Enzymatic Racemization of Leucine and α-Aminobutyrate

Part III. Properties of Partially Purified Racemase*

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A new amino acid racemase catalyzing the conversion of either D or L enantiomorph of leucine and α-aminobutyrate to the racemates, was partially purified from the cell-free extract of Pseudomonas striata. Both the racemase reactions are suggested to be catalyzed by a single enzyme because of the constant ratio between the activities during the purification, and of their very resemble behavior to pH, temperature and heating the enzyme. Pyridoxal phosphate functions as the coenzyme for this racemase.

Since an enzyme catalyzing the racemization of alanine was discovered and isolated from Streptococcus faecalis,1) several amino acid racemases such as glutamate racemase,2)–4) lysine racemase5)–6) and arginine racemase7) have been found in bacteria. Recently, evidence has been obtained for the occurrence of a new amino acid racemase catalyzing the conversion of either D or L isomer of leucine and α-aminobutyrate to the racemates in the cell-free extract of Pseudomonas striata.8) The distribution of amino acid racemase activities, and a convenient and sensitive assay procedure of the enzyme with D-amino acid oxidase and 3-methyl-2-benzothiazolone hydrazine hydrochloride (MBTH) were reported in the preceding paper.9)

In the present communication, the preparation of partially purified enzyme racemizing leucine and α-aminobutyrate and some of its properties are described.

EXPERIMENTAL

Materials. L-α-Aminobutyric acid was prepared from DL-α-aminobutyric acid with hog renal acylase10) L-Leucine was a product of Kyowa Hakko Kogyo Co., Tokyo. MBTH was purchased from Aldrich Chemical Company, Inc., Wisconsin, U.S.A. TEAE-cellulose was a product of Serva Entwicklungslabor, Heidelberg, Germany. The other chemicals were analytical grade reagents. Crude D-amino acid oxidase was prepared from hog kidney as described previously.5)

Microbiological methods. Pseudomonas striata isolated from soil1) was employed throughout. The composition of medium and the culture conditions were also described in the preceding paper.5)

Assay procedure of racemase. The activity of enzyme was determined by the manometric procedure or the

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MBTH procedure as described in the preceding paper.\(^9\) One unit of the enzyme is defined as the amount that catalyzes the formation of 1 \( \mu \)mole of the antipode per min. Specific activity is expressed as units per mg of protein. Protein was determined by the method of Lowry et al.,\(^{11} \) using egg albumin as standard.

RESULTS

Purification of enzyme

The enzyme catalyzing the racemization of \( \alpha \)-aminobutyrate and leucine was partially purified from the cell-free extract of Pseudomonas striata as described below. All operations, unless otherwise stated, were carried out at 0–5°C.

Step 1. Enzyme extraction. The washed cells were suspended in 0.05M potassium phosphate buffer, pH 8.0, containing 0.01% 2-mercaptoethanol and sonicated in a 19Kc Kaijo Denki oscillator for 5 min. The intact cells and cell debris were removed by centrifugation at 17,000 \( \times \) \( g \) for 40 min.

Step 2. Protamine treatment. To the supernatant was added 1 ml of 1.5% protamine sulfate solution per 100 mg of enzyme protein with stirring. The bulky inactive precipitate was removed by centrifugation.

Step 3. Ammonium sulfate fractionation. The supernatant was brought to 30% saturation with ammonium sulfate. After 30 min, the precipitate formed was removed by centrifugation at 17,000 \( \times \) \( g \) for 20 min. The supernatant obtained was adjusted to 50% saturation with ammonium sulfate. After 30 min, the precipitate was collected by centrifugation at 17,000 \( \times \) \( g \) for 20 min, dissolved in a small volume of 0.01M potassium phosphate buffer, pH 7.2, and dialyzed against the same buffer overnight. The inactive precipitate formed during dialysis was centrifuged off.

Step 4. TEAE-Cellulose chromatography. The dialyzed enzyme solution was placed on a column of TEAE-cellulose (2.2 \( \times \) 30 cm) equilibrated with 0.01M potassium phosphate buffer, pH 7.2. Elution was carried out stepwise with the same buffer containing various concentrations of sodium chloride. The flow rate was 25 ml per hour and 10 ml aliquots of eluate were collected. The elution of protein was followed by measuring the absorbance at 280 m\( \lambda \). Fig. 1 shows the typical elution pattern of enzyme.

