On the Reaction of D-Amino Acid Oxidase with β-Chloroalanine

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D-amino acid oxidase, D-amino acid oxidase apoenzyme, β-chloro-L-alanine, β-chloro-D-alanine, glutathione

The reactions of D-amino acid oxidase with β-chloro-D-alanine and β-chloro-L-alanine have been described. Both the holoenzyme and the apoenzyme catalyse the deamination of β-chloro-L-alanine to keto acid without any consumption of oxygen or oxidation-reduction of the enzyme-bound FAD. The anaerobic reaction of the holoenzyme with β-chloro-D-alanine involves three phases with respect to the reduction of the enzyme-bound FAD, the rapid formation of an intermediate spectral species followed by a steady state of this intermediate and the last decay of the intermediate to the fully reduced enzyme. The rate of keto acid formation decreases as the reaction proceeds and ceases at the beginning of the conversion of the intermediate to the fully reduced enzyme. The aerobic reaction of the holoenzyme with β-chloro-D-alanine is biphasic. The first rapid oxygen consumption is followed by the second slow oxygen consumption. The second slow phase disappears by treating the enzyme with glutathione before the addition of substrate.

Recently, studies on the reaction of D-amino acid oxidase with β-chloro-D-alanine have demonstrated that β-chloro-D-alanine was oxidatively deaminated to keto acid and two intermediate states of the enzyme, which were not observed as far as D-alanine was used, were detected. It has been also shown that the keto acid was chloropyruvate when the reaction is carried out under 100% oxygen, while it was pyruvate under anaerobic conditions. These results indicate that the enzyme catalyses not only the oxidative deamination of substrate but the elimination reaction without oxidation process. The present communication deals with those elimination and oxidation reactions.

D-amino acid oxidase was purified by the procedure of KUBO et al. The enzyme preparations were treated further with hydroxylapatite by the procedure of MASSEY et al. Benzoate bound to the enzyme preparations was removed by passing the preparations through a Sephadex G-100 column immediately after the addition of a small amount of 1 M DL-alanine to the column. The apoenzyme was purified by the procedure of MIYAKE et al. β-Chloro-D-alanine-HCl and β-chloro-L-alanine-HCl were kindly supplied by Dr. S. IMAMOTO, Food Research Institute, Osaka. Because of instability of these materials at pH 8.3, a freshly prepared solution was used for each experiment. All other chemicals used were analytical grade. Protein was determined by the method of GORNALL et al. or from absorbancy at 280 μm. FAD was determined by the method of WHITBY. The enzymatic activity was measured by the use of a Bioxygraph equipped with a Clark type Galvani electrode, Kyusui Kagaku Co., Tokyo. The enzyme reaction was initiated by the addition of substrate to the enzyme solution containing 10 μM FAD. The oxygen consumption at the initial 1 min was taken as the velocity of the enzyme reaction. The reaction was carried out at 38°C. A Hitachi two wavelength double beam spectrophotometer model 356 or a Hitachi spectrophotometer model 124 was used for measurements of absorption spectra or changes in absorbancy.

When β-chloro-L-alanine was added to the apoenzyme solution, absorbancy at 315 μm region gradually increased. The absorption spectrum of the reaction mixture showed a broad band at 315 μm region after incubation for 60 min and at 20°C. Such a spectral change was not observed by the incubation of β-chloro-L-alanine in the absence of the apoenzyme or in the presence of the heat denatured apoenzyme. As the spectral pattern obtained after the incubation for 60 min was similar to that of pyruvate, the apoenzyme reaction was carried out in the presence of lactate dehydrogenase and NADH. The reaction product was also analysed according to the method of FRIEDMANN and HAUGEN as modified by WADA and SNELL. The addition of β-chloro-L-alanine to the apoenzyme...
solution containing lactate dehydrogenase and NADH resulted in the decrease in absorbancy of NADH at 340 m\text{m} \mu, and the formation of a hydrazone derivative of keto acid was also detected, indicating that a keto acid, probably pyruvate, was formed as the reaction product. Oxygen consumption was not observed during the apoenzyme reaction. These results showed that the apoenzyme catalyzes the deamination of \( \beta \)-chloro-L-alanine. The apoenzyme was inactive toward \( \beta \)-chloro-D-alanine as well as L-alanine, D-alanine, L-serine and L-threonine. The Michaelis constant for \( \beta \)-chloro-L-alanine and the maximum velocity of the apoenzyme reaction were obtained from double reciprocal plots of the reaction velocity versus the concentration of the substrate, and the values of 26 mM for the Michaelis constant and 0.41 \( \mu \)mole/min per mg protein for the maximum velocity were obtained, respectively.

The rate of the apoenzyme reaction was constant irrespective to the presence or the absence of FAD, indicating that \( \beta \)-chloro-L-alanine is also catalysed by the holoenzyme. In this case also, the reaction did not accompany oxygen consumption nor oxidation-reduction of the enzyme-bound FAD even in anaerobic conditions. Accordingly, the deamination of \( \beta \)-chloro-L-alanine by the holoenzyme is similar to that by the apoenzyme. In fact, the rate of the holoenzyme reaction was close that of the apoenzyme, and the values of the Michaelis constant and the maximum velocity were 31 mM and 0.35 \( \mu \)mole/min per mg protein, respectively.

