Studies on the Zn^{2+}/Co^{2+} Exchange with Acylamino Acid Amidohydrolase from Pig Kidney

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Dedicated to Prof. H. Zahn *on the Ocassion of His 65th Birthday*

Co^{2+}-Acylamino Acid Amido Hydrolase, Chelating Ligands, Co^{2+} Dissociation Constant, Apo-Enzyme

The kinetics of inactivation of the Zn^{2+}-metalloenzyme acyl-amino acid amido hydrolase by chelating ligands were studied. The rate of inactivation by 1,10-phenanthroline is enhanced by histidine and inhibited in the presence of phenyl-alanine. Removal of the metal ion increases the heat stability and decreases the pH stability of the enzyme. Reactivation of the inactive metal free enzyme is possible with Zn^{2+} and Co^{2+}. Titration of the metal free protein with a Co^{2+}/nitrilotriacetate metal buffer revealed a dissociation constant of about 10^{-7} M for the Co^{2+} enzyme (Zn^{2+} enzyme = 10^{-10} M). The Co^{2+} substituted enzyme is less stable than the Zn^{2+} enzyme. Histidine and phenylalanine protect the Co^{2+} enzyme against inactivation by 1,10-phenanthroline.

In a previous communication we have shown that acylaminoacid amido hydrolase (amino acylase) from pig kidney is a metallo enzyme containing 2 Zn^{2+} per molecule which are essential for the catalytic activity of the protein [1]. The Zn^{2+} can be removed by dialysis against metal chelating agents such as 1,10-phenanthroline with concomitant loss of activity [1]. Restoration of activity is possible with Zn^{2+} and Co^{2+} [2, 3]. By chemical modification experiments with diethylpyrocarbonate and photo-oxidation, histidine residues have tentatively been identified as ligands of the metal ions [2].

Cobalt linked absorption spectra and other physical properties of Co^{2+} enzymes have been utilized in attempts to elucidate relations between structure and function of metallo enzymes. The environmentally-sensitive physical parameters have furnished essential information as to the role of the metal ions in the catalytic reaction [4]. We therefore have started to investigate Co^{2+} substituted aminoacylase from pig kidney. In the present paper we describe our experiments on the Zn^{2+}/Co^{2+} exchange and some properties of the Co^{2+}-enzyme.

Materials and Methods

Pig kidney amino acylase was prepared as described earlier [5]; final purification was performed electrophoretically up to a spec. act. of 230 ± 20 U/mg protein with N-chloroacetyl alanine as substrate. The purified enzyme was homogeneous as judged by disc gel electrophoresis.

Activity measurements were performed spectrophotometrically with a Zeiss PMQ II [5]. N-chloroacetyl-L-alanine and N-chloroacetyl-L-leucine were synthesized according to Greenstein *et al.* [6].

Removal of the Zn^{2+} or Co^{2+} was performed by dialysis of the enzyme (1 – 10 μM) in 0.3 M phosphate buffer pH 7.3 against 1 mM of the complexing ligand at 24° [1]. The effect of amino acids on the rate of inactivation by phenanthroline was studied by addition of 1 mM histidine or phenylalanine. Reactivation of metal free inactive enzyme in a cobalt ion buffer was performed as described in [1] using a stability constant for Co^{2+}-nitrilotriacetate of 10.8 at 20°, 150 mM KCl and 10 mM Tris pH 7.0.

Results and Discussion

Removal of the Zn^{2+} by chelating ligands

The effects of different metal chelating ligands on the activity of aminoacylase are illustrated in Fig. 1. The rate of inactivation of the enzyme by the bidentate or tridentate ligands 1,10-phenanthroline and tris-(2-amino-ethyl)-amine is first order in ligand concentration, the rate constants being 3×10^{-3} sec^{-1} and 3.3×10^{-4} sec^{-1} respectively. Other

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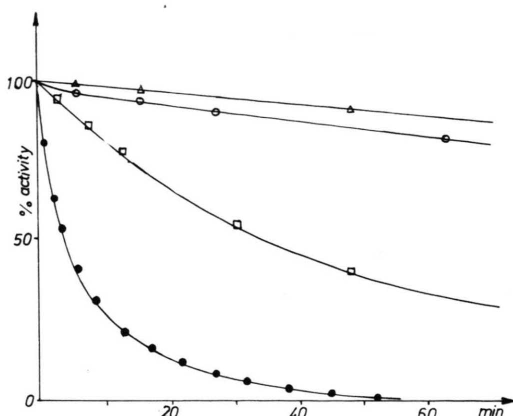