![Fig. 1. Elution Pattern of the Enzyme from TEAE-Cellulose Column.](image)

A summary of the purification procedure is given in Table I. The ratio of leucine racemase activity to \( \alpha \)-aminobutyrate racemase activity was determined at every step of the purification. The ratio remained approximately constant. This finding suggests that

TABLE I. SUMMARY OF PURIFICATION OF ENZYME*

<table>
<thead>
<tr>
<th>Step</th>
<th>Total protein (mg)</th>
<th>Specific activity</th>
<th>Yield (%)</th>
<th>Leucine**</th>
<th>(\alpha)-ABA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Enzyme extraction</td>
<td>1748</td>
<td>692</td>
<td>0.396</td>
<td>100</td>
<td>0.61</td>
</tr>
<tr>
<td>2. Protamine treatment</td>
<td>1380</td>
<td>553</td>
<td>0.400</td>
<td>80</td>
<td>0.63</td>
</tr>
<tr>
<td>3. Ammonium sulfate fractionation</td>
<td>524</td>
<td>348</td>
<td>0.665</td>
<td>50.3</td>
<td>0.58</td>
</tr>
<tr>
<td>4. TEAE-Cellulose chromatography</td>
<td>13.7</td>
<td>75</td>
<td>5.48</td>
<td>10.8</td>
<td>0.60</td>
</tr>
</tbody>
</table>

* \(\alpha\)-Aminobutyrate was employed as a substrate.

** The ratio of leucine racemase activity to \(\alpha\)-aminobutyrate racemase activity.

Leucine and \(\alpha\)-aminobutyrate may be racemized by a single enzyme or very resemble enzymes.

Properties of enzyme

Effect of incubation time and enzyme concentration. With the partially purified enzyme, the enzyme activity as function of incubation time and enzyme concentration was investigated. The formation of p-\(\alpha\)-aminobutyrate and p-leucine from their L isomers proceeded as a function of time and enzyme concentration as indicated in the preceding paper.

Effect of pH on enzyme activity. The effect of pH on the activity of enzyme is shown in Fig. 2. Optimal activities for \(\alpha\)-aminobutyrate racemization and leucine racemization were observed at pH about 8.5. The racemization of leucine was less sensitively influenced by the pH of reaction mixtures than the racemization of \(\alpha\)-aminobutyrate.

Effect of temperature on enzyme activity. The effect of incubation temperature on both the activities was demonstrated in Fig. 3. Maxi-

FIG. 2. Effect of pH on Enzyme Activity.
(a) \(\alpha\)-aminobutyrate racemase reaction. (b) leucine racemase reaction.

\[\text{Specific activity vs. pH}\]

\[\text{pH}\]

FIG. 3. Effect of Incubation Temperature on Enzyme Activity.

\[\text{Specific activity vs. Temperature (°C)}\]

\[\text{Temperature (°C)}\]
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Enzymatic activities were observed to be at 50°C for both the reactions.

Effect of heating on enzyme activity. On heating, the enzyme was stable up to 37°C, but it was markedly inactivated above 47°C (Fig. 4). The enzyme activity racemizing α-aminobutyrate and the activity racemizing leucine were very stable in the pH region 6.5 to 8.0 and was considerably labile above pH 8.0, when the enzyme was heated at 50°C for 10 min. Both the pH-stability curves showed close similarity.

The enzyme was heated in 0.1 M pyrophosphate buffer, pH 8.3, at 50°C and 55°C for a given period. After being cooled rapidly, the enzyme activity was determined. As shown in Fig. 5, on heating the enzyme, α-aminobutyrate racemase activity decreased in parallel with leucine racemase activity.

Effect of substrate concentration on enzyme activity. The enzyme activities catalyzing the racemization of α-aminobutyrate and leucine were investigated in various concentrations of substrates. A plot of the reciprocal of reaction velocity against the reciprocal of substrate concentration is shown in Fig. 6. The apparent Michaelis constants for α-aminobutyrate and leucine were calculated by the method of Lineweaver and Burk12) to be 1.8×10⁻² M and 2.0×10⁻² M, respectively.

