 min of incubation with trypsin the original 79 000 subunit disappeared and only one band of mol wt 72 000 ± 2000 was found (fig 6)

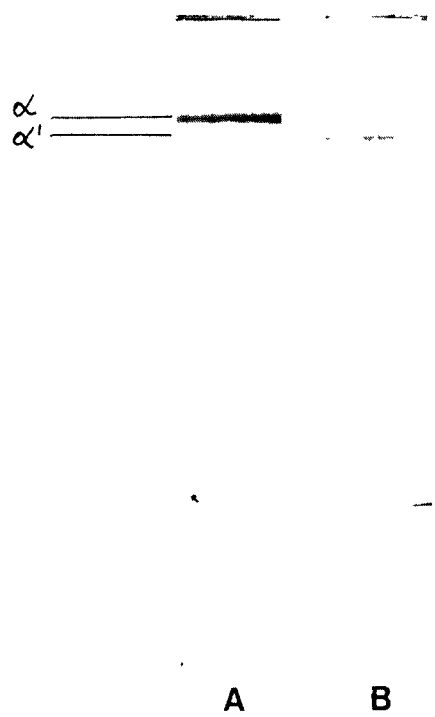

Fig 6 Sodium dodecyl sulphate gel electrophoresis of native and trypsinized AMP deaminase Native (A) and trypsin-digested AMP deaminase (B) were subjected to 10% polyacrylamide gel electrophoresis as in section 2

4. Discussion

Skeletal muscle AMP deaminase at substrate concentration lower than K_m (0.4–0.5 mM AMP [1,2]) is activated by monovalent cations, the most effective being K^+ [8,13]. This activation causes the sigmoid substrate–velocity curve shown by the enzyme in the absence of salt to revert to a hyperbolic one [1,4,5]. The same effect can be achieved by adding ADP to the enzyme in the presence of KCl concentrations lower than optimal [4,5]. We have shown here that on incubation with trypsin a conversion of skeletal muscle AMP deaminase occurs to a form exhibiting hyperbolic kinetics even at low K^+ concentration and in the absence of ADP. As shown by sodium dodecylsulphate gel electrophoresis, this effect results from limited proteolysis of the enzyme subunit, which is degraded to a product of similar size to the minor contaminant of native AMP deaminase. Since, at saturating substrate concentrations, the specific activity of the digested enzyme is $\leqslant 20\%$ lower than that of the native enzyme, a plausible interpretation of the kinetic data is that trypsin, in the conditions adopted here, irreversibly removes from AMP deaminase a fragment that possesses a regulatory function but appears to bear little importance in the formation of the enzyme catalytic center. According to this hypothesis the loss of sensitivity towards inhibition by ATP shown by AMP deaminase after trypsin treatment suggests that the adenine nucleoside di- and tri-phosphates presumably bind at a common regulatory site (or sites) on which they act antagonistically. This is particularly significant since on the basis of kinetic studies it has been suggested that the most important factor in the regulation of skeletal muscle AMP deaminase is the removal of ATP inhibition which occurs in muscle under conditions of strong tetanic contractions when ADP accumulates and the energy charge falls [3,5].

That no noticeable change in AMP deaminase sensitivity towards GTP inhibition is observed after the trypsin treatment also deserves consideration. In fact the observation of a quite different behaviour of the digested enzyme towards GTP and ATP strongly suggests that these nucleotides bind to different sites in rabbit skeletal muscle AMP deaminase. This is in good agreement with the observation that 3.8 binding sites for ATP but only 2.0 for GTP exist in the enzyme and *p*-mercuribenzoate selectively abolishes binding of GTP [6].

We have shown [11,14] that the inhibitory effects of ATP and GTP on rat skeletal muscle AMP deaminase are qualitatively different and we related the kinetic results to marked quantitative differences in the binding of periodate-oxidized ATP and GTP to the enzyme. In fact we found 3.2 binding sites for oxidized ATP, with an app. K_d 86 μM, but only 1.9 for oxidized GTP, with an app. K_d 6.8 μM.

Acknowledgements

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References

[1] Smiley, K. L., jr and Suelter, C. H. (1967) J. Biol. Chem. 242, 1980–1981.
[2] Ronca, G., Raggi, A. and Ronca-Testoni, S. (1968) Biochim. Biophys. Acta 167, 626–629.
[3] Ronca-Testoni, S., Raggi, A. and Ronca, G. (1970) Biochim. Biophys. Acta 198, 101–112.
[4] Ronca, G., Raggi, A. and Ronca-Testoni, S. (1972) Ital. J. Biochem. 21, 305–321.
[5] Coffee, C. J. and Solano, C. (1977) J. Biol. Chem. 252, 1606–1612.
[6] Tomozawa, Y. and Wolfenden, R. (1970) Biochemistry 9, 3400–3404.
[7] Ashman, L. K. and Atwell, J. L. (1972) Biochim. Biophys. Acta 258, 618–625.
[8] Smiley, K. L., jr, Berry, A. J. and Suelter, C. H. (1967) J. Biol. Chem. 242, 2502–2506.
[9] Raggi, A., Bergamini, C. and Ronca, G. (1975) FEBS Lett. 58, 19–23.
[10] Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406–4412.
[11] Raggi, A. and Ranieri-Raggi, M. (1979) Biochim. Biophys. Acta 566, 353–361.
[12] Boosman, A. and Chilson, O. P. (1976) J. Biol. Chem. 251, 1847–1852.
[13] Ronca-Testoni, S., Ranieri, M., Raggi, A. and Ronca, G. (1970) Ital. J. Biochem. 19, 262–276.
[14] Ranieri-Raggi, M. and Raggi, A. (1976) Biochim. Biophys. Acta 445, 223–233.