Fig. 1. Inactivation of acyl amino acid amido hydrolase (1.2 µM) by metal chelating ligands (1 mM). △—△ Diethyldithiocarbamate (11.6*); ○—○ Nitrilotriacetate (10.45); ■—■ Tris-(2-amino-ethyl)-amine (14.53); ●—● 1,10 Phenanthroline (6.2).

complex forming agents such as diethyldithiocarbamate and the negative charged nitrilotriacetate are by far less effective. Presumably these compounds in the applied concentration do not inactivate the enzyme by direct interaction with the enzyme bound metal ion but rather by rapid scavenging of the dissociated Zn^{2+} thus preventing the back reaction. The inactivation curve therefore reflects in these cases primarily the kinetic stability of the metallo enzyme. This interpretation is supported by the fact, that the rate of inactivation by the complexing ligands does not correlate with the stability constants of their Zn^{2+} chelate complexes (see Fig. 1). Other factors than the power of Zn^{2+} binding are important for the interaction of the ligands with the metal ion of the active center, namely charge, hydrophobicity and spatial arrangement. This is well known from other Zn^{2+} dependent enzymes [4].

The inactivation of amino acylase by complexing agents is significantly affected by amino acids. As is shown in Fig. 2, the rate of inactivation of the enzyme by 1.10 phenanthroline is enhanced by histidine and inhibited by phenylalanine after a lag period. Catalysis and inhibition by amino acids of the transfer of the Zn^{2+} from carboxypeptidase A to a chelating ligand (EDTA) was also observed by Billo [7]. Referring to his kinetic data, he postulated a ternary complex amino acid-Zn^{2+}-enzyme as a kinetic intermediate of the reaction.

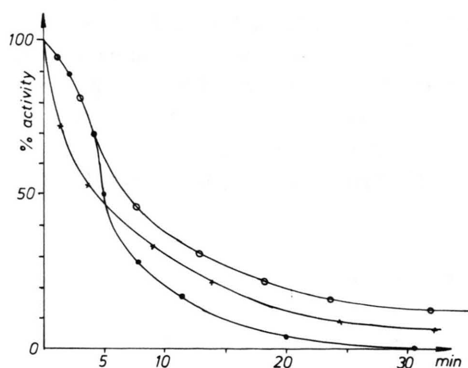

Fig. 2. Effect of 1 mM His ●—● and 1 mM Phe ○—○ on the inactivation of Zn^{2+} acylamino acid amido hydrolase by 1 mM 1,10-phenanthroline ×—×. Protein concentration 2 µM. Further details see Methods.

Some properties of the apoprotein

Removal of the Zn^{2+} does not alter the immunological properties of the enzyme as is shown in Fig. 3. No significant differences between the active Zn^{2+}-enzyme and the apoenzyme can be observed in the Ouchterlony immunodiffusion test. Significant differences between the two protein species were, however, found in the pH- and heat-stability and the reactivity of SH-groups. A reduced pH-stability of the apoenzyme, which is most evident between pH 7–8, can be detected in Fig. 4. A somewhat unexpected feature shows the heat stability of the enzyme before and after removal of the metal

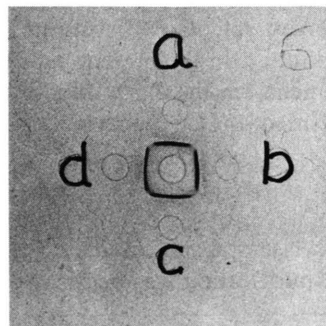

Fig. 3. Ouchterlony immunodiffusion. Native enzyme A, C.; metal free apo enzyme B, D.

* Stability constants of the Zn^{2+} complexes [10] for further details see Methods.

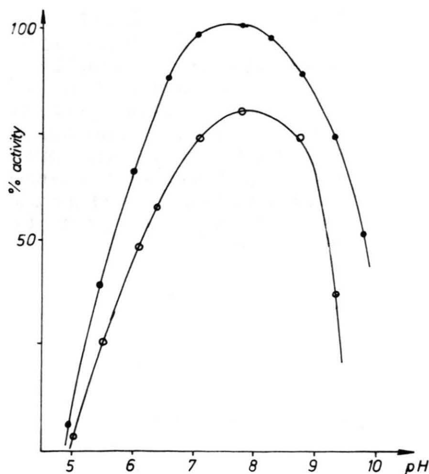

Fig. 4. pH-stability of the active Zn^{2+}-enzyme ●—● and the metal free apo enzyme ○—○. 50 µl Zn^{2+} enzyme. (0.2 mg/ml) and apo-enzyme solution were incubated for 10 min in 0.5 ml 0.05 M phosphate-borate-acetate buffer of the desired pH value. Thereafter the activity was measured with N-chloroacetyl-alanine as substrate after addition of Zn^{2+} (final concentration 2×10^{-4} M) to the metal free sample.

