Co^{+2}-Substituted Acylamino Acid Amido Hydrolase from *Aspergillus oryzae*

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

Zn^{+2}/Co^{+2} Exchange, Microbial Acylamino Acid Amido Hydrolase, *Aspergillus oryzae*, Kinetic Properties

The inactivation of the Zn^{+2} metallo enzyme acylamino acid amido hydrolase from *Aspergillus oryzae* by ethylendiamine-tetraacetate (EDTA) and nitrilotriacetate (NTA) and the effects of Phe and His on this process were studied. Reactivation of the enzyme by Zn^{+2}- or Co^{+2}-NTA buffer revealed a dissociation constant for the Zn^{+2}-enzyme of 10^{-10} M and for the Co^{+2}-enzyme of $10^{-7.5}$ M. The kinetic properties of the Zn^{+2} and Co^{+2} enzyme were compared for a series of substrates. Substitution of Co^{+2} for Zn^{+2} reduces substrate specificity of the enzyme.

Acylamino acid amido hydrolase (aminoacylase) from *Aspergillus oryzae* is a metallo enzyme, containing essential Zn^{+2} ions [1, 2]. Removal of the Zn^{+2} is possible by chelating ligands with concomitant loss of activity; restoration of activity is achieved in the presence of Zn^{+2} [2]. Since Co^{+2} has been shown to be a valuable reporter group, sensing the structure of the active site and certain steps in the catalytic process [3], we have studied the Zn^{+2}/Co^{+2} exchange with acylamino acid amido hydrolase from *Aspergillus oryzae*. In the present communication we report the results of the evaluation of the dissociation constants of the Zn^{+2}- and Co^{+2}-enzyme and present a comparison of some of their kinetic properties.

Materials and Methods

Amino acylase from *Aspergillus oryzae* was purified starting with a commercially available material as described earlier [2]. The spec. activity of the enzyme, which was homogeneous as judged by gel electrophoresis, was 130 ± 15 U/mg. The provenance of the substrates is described in reference [1].

Buffer substances and other chemicals were p. a. grade from Merck, Darmstadt. Kinetic parameters were determined as described in [2] and [4].

Abbreviations: NTA, nitrilotriacetate; EDTA, ethylen diamine-tetraacetate.

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The effects of chelating ligands on the activity were tested by incubating the enzyme (1 mg protein/ml), which was dialyzed over night against a 30 mM phosphate buffer pH 7.0, with NTA (1 and 10 mM), and EDTA (1,5 and 10 mM) at pH 7.0 and 22°. Activity measurements were performed in 10 µl samples with N-chloroacetyl alanine as substrate. The inactivation experiments with EDTA in the presence of histidine and phenylalanine were accomplished in phosphate buffer at pH 8.0.

For the determination of the dissociation constant of the Zn^{+2} enzyme a metal ion buffer described by Cohen and Wilson [5] was used. Further details of the method are to be found in references [4] and [5]. A suitable Co^{+2}-buffer system for the evaluation of the dissociation constant of the Co^{+2}-aminoacylase consists of 10 mM Tris/HCl, 150 mM KCl, 10 mM NTA and different concentration of $CoSO_4$, pH 7.0. Activity measurements must be performed immediately after incubation of the inactive metal free enzyme in the Co^{+2} buffer; prolonged incubation inactivates the enzyme. The activity of the Co^{+2}-enzyme was determined with N-Chloroacetyl-alanine [4].

Results and Discussion

Inactivation and reactivation studies

The inactivation of the microbial aminoacylase by the chelating ligands NTA and EDTA is demonstrated in Fig. 1. EDTA is a more effective inactiva-

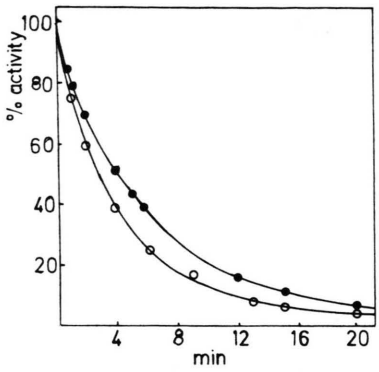

Fig. 1. Inactivation of Zn^{+2}-aminoacylase from *Aspergillus oryzae* by 10 mM nitrilotriacetate ●——● and 1 mM EDTA ○——○ at pH 7.0 and 22 °C in phosphate buffer.