In the anaerobic reaction of the holoenzyme with \( \beta \)-chloro-D-alanine, an intermediate spectral species appears in the early stage of the reaction showing a slight decrease in the 455 m\text{m} \mu absorption maximum of the oxidized enzyme and the appearance of a broad band at around 550 m\text{m} \mu. The rate of decay of the spectral species is very slow with complete reduction of the enzyme being attained after reaction for more than 2 hrs\(^1\). It was found from the present experiments that the slow decay of the intermediate species became biphasic when the concentration of the substrate was limited, namely the presence of a steady state of the intermediate species was observed before the conversion to the fully reduced form. Therefore, the anaerobic reaction of the holoenzyme with \( \beta \)-chloro-D-alanine involves three phases with respect to the state of the enzyme-bound FAD, the initial rapid formation followed by the steady state of the intermediate and the reduction of the intermediate to the fully reduced enzyme. The rate of keto acid formation decreased as the reaction proceeded and ceased at the beginning of the third phase.

The aerobic reaction of the holoenzyme with \( \beta \)-chloro-D-alanine was biphasic with respect to oxygen consumption. It was observed that the rate of oxygen consumption decreased remarkably after the initial rapid phase. The biphasic feature was dominated as the concentration of the enzyme increased at a fixed substrate concentration. The suppressed oxygen consumption does not mean that oxygen is exhausted but it is observed even when oxygen in the reaction mixture still remains to some extents. The biphasic oxygen consumption disappeared when the enzyme was treated with 2 mM glutathione before the addition of substrate. This effect of glutathione, however, was not observed when it was added to the enzyme solution after the addition of substrate.

We have shown several types of reactions of D-amino acid oxidase holoenzyme and the apoenzyme with \( \beta \)-chloro-L-alanine and \( \beta \)-chloro-D-alanine. These are summarized in Table I. In these reactions, it is interesting to note that the apoenzyme showed catalytic activity when substrate was properly chosen and it is specific for L-configuration of

<table>
<thead>
<tr>
<th>Forms of enzyme</th>
<th>Substrates</th>
<th>Types of reactions</th>
<th>Products</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 apoenzyme</td>
<td>( \beta )-chloro-L-alanine</td>
<td>deaminase</td>
<td>pyruvate</td>
</tr>
<tr>
<td>2 holoenzyme</td>
<td>( \beta )-chloro-L-alanine</td>
<td>deaminase</td>
<td>pyruvate</td>
</tr>
<tr>
<td>3 holoenzyme</td>
<td>( \beta )-chloro-D-alanine</td>
<td>deaminase</td>
<td>pyruvate</td>
</tr>
<tr>
<td>a* anaerobic</td>
<td>( \beta )-chloro-D-alanine</td>
<td>deaminase</td>
<td>pyruvate</td>
</tr>
<tr>
<td>b aerobic</td>
<td>( \beta )-chloro-D-alanine</td>
<td>oxidase</td>
<td>chloropyruvate</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>and pyruvate*</td>
</tr>
</tbody>
</table>

\* see ref. 2.
the substrate. Though the details on the reaction mechanism is unknown, it might present unique informations for the complete elucidation of the reaction mechanism of D-amino acid oxidase. Several lines of evidence on aerobic and anaerobic reactions of the holoenzyme with β-chloro-D-alanine suggest that some special type of reaction is involved during catalysis. When these results are referred to recent report by Walsh et al. 2 that both pyruvate and chloropyruvate are formed as the reaction product and the ratio of the formation is dependent on oxygen tension, it is considered that a certain intermediate state of the enzyme may play an important role for those β-elimination and oxidation reactions. The facts that the aerobic and anaerobic reactions of the holoenzyme with β-chloro-D-alanine had two or three phases, may be related with those elimination and oxidation reactions.


Untersuchungen zur Konfiguration von 4-Nitroimidazolnucleosiden

Investigations on the Configuration of 4-Nitro Imidazole Nucleosides

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Nucleosides, Circular Dichroism, Nuclear Magnetic Resonance, Carbohydrate Copper Complexes

It is demonstrated that the 220—225 nm Cotton effect of 4-nitro imidazole nucleosides can be used for the determination of the configuration of this class of compounds. A more detailed picture about conformation and configuration of the sugar residue is received from the copper-ammonia complexes of these nucleosides.

The pH-dependency of the stability and the complex ratio are discussed for a furanose moiety. Correlation between signs and intensities of the Cotton effects and configurations and conformations of the diol structures are discussed.

4-Nitroimidazolnucleoside bestimmter Struktur hemmen die DNS-abhängige RNS-Polymerase aus E. coli und zeigen bakteriostatische Wirkung1,2. Über die Hemmechanismen dieser Nucleoside ist jedoch noch wenig bekannt. Zur Aufklärung dieser Wirkungsmechanismen ist die genaue Kenntnis von Konfiguration und Konformation dieser Nucleosid-Substrate jedoch Voraussetzung.

Die Konfigurationen und Konformationen von Nucleosiden werden heute hauptsächlich durch physikalisch-chemische Strukturanalytiken wie Massenspektrometrie3—6, Protonenresonanz7,8, 13C-Resonanz9—11, Fluorresonanz12, Circulardichroismus13,14 und magnetischen Circulardichroismus untersucht15,16.

In dieser Arbeit wird beschrieben, welche Aussagen der Circulardichroismus über die Stereochemie von 4-Nitroimidazolnucleosiden, potentiellen Cytostatica, zu machen vermag.