

## INTERACTION OF RAT MUSCLE AMP AMINOHYDROLASE WITH CHELATING AGENTS AND METAL IONS

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Received 10 July 1970

### 1. Introduction

AMP deaminase (AMP aminohydrolase, EC 3.5.4.6) from rat skeletal muscle behaves as a regulated enzyme. It is activated by some monovalent cations, the cation-activated enzyme is inhibited by nucleoside triphosphates, creatine phosphate and phosphate; ADP removes the inhibition by these compounds [1]. Some organic anions as lactate, acetate and citrate are also inhibitors. In the presence of citrate the enzyme becomes insensitive to the nucleoside triphosphate inhibition [2].

Recently Zielke and Suelter [3, 4] reported that rabbit AMP deaminase which showed similar kinetic and regulatory properties to the rat enzyme [1, 2, 5, 6] was a metallo protein containing  $Zn^{2+}$ . This paper gives evidence that  $Zn^{2+}$  is a firmly bound component (2 atoms per mole of enzyme) of the rat AMP deaminase and is essential for the enzyme activity. The metal ion can be dissociated from the protein by incubation with chelating agents; some enzyme effectors affect the rate of inactivation by chelating compounds. The inactive apoenzyme can be reactivated by the addition of  $Zn^{2+}$ . Some other bivalent metal ions can also partially reactivate AMP deaminase.

### 2. Materials and methods

Homogeneous rat muscle AMP deaminase was prepared essentially by the method of Smiley et al.

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[6] for the purification of rabbit enzyme. The specific activity of the homogeneous enzyme was 1120  $\mu$ moles of AMP deaminated per min per mg of protein at 20° in 50 mM imidazole-HCl (pH 6.5), 100 mM KCl and 2 mM substrate.

Metal analysis was performed by atomic absorption spectrophotometry using a Perkin-Elmer 303 spectrophotometer. The procedure suggested by Fuwa et al. [7] for the determination of metals in proteins was followed. The buffer used for the metal analysis and for the preparation of the apoenzyme was freed of metals by extraction with 0.003% dithizone in redistilled  $CCl_4$ . Before metal analysis the enzyme (3–5 mg/ml) was dialyzed for 36 to 48 hr against 30 mM potassium phosphate (pH 6.5) containing 1 M KCl; the buffer used for dialysis was also analysed. Precautions to prevent contamination by adventitious metal ions were taken as described by Thiers [8]. The enzyme activity was routinely determined spectrophotometrically following the decrease in absorbancy at 265 nm according to Kalckar [9]. The assay mixture contained 50 mM imidazole-HCl (pH 6.5), 150 mM KCl and 0.1 mM AMP; when it was necessary dithizone-treated assay mixtures were used.

### 3. Results

When rat AMP deaminase is incubated with 1 mM EDTA at pH 6.5, the activity is lost at a first order rate (fig. 1). The rate of inactivation strongly depends on potassium phosphate and KCl concentration in the incubation mixture; phosphate ( $P_i$ ) and KCl are enzyme effectors [1, 2]. 50% inactivation is observed after 22,