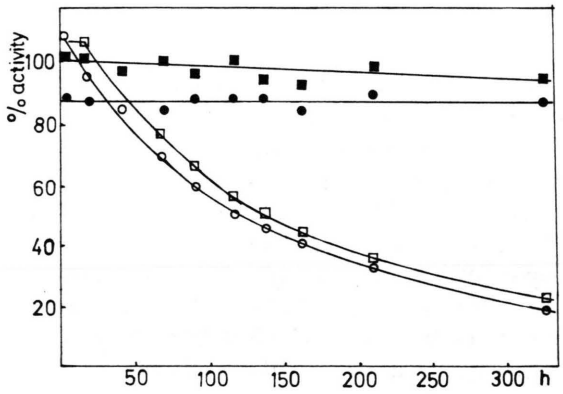

Fig. 2. Irreversible inactivation of microbial aminoacylase by prolonged incubation with 10 mM EDTA at 0 °C and pH 7.0 □——□, at pH 6.5 ○——○; ■——■ control at pH 7.0, ●——● control at pH 6.5.

tor than NTA, which correlates with their complex forming power. (Stability constants of their Zn^{+2} complexes are 16.5 and 10.4 respectively [6].) Prolonged incubation of the enzyme with chelating agents causes irreversible inactivation as is shown in Fig. 2. This diagram in reality reflects the loss of stability of the protein after removal of the essential metal ions.

The effects of the amino acids histidine and phenylalanine on the inactivation of the enzyme by EDTA is illustrated in Fig. 3. A significant decrease of the rate of inactivation by phenylalanine is observed, while histidine catalyses the transfer of the metal ion from the enzyme to the chelating ligand.

Catalysis and inhibition by amino acids of the removal of the essential Zn^{+2} from carboxypeptidase A by EDTA is also described by Billo [7]. This author postulates a ternary complex – amino acid-Zn^{+2}-enzyme – as a kinetic intermediate of the inactivation reaction.

Reactivation of the metal free protein is possible by Zn^{+2} and Co^{+2} without a lag phase. Using a Zn^{+2}-buffer described by Cohen and Wilson [5] we have measured the activity of the metal free inactive enzyme after equilibration with different known concentrations of Zn^{+2} ions. Plotting the activity against the negative logarithm of the Zn^{+2} ion concentration of the buffer, we obtained the titration curve shown in Fig. 4. This diagram demonstrates that the activity of the enzyme with respect to the Zn^{+2}-binding is controlled by a dissociation constant of about 10^{-10} M at pH 8.5 and 25 °C. Fig. 4 shows also the titration curve obtained

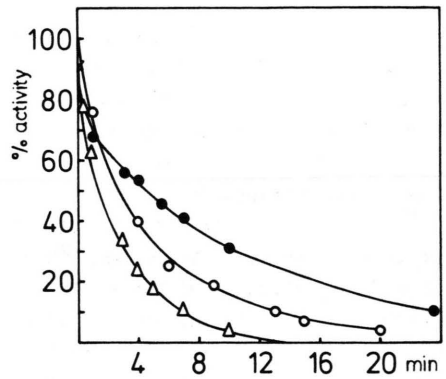

Fig. 3. Inactivation of aminoacylase by 1 mM EDTA ○——○; 1 mM EDTA + 1 mM phenylalanine ●——●; 1 mM EDTA + 1 mM histidine △——△.

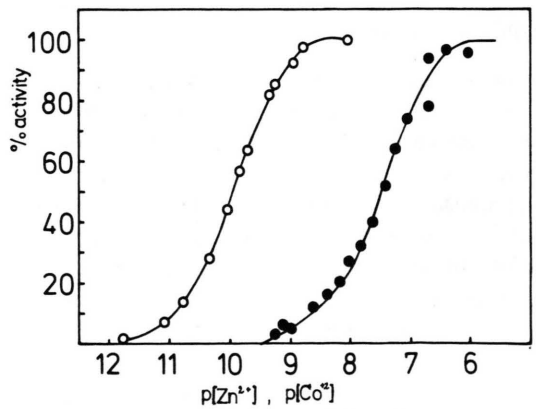

Fig. 4. Relation between the activity of metal free aminoacylase and the metal ion concentration in a Zn^{+2} ○——○, and a Co^{+2} ●——● ion buffer; for further details see Methods and references [4] and [5].

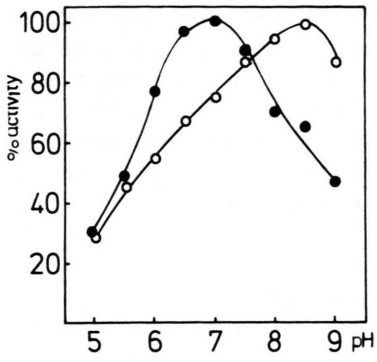

Fig. 5. pH-dependence of microbial aminoacylase with N-chloroacetyl alanine as substrate. ○——○ Zn^{+2}-enzyme, ●——● Co^{+2}-enzyme.

with a Co^{+2}-NTA buffer at pH 7.0; from this curve we obtain a dissociation constant of Co^{+2}-acylase of $10^{-7.5}$ M. The catalytically active Co^{+2}-enzyme is less stable than the Zn^{+2}-enzyme; a significant concentration of free metal ions must be present to achieve saturation and maximal activity. The Zn^{+2}- as well as Co^{+2}-dissociation constant of the microbial acylase are in the same range as those found for other Zn^{+2}- and Co^{+2} enzymes [8].

