Co\textsuperscript{2+}-Substituted Acylamino Acid Amido Hydrolase from 
Aspergillus oryzae

Ingrid Gilles, Hans-Gerhard Löffler, and Friedh. Schneider

Physiologisch-Chemisches Institut II der Philipps-Universität Marburg,
Lahnberge, D-3550 Marburg

Z. Naturforsch. 36 c, 751–754 (1981); received June 11, 1981

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

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

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

Acylamino acid amido hydrolase (aminoacylase) from Aspergillus oryzae is a metallo enzyme, containing essential Zn\textsuperscript{2+} ions [1, 2]. Removal of the Zn\textsuperscript{2+} is possible by chelating ligands with concomitant loss of activity; restoration of activity is achieved in the presence of Zn\textsuperscript{2+} [2]. Since Co\textsuperscript{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\textsuperscript{2+}/Co\textsuperscript{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\textsuperscript{2+} and Co\textsuperscript{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, ethylenediamine-tetraacetate.

Reprint requests to Prof. Dr. Fr. Schneider.
0341-0382/81/0900-0751 $ 01.00/0

The effects of chelating ligands on the activity were tested by incubating the enzyme (1 mg protein/ml), which was dialyzed overnight 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\textsuperscript{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\textsuperscript{2+}-buffer system for the evaluation of the dissociation constant of the Co\textsuperscript{2+}-aminoacylase consists of 10 mM Tris/HCl, 150 mM KCl, 10 mM NTA and different concentration of CoSO\textsubscript{4}, pH 7.0. Activity measurements must be performed immediately after incubation of the inactive metal free enzyme in the Co\textsuperscript{2+} buffer; prolonged incubation inactivates the enzyme. The activity of the Co\textsuperscript{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-
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. 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 micro-

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

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Relative activity [%]</th>
<th>Zn(^{2+})-Enzyme</th>
<th>Co(^{2+})-Enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-Chloro-acetyl-Ala</td>
<td>100</td>
<td>127</td>
<td></td>
</tr>
<tr>
<td>N-Chloro-acetyl-Met</td>
<td>286</td>
<td>163</td>
<td></td>
</tr>
<tr>
<td>N-Chloro-acetyl-Norleu</td>
<td>326</td>
<td>191</td>
<td></td>
</tr>
<tr>
<td>N-Chloro-acetyl-Leu</td>
<td>60</td>
<td>153</td>
<td></td>
</tr>
<tr>
<td>N-Chloro-acetyl-Phe</td>
<td>215</td>
<td>195</td>
<td></td>
</tr>
<tr>
<td>N-Acetyl-Glu</td>
<td>1</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td>N-Acetyl-Gln</td>
<td>12</td>
<td>59</td>
<td></td>
</tr>
<tr>
<td>N-Acetyl-Ala</td>
<td>16.5</td>
<td>39</td>
<td></td>
</tr>
<tr>
<td>N-Acetyl-Lys</td>
<td>1</td>
<td>6.5</td>
<td></td>
</tr>
</tbody>
</table>

* Substrate concentration was 7.5 mM.

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.
Comparison of \( k_{\text{cat}}/K_M \) 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 \( k_{\text{cat}}/K_M \) maximally differs 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].