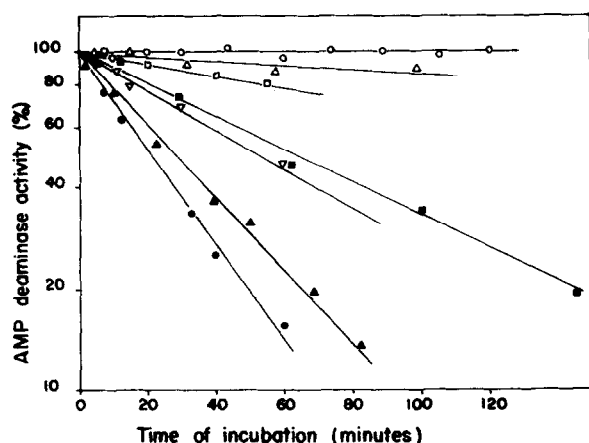


Fig. 1. Inactivation of AMP deaminase by EDTA as a function of time. The enzyme ( $2 \times 10^{-7}$  M) was incubated at  $20^\circ$  in 50 mM imidazole-HCl (pH 6.5), 1 mM EDTA and 180 mM ( $\Delta$ ) or 530 mM ( $\blacksquare$ ) KCl or 2 mM ( $\bullet$ ), 4 mM ( $\nabla$ ) or 22 mM ( $\square$ ) potassium phosphate (pH 6.5). Controls ( $\circ$ ) without EDTA with 2 mM, 4 mM or 22 mM potassium phosphate or with 530 mM KCl; control ( $\triangle$ ) without EDTA with 180 mM KCl. For the assay, aliquots were removed at the time indicated and immediately diluted 200 fold into the assay mixture described under Materials and methods.

50 and 185 min in the presence of 2 mM, 4 mM and 22 mM potassium phosphate, respectively; the last value is obtained by extrapolation. With 180 mM and 530 mM KCl the 50% inactivation is obtained after 27 and 64 min, respectively.

In fig. 2 the effect of the enzyme incubation with 1,10-phenanthroline (OP) is reported. It must be pointed out that during the enzymatic assay the OP-treated AMP deaminase slowly reactivates. For this reason the initial velocity and the maximal activity reached during the assay are reported in the figure; in both cases the loss of the activity does not follow a first order rate. If, however, 5  $\mu$ M EDTA is added to the assay mixture or if a dithizone-treated assay mixture is used, no reactivation is observed during the assay and a straight line is obtained when the log (percentage of AMP deaminase activity remaining) is plotted against time. The reactivation is probably due to the metal ion contamination of the assay mixture. Reactivation is not observed with the EDTA-treated enzyme, however in this case, due to the 200-fold dilution of the incubation mixture in the assay

medium, 5  $\mu$ M EDTA instead of 5  $\mu$ M OP is present during the assay of the enzyme activity; 5  $\mu$ M EDTA or OP in the assay does not affect the activity of the native enzyme.  $P_i$  and KCl also protect the enzyme from OP inactivation.

The rate of EDTA and OP inactivation in the presence of other enzyme effectors was also investigated. In fig. 3 the effect of ADP, ATP, GTP and pyrophosphate ( $PP_i$ ) is reported. ADP (40  $\mu$ M) protects from EDTA inactivation; ATP at the same concentration is without effect, while  $PP_i$  (0.5 mM) and GTP (16  $\mu$ M) increase the rate of inactivation. In the absence of EDTA these compounds do not inactivate the enzyme. The increasing inactivation rate determined