(Fig. 5). Up to about 45° the apoenzyme is less heat sensitive than the active holoenzyme. Studies on the kinetics of the heat inactivation of Zn^{2+}-containing porcine kidney aminoacylase were recently published by Szajani [8]. As was emphasized by this author, the process of inactivation does not exhibit simple first order kinetics, but is of more complex nature. From his data he concluded, that heat inactivation of the enzyme precedes a dissociation into the less heat stable subunits.

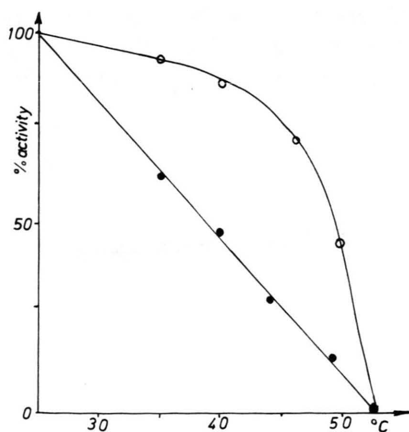

Fig. 5. Heat stability of active ●—● and metal free ○—○ amino acylase. 100 µl enzyme solution (0.2 mg/ml) in 0.05 M phosphate buffer pH 7.3 were incubated in a water bath for 10 min at the desired temperature and rapidly cooled to room temperature; activity was measured as described above. The enzyme was freed from Zn^{2+} by dialysis against 1 mM 1,10-phenanthroline.

Further information of structural differences between the active Zn^{2+} enzyme and the metal free inactive protein were obtained from measurements of the rate of reaction of the SH-groups with Ellman's reagent. The results of these experiments are demonstrated in Fig. 6. From this figure it becomes evident that the reactivity of the SH-groups of the enzyme is considerably affected by the metal ions; a threefold increase in the initial rate of reaction of the fast reacting SH group is observed after removal of the Zn^{2+} [5]. In this experiment only stoichiometric amounts of Zn^{2+} were present: (2 Zn^{2+} per molecule [1]); as yet there are no indications for the involvement of SH groups in the binding of the metal ion. Further experiments are underway to clarify this question.

Reactivation by Co^{2+}

Reactivation of the metal free, inactive protein is possible by Zn^{2+} and Co^{2+} [1, 3]. Our previous studies on the reactivation of the enzyme by Zn^{2+} had shown, that the activity of the enzyme with respect to the Zn^{2+} binding is controlled by a dissociation equilibrium with a K_d of about 10^{-10} M at pH 7.8 and room-temperature.

Using a Co^{2+} nitrilotriacetate buffer [9] we have measured the activity of the metal free inactive enzyme after equilibration with different known concentrations of Co^{2+}. Plotting the activity against the negative logarithm of the Co^{2+} concentration of the buffer we obtained the titration curve of Fig. 7. This diagram delivers a Co^{2+}-dissociation constant of about 10^{-7} M at pH 7.0 and 22°. This constant is in the same range as those found for other Co^{2+} metallo enzymes for instance carboxypeptidase A (10^{-7} M) [4] and human carbonic anhydratase B ($10^{-7.2}$ M) [4].

The catalytically active Co^{2+} enzyme is less stable than the Zn^{2+} enzyme; a non negligible concentration of free metal ions must be present to achieve saturation and maximal activity. A mixture of complexed species may result, especially non specific binding to secondary sites of the protein. It therefore was not possible to obtain a Co^{2+} amino acylase with a stoichiometric content of metal ions. Dialysis of the Co^{2+} enzyme of maximal activity

