Renal Aminoacylase, a Zinc Enzyme

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Aminoacylase, Zinc Content, Zinc Dissociation Constant

Renal aminoacylase is inactivated by dialysis against metal complexing agents such as *o*-phenanthroline. Activity can be restored by addition of zinc ions. A zinc dissociation constant of about 10^{-10} M at pH 7.8 is obtained by titration of the enzyme with a metal ion buffer. The reactivity of the SH groups of the enzyme is considerably affected by zinc ions. The enzyme contains two essential zinc ions per molecule.

In 1952 Greenstein and coworkers [1] observed that the activity of renal aminoacylase is influenced by Co^{2+} and that the effects varied with the nature of the substrate: an activation as well as an inhibition was observed depending on the structure of the N-acylamino acids. These findings were confirmed in a later publication [2] which revealed also that the enzyme is active in the absence of Co^{2+}; no Co^{2+} could be detected in enzyme preparations which were isolated under Co^{2+} free conditions. The question wether aminoacylase is a real metallo enzyme or if the metal is only an effector which varies the activity of the metal free enzyme remained obscure. Within the scope of our investigations on the catalytic mechanism and the physiological function of aminoacylase [3–6], we have also studied the effects of metal ions on the activity of the enzyme. In the present communication we report the results of these experiments, which have shown that aminoacylase is a zinc containing metallo enzyme.

Materials and Methods

Pig kidney aminoacylase was a gift of Boehringer Mannheim. The enzyme (spec. activity about 34 U/mg protein) was purified by chromatography on Sephadex G 150 and DEAE cellulose up to a spec. activity of 250 U/mg protein [3]. The purified enzyme was homogeneous as judged by disc gel electrophoresis and immunoelectrophoresis.

Buffer substances, ethylenediamine tetraacetate (EDTA), nitrilotriacetic acid (NTA), *o*-phenanthroline, dithizone were from Merck, Darmstadt. Ellman's reagent was from Serva, Heidelberg. Sephadex G 25 was from Pharmacia, Frankfurt. N-chloroacetyl-L-alanine was synthesized according to Greenstein *et al.* [7].

Activity measurements were performed spectrophotometrically with a Zeiss PMQ II or a Beckman Acta III spectrophotometer. Hydrolysis of N-chloroacetyl-L-alanine was followed at 238 nm.

Reactivation of metal free inactive aminoacylase in a zinc ion buffer

Aminoacylase (3 mg/ml) was dialzyed at 4 °C against a 0.1 M Tris buffer pH 7.8 containing 1 mM *o*-phenanthroline. After two days *o*-phenanthroline was removed by filtration through a Sephadex G 25 column, equilibrated with 0.1 M Tris buffer pH 7.8 which was 10 mM in NTA. The metal free enzyme was diluted 50 fold with Tris buffer and 2 ml aliquotes were equilibrated with definite concentrations of Zn^{2+} ions maintained by a zinc ion buffer as described by Cohen and Wilson [8]. After 60 min at room temperature the activity was measured as described above.

Inactivation of aminoacylase by metal chelating agents.

The enzyme was incubated at 0 °C in a 0.1 M Tris buffer pH 7.8 with 1 mM *o*-phenanthroline or 10 mM EDTA or 10 mM NTA respectively. After appropriate time intervals the activity was measured in 1 ml aliquotes after addition of 50 μl 0.2 M N-chloroacetyl-L-alanine in the same buffer. The activity of the controls in the absence of chelating agents was considered to be 100%.

Effect of zinc ions on the reactivity of the SH-groups of aminoacylase with Ellman's reagent [9]

The reaction of aminoacylase with Ellman's reagent in the presence and absence of zinc was performed as described in ref. 5. For the removal of zinc ions the enzyme was dialyzed against *o*-phenanthroline.

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Zinc analysis of aminoacylase

The enzyme was dialyzed against a 0.1 M phosphate buffer pH 6.5 which was deprived of metal ions by extraction with dithizone. The protein concentration was adjusted to about 0.5 mg/ml. The zinc concentration was determined by atomic absorption spectrophotometry with a Perkin-Elmer 300 SG photometer equipped with graphite cell accessory HGA 70.

Results and Discussion

The zinc requirement of aminoacylase

The activity of aminoacylase depends on the presence of metal ions. This conclusion may be drawn from inactivation experiments with metal chelating agents such as *o*-phenanthroline, EDTA or NTA. The results of a typical experiment are given in Fig. 1, which illustrates the relation between the

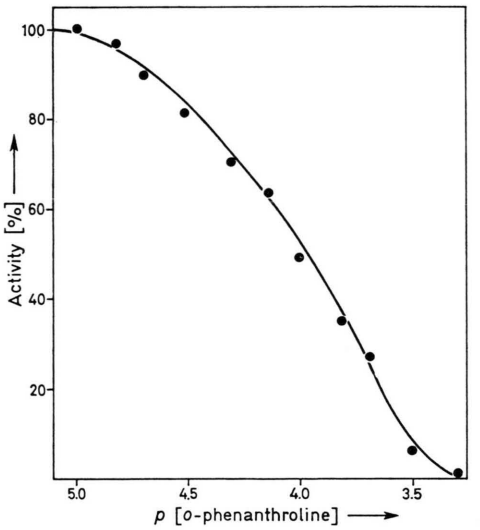

Fig. 1. Effect of increasing concentrations of *o*-phenanthroline on the activity of aminoacylase. For reaction conditions see Material and Methods.

fractional activity and the concentration of *o*-phenanthroline. Incubation of the enzyme with about 4×10^{-3} M of the chelating agent completely abolishes the catalytic activity. Less effective in inactivating the enzyme are EDTA and NTA (see Table I).