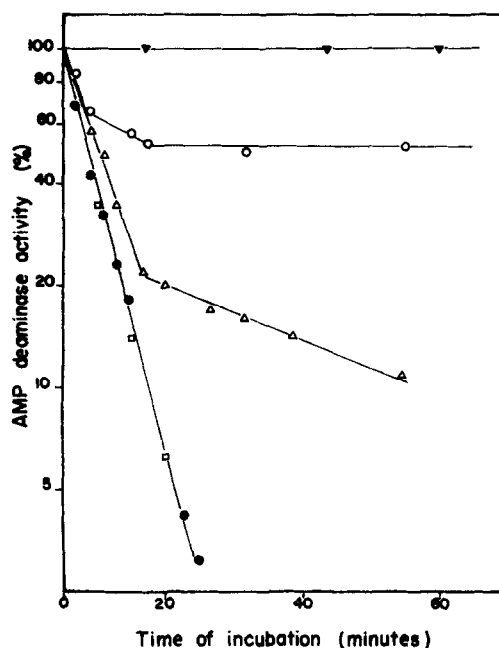


Fig. 2. Inactivation of AMP deaminase by 1,10-phenanthroline as a function of time. The enzyme ( $2 \times 10^{-7}$  M) was incubated at  $20^\circ$  in 50 mM imidazole-HCl (pH 6.5) and 530 mM KCl. Control without chelating agent ( $\nabla$ ); 1 mM 1,10-phenanthroline (initial velocity) ( $\Delta$ ); 1 mM 1,10-phenanthroline (maximal velocity reached during the assay; see the text) ( $\circ$ ); 1 mM 1,10-phenanthroline (assayed in dithizone-treated assay mixtures) ( $\square$ ); 1 mM 1,10-phenanthroline (5  $\mu$ M EDTA in the assay mixture) ( $\bullet$ ). The enzymatic activity was assayed as described under fig. 1; 5  $\mu$ M EDTA in the assay mixture does not affect the assay of the native enzyme.

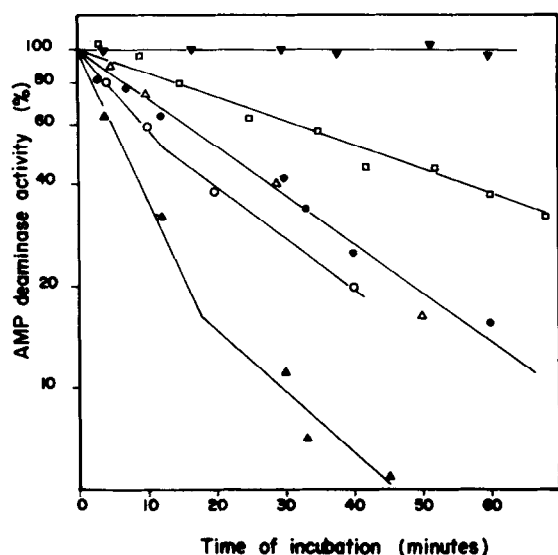


Fig. 3. Inactivation of AMP deaminase by EDTA as a function of time in the presence of enzyme effectors. The enzyme ( $2 \times 10^{-7}$  M) was incubated at  $20^\circ$  in 50 mM imidazole-HCl (pH 6.5), 1 mM EDTA and 2 mM potassium phosphate (pH 6.5) (●); 40  $\mu$ M ADP added (□); 40  $\mu$ M ATP added (△); 0.5 mM potassium pyrophosphate (pH 6.5) added (○); or 16  $\mu$ M GTP added (▲). Control samples containing the enzyme effectors without EDTA (▼). The enzymatic activity was assayed as described under fig. 1.

by GTP cannot be due to the increasing concentrations of the chelating compounds in the incubation mixture: nucleotides bind metal ions but the concentration of GTP is low in comparison to that of EDTA. Furthermore ATP and  $PP_i$  at concentrations higher than GTP have no little effect. It must be noted that GTP and ATP both inhibit the enzyme to a limited extent and seem to bind at the same site, however they inhibit the enzyme to a different extent [1].

The metal analysis carried out on a number of different enzyme preparations gives a value of  $1.95 \pm 0.2$  atoms of  $Zn^{2+}$  per mole of enzyme (M.W. 290,000); traces of  $Cu^{2+}$  (0.06 atoms per mole of protein) are also present. Dialysis of the enzyme against EDTA (1 mM) or OP (1 mM) results in a concomitant loss of zinc and of enzymatic activity. An instantaneous and complete reactivation is observed when the enzyme inactivated by chelating agents is treated with zinc. Partial reactivations are obtained with  $Cu^{2+}$ ,  $Co^{2+}$ ,  $Mn^{2+}$  and  $Fe^{2+}$ ; in contrast  $Mg^{2+}$ ,  $Ca^{2+}$ ,  $Ni^{2+}$  and

Table 1  
Reversal of EDTA inactivation of rat muscle AMP deaminase by metal ions.