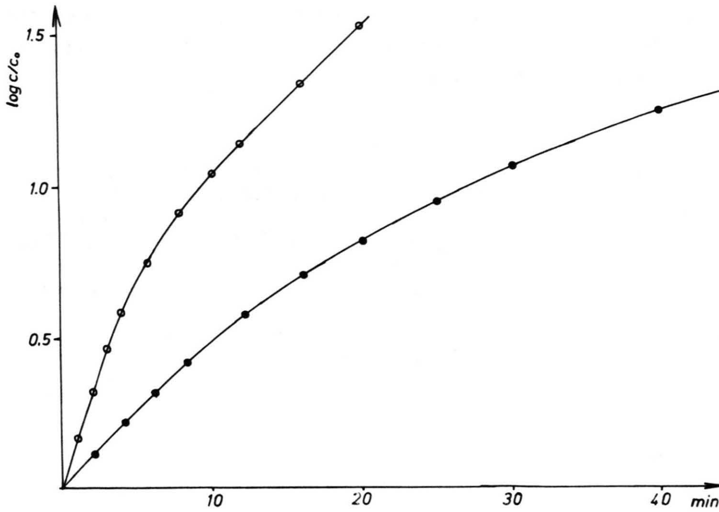

Fig. 6. Rate of reaction of amino acylase with Ellman's reagent. ●—● active Zn^{2+}-enzyme, ○—○ metal free inactive enzyme. 1 ml of the native enzyme and the metal free protein (2 mg/ml) in Tris buffer pH 8.0 were mixed with 100 µl 5 mM Ellman's reagent and the reaction followed spectrophotometrically at 412 nm; for further details see 5.

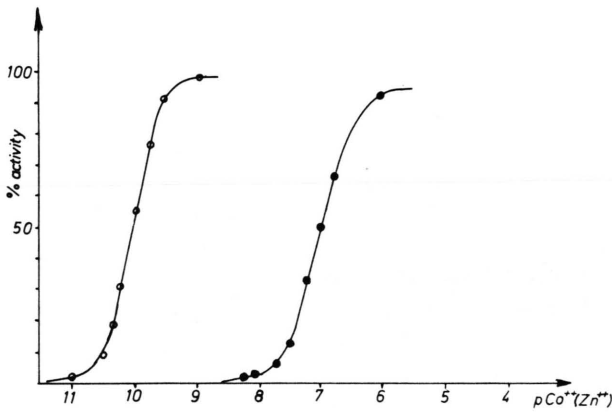

Fig. 7. Relation between the activity of metal free acyl amino acid amido hydrolase and metal ion concentration in a Zn^{2+} ○—○ and Co^{2+} ●—● buffer (see Methods).

against metal free buffer gives rise to considerable inactivation within one hour (see Fig. 8). Under the same conditions, the Zn^{2+} enzyme is completely stable.

The inactivation of Co^{2+}-amino acylase by 1,10-phenanthroline and the effect of the amino acids phenylalanine and histidine on this process are demonstrated in Fig. 9. A first order rate constant of inactivation by 1,10-phenanthroline of $k = 1.1 \times 10^{-2}$ sec^{-1} was found. The amino acids tested do not only protect the Co^{2+} enzyme against the inactivation by the chelating ligand, but bring about an activation by about threefold.

The totally different effects of the amino acids on the removal of the Zn^{2+} respectively Co^{2+} ions by phenanthroline (Figs. 2 and 9) demonstrate the sig-

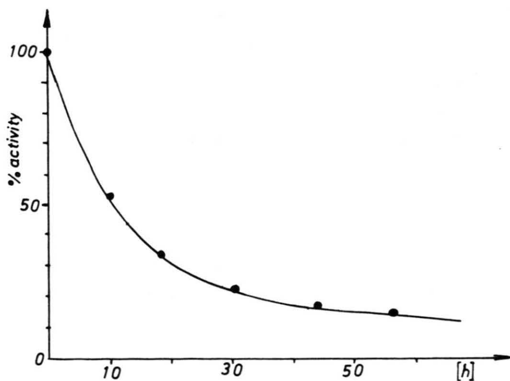

Fig. 8. The effect of dialysis against metal free phosphate buffer pH 7.3 on the activity of Co^{2+} acyl amino acid amido hydrolase.

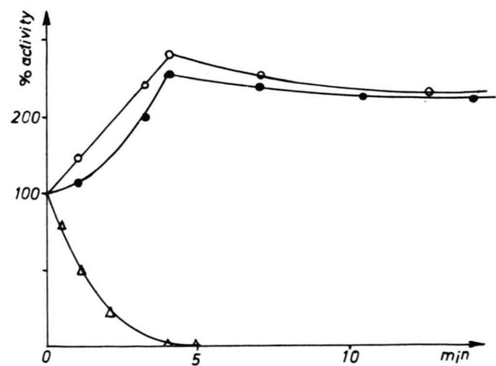

Fig. 9. Effect of 1 mM histidine ○—○ and 1 mM phenylalanine ●—● on the inactivation of Co^{2+} amino acid amido hydrolase by 1 mM 1,10-phenanthroline △—△.

nificant changes in the structure of the active site and the different kind of respond to effectors, produced by the exchange of Zn^{2+} for Co^{2+} in the enzyme. Further investigations on this problem and on the spectroscopic properties of the Co^{2+} enzmye are under way.

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