The effectivity of the complex forming agents seems to be not only a function of their complex constants but also of their hydrophobicity.

Table I. Inactivation of aminoacylase by metal chelating agents.

Time of incubation [h]	Remaining activity 1 mM *o*-phenanthroline	10 mM EDTA	10 mM NTA
24	0%	67%	86%
48	0%	53%	77%
168	0%	6%	50%

Inactivation of the enzyme by chelating agents is completely reversible; activity can easily be restored by addition of zinc ions without a lag period.

Zinc analysis of aminoacylase

The metal analysis of the enzyme, performed with a preparation which was extensively dialyzed against a zinc free buffer revealed that the enzyme contained 1.9 ± 0.5 atoms zinc/86000 g. This value is a good reason to believe that aminoacylase contains one atom zinc per subunit.

Determination of the zinc dissociation constants of aminoacylase

Using a zinc ion buffer described by Cohen and Wilson [8] we have measured the activity of the metal free inactive enzyme after equilibration with different known concentrations of zinc ions. Plotting the activity against the negative logarithm of the

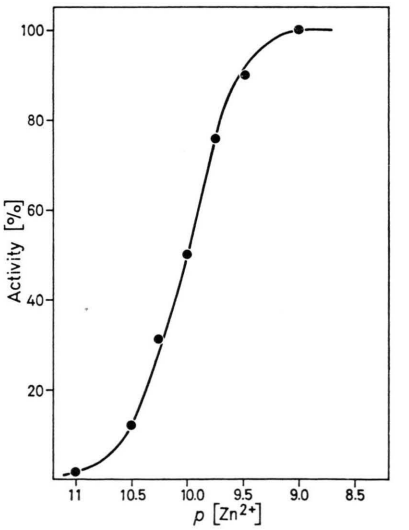

Fig. 2. Relation between the activity of metal free aminoacylase and the metal ion concentration in a zinc ion buffer. For further details see Methods.

zinc ion concentration of the buffer we obtained the titration curve of Fig. 2. This diagram demonstrates that the activity of the enzyme with respect to the zinc binding is controlled by a dissociation equilibrium with a zinc dissociation constant of about 10^{-10} M at pH 7.8 and room temperature. This dissociation constant is in the same range which is found with other zinc dependent enzymes, for instance alkaline phosphatase [8]. Since we could not find a significant deviation from a normal titration curve, we assume that the dissociation constants of the two zinc ions are nearly identical and that each of the two subunits contains one zinc ion in its active center.

Effect of zinc ions on the reactivity of the SH groups of aminoacylase

As we have shown in previous experiments [3, 5], aminoacylase contains two essential SH groups per subunit which are presumably part of the active site of the enzyme. In order to get some information on the relation between the SH groups and the metal ions or on a possible direct interaction, we have compared the reactivity of the thiol functions in the presence and absence of zinc ions. We therefore have studied the rate of reaction of Ellman's reagent [9] with the sulfhydryl groups of aminoacylase in the zinc free inactive, and the zinc containing enzyme of maximal activity. The results of these experiments are shown in Fig. 3. From this figure it becomes evident that the reactivity of the SH groups of the enzyme is considerably affected by zinc ions. A 3 fold increase in the initial rate of reaction is observed after removal of the metal ions. The two phase reaction observed with the intact enzyme [3, 5] is even more pronounced after deprivation of the metal ions. At this point it is important to mention that above a zinc ion concentration of 10^{-3} M the metal inactivates the enzyme as is to be expected if essential SH groups are blocked by the zinc ions.

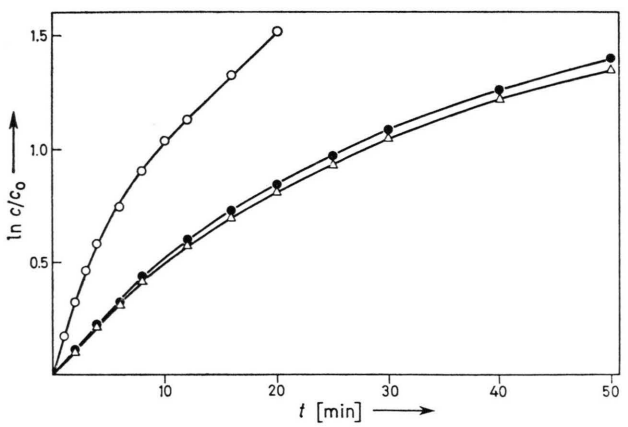

Fig. 3. Rate of reaction of aminoacylase with Ellman's reagent. △—△ intact enzyme, ○—○ metal free inactive enzyme, ●—● metal free inactive enzyme in the presence of 10^{-3} M DL-norleucine. Reaction conditions see ref. 3, 5.

We have further investigated the action of the competitive inhibitor DL-norleucine on the reaction of the enzyme with Ellman's reagent in the presence and absence of zinc (Fig. 3). These experiments have shown, that the competitive inhibitor DL-norleucine alone and the zinc ions alone have the same effect on the reactivity of the SH groups of the zinc free enzyme. From these results we may conclude, that zinc ions are not required for the binding of the competitive inhibitor and that the metal ions play no important role in maintaining the conformation and stability of the enzyme. This conclusion is confirmed by the observation, that removal of zinc ions does also not impair the interaction of the subunits [10].

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