Metal ion	Relative activity in the presence of the following metal ion concentrations:		
	5 $\mu$ M	10 $\mu$ M	25 $\mu$ M
$Zn^{2+}$	78	98	72
$Mn^{2+}$	25	31	26
$Cu^{2+}$	29	7	4
$Fe^{2+}$	25	19	13
$Co^{2+}$	20	25	25
$Mg^{2+}$	1	1	1
$Ca^{2+}$	1	1	1
$Ni^{2+}$	1	1	1
$Cd^{2+}$	1	1	1

The enzyme ( $1.5 \times 10^{-7}$  M) was incubated with 1 mM EDTA in 50 mM imidazole-HCl (pH 6.5), 530 mM KCl and 0.5 mM  $\beta$ -mercaptoethanol for 5 hr, then diluted 400 fold into the assay mixture containing metal ions at the concentrations reported in the table. The metal ions were added as chloride except  $Fe^{2+}$  which was added as sulphate. The assay mixture is described under fig. 1. An arbitrary value of 100 is assigned to the activity of the enzyme not treated with EDTA; the activity of the EDTA-treated enzyme without addition of metals was 1% of the original activity.

$Cd^{2+}$  fail to restore the activity to any measurable extent (table 1). Similar results are obtained with the apoenzyme prepared by dialysis against 1 mM EDTA and then against 50 mM imidazole-HCl (pH 6.5), 1 M KCl and 1 mM  $\beta$ -mercaptoethanol. From table 1 it also results that  $Zn^{2+}$ ,  $Cu^{2+}$  and  $Fe^{2+}$  concentrations higher than those required for maximal reactivation inhibit. Inhibition by these ions is observed also with native enzyme.  $Co^{2+}$  does not show this effect.

#### 4. Discussion

AMP deaminase from rat skeletal muscle can be classified as a metallo enzyme [10]. In fact it contains 2 atoms of zinc per mole of protein which are bound firmly enough to remain associated with the enzyme during the purification process; zinc is reversibly removed from the protein with concomitant loss and

restoration of the enzymatic activity. As observed with other metallo enzymes [10] the metal binding site has not an absolute specificity for zinc; the apoenzyme can be also reactivated by  $\text{Co}^{2+}$ ,  $\text{Fe}^{2+}$ ,  $\text{Cu}^{2+}$  and  $\text{Mn}^{2+}$ , however with these ions the activity is lower than that of the zinc-reactivated enzyme.  $\text{Co}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Fe}^{2+}$ , but not  $\text{Cu}^{2+}$ , partially reactivate AMP deaminase from rabbit skeletal muscle [4].

Although the results show the necessity of zinc for the AMP deaminase activity, its participation to the constitution of the active site remains to be demonstrated. The metal ions, in fact, besides a catalytic role can have a structural role [10, 11]; the possibility that the zinc ion is bound to regulatory site(s) may be also considered.

Of interest is the variation of the inactivation rate by chelating agents determined by some enzyme effectors, either activators as ADP and KCl or inhibitors as phosphate and GTP. The protection of alkaline phosphatase from *E. coli* by *p*-nitrophenyl-phosphate and phosphate (a substrate and a competitive inhibitor) against inactivation by chelating agents has been reported [12, 13]. On the basis of spectral changes observed by adding phosphate to Co-alkaline phosphatase, it has been also suggested that phosphate might be bound to the catalytically active metal ion [11]. Although the binding of the effectors to metal ion might explain the protection, other mechanisms must be considered, at least in the case of AMP deaminase, e.g. the binding of effectors to sites topologically distinct from metal site. In fact the effectors can modify the catalytical properties of AMP deaminase inducing conformational changes and such modifications might be responsible also for the variations of the inactivation rate by chelating agents.

The active AMP deaminases containing transition metals with more favorable probe characteristics than zinc [14] will be useful for the further studies on the role of metal in this enzyme.

### Acknowledgement

This work was supported by Italian C.N.R.

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