Table I. Relative activities of the Zn^{+2}- and Co^{+2}-aminoacylase from *Aspergillus oryzae* with different substrates.

Substrates [a]	Relative activity [%]	
	Zn^{2+}-Enzyme	Co^{2+}-Enzyme
N-Chloro-acetyl-Ala	100	127
N-Chloro-acetyl-Met	286	163
N-Chloro-acetyl-Norleu	326	191
N-Chloro-acetyl-Leu	60	153
N-Chloro-acetyl-Phe	218	195
N-Acetyl-Glu	1	2.5
N-Acetyl-Gln	12	59
N-Acetyl-Ala	16.5	39
N-Acetyl-Lys	1	6.5

[a] Substrate concentration was 7.5 mM.

Comparison of the kinetic properties of the Zn^{+2}- and Co^{+2}-enzyme

The kinetic properties of the microbial aminoacylase are significantly changed by the substitution of Co^{+2} for Zn^{+2}. As is illustrated in Fig. 5, the pH-optimum of the activity with N-chloroacetyl-alanine as substrate is shifted from pH 8.5 for the Zn^{+2} enzyme to pH 7.0 for the Co^{+2}-enzyme. This shift of the pH-optimum was also observed with Co^{+2}-acylase from pig kidney [9].

The relative activites of the Zn^{+2}- and Co^{+2}-enzyme with different substrates are summarized in Table I. In most cases the activity of the enzyme is enhanced by the Zn^{+2}/Co^{+2} exchange. This effect has been described also for other Co^{+2} substituted enzymes [3]; it may, among other things, result from the different behaviour of Zn^{+2}- and Co^{+2} as Lewis acids.

A comparison of the kinetic parameters of the Zn^{+2} and Co^{+2}-enzyme for different substrates is given in Table II. From these parameters it becomes evident, that the binding of the substrates, as far it can be derived from the K_M values, is not significantly affected by the Zn^{+2}/Co^{+2} exchange, while the catalytic activity increases or decreases depending on the nature of the substrate.

A commonly used measure of the relative substrate specificity of an enzyme is the ratio of k_{cat}/K_M. This parameter is the apparent second order rate constant for the reaction of the enzyme with the substrate and the comparison of k_{cat}/K_M values truly give a fair measure of an enzyme's overall preference for a particular substrate.

Table II. Comparison of the kinetic parameters of the Zn^{+2}- and Co^{+2}-aminoacylase for different substrates.

Substrates	Zn^{2+}-enzyme			Co^{2+}-enzyme		
	K_M [$\cdot 10^3$ M]	k_{cat} [$\cdot 10^{-2}$ sec^{-1}]	k_{cat}/K_M [$\cdot 10^{-5}$ M^{-1} sec^{-1}]	K_M [$\cdot 10^3$ M]	k_{cat} [$\cdot 10^{-2}$ sec^{-1}]	k_{cat}/K_M [$\cdot 10^{-5}$ M^{-1} sec^{-1}]
N-Chloro-acetyl-Ala	6.3	1.8	0.28	4.0	7.3	1.82
N-Chloro-acetyl-Leu	10.0	1.0	0.10	6.6	7.0	1.06
N-Chloro-acetyl-Met	1.5	22.0	14.6	2.5	12.4	5.0
N-Chloro-acetyl-Phe	0.7	36.0	51.0	0.7	7.0	10.0
N-Chloro-acetyl-Trp	1.0	19.0	19.0	0.6	12.6	21.0
N-Dichloro-acetyl-Nle	2.5	0.4	0.16	4.0	3.3	0.82
N-Acetyl-Gln	6.6	2.1	0.31	6.6	6.8	1.1

Comparison of k_{cat}/K_M of the Zn^{+2} and Co^{+2} enzyme shows, that the relative substrate specificity and preference for a special substrate is reduced in the Co^{+2} enzyme as compared to the Zn^{+2} enzyme. While in the case of the Zn^{+2} enzyme k_{cat}/K_M maximally differes by a factor of 510 (N-chloroacetyl-Phe 51 : N-chloroacetyl-Leu 0,1), this factor is only 25 for the Co^{+2} enzyme. (N-chloroacetyl-Trp 21 : N-Dichloroacetyl-Nle 0.8). The active site of the Co^{+2} enzyme seems to be more "susceptible" to substrates of different structure. A loss of stereospecificity of aminoacylase from pig kidney after addition of Co^{+2} to their enzyme preparations was already observed by Greenstein *et al.* [9].

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