Effect of pyridoxal phosphate. The effect of pyridoxal phosphate on the enzyme activities was investigated. The activities were enhanced approximately 20% by addition of pyridoxal phosphate (final concentration: $10^{-4}$ M). This fact suggests that the enzyme may require pyridoxal phosphate for the maximal activity and that the preparation may be partially resolved, although further work is needed to elucidate the content and binding of the coenzyme.

Effect of inhibitors on enzyme activities. The effect of several inhibitors on the activities were investigated (Table II). Hydroxylamine and D-cycloserine inhibited both the activities approximately 70% and 30%, respectively. L-Penicillamine showed slightly inhibitory effect at a concentration of $10^{-3}$ M, and none of isonicotinic acid hydrazide, semicarbazide, $p$-chloromercuribenzoic acid (PCMB), monooiodoacetic acid, arsenite, sodium fluoride, ethylenediaminetetraacetic acid (EDTA) and riboflavin had influence on the activities. Inhibition caused by D-cycloserine was partially reduced with pyridoxal phosphate as reported previously. Inhibition by hydroxylamine was also eliminated by addition of higher concentration of pyridoxal phosphate (Table III). When the enzyme was incubated with hydroxylamine ($10^{-2}$ M, pH 7.0) at room temperature for a few min, followed by dialysis against 0.01 M potassium phosphate buffer, pH 7.2, at 4°C, both the activities were lost in the absence of the coenzyme. However, the almost full activities were recovered by addition of pyridoxal phosphate ($10^{-3}$ M).

### Table II. Effect of inhibitors on enzyme activity

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration ($\times 10^{-3}$ M)</th>
<th>Inhibition (%)</th>
<th>Leucine racemase reaction</th>
<th>$\alpha$-Amino butyrate racemase reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isonicotinic acid hydrazide</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Semicarbazide</td>
<td>1</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>L-Penicillamine</td>
<td>1</td>
<td>6</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>D-Cycloserine</td>
<td>1</td>
<td>35</td>
<td>33</td>
<td>33</td>
</tr>
<tr>
<td>Hydroxylamine</td>
<td>1</td>
<td>73</td>
<td>68</td>
<td>68</td>
</tr>
<tr>
<td>PCMB</td>
<td>0.1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Monooiodoacetic acid</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Arsenite</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Sodium fluoride</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>EDTA</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>0.1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

The enzyme activity was determined manometrically.

### Table III. Effect of hydroxylamine and pyridoxal phosphate

<table>
<thead>
<tr>
<th>Concentration ($\times 10^{-3}$ M)</th>
<th>Hydroxylamine</th>
<th>Pyridoxal phosphate</th>
<th>$\alpha$-Amino butyrate racemase reaction</th>
<th>Leucine racemase reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>53.9</td>
<td>53.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>90.0</td>
<td>89.2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The enzyme activity was determined manometrically.

and D-cycloserine inhibited both the activities approximately 70% and 30%, respectively. L-Penicillamine showed slightly inhibitory effect at a concentration of $10^{-3}$ M, and none of isonicotinic acid hydrazide, semicarbazide, $p$-chloromercuribenzoic acid (PCMB), monooiodoacetic acid, arsenite, sodium fluoride, ethylenediaminetetraacetic acid (EDTA) and riboflavin had influence on the activities. Inhibition caused by D-cycloserine was partially reduced with pyridoxal phosphate as reported previously. Inhibition by hydroxylamine was also eliminated by addition of higher concentration of pyridoxal phosphate (Table III). When the enzyme was incubated with hydroxylamine ($10^{-2}$ M, pH 7.0) at room temperature for a few min, followed by dialysis against 0.01 M potassium phosphate buffer, pH 7.2, at 4°C, both the activities were lost in the absence of the coenzyme. However, the almost full activities were recovered by addition of pyridoxal phosphate ($10^{-3}$ M).

**DISCUSSION**

A new amino acid racemase catalyzing the conversion of either $D$ or $L$ enantiomorph of leucine and $\alpha$-aminobutyrate to the racemates was partially purified from the cell-free extract of *Pseudomonas striata*. The ratio of leucine racemase activity to $\alpha$-aminobutyrate racemase activity was kept constant during the purification. The effects of the pH of reaction system and the incubation temperature on the $\alpha$-aminobutyrate racemization bear a close parallel to those on the leucine racemization. In addition, these activities of enzyme were diminished in parallel by heating the enzyme and the similar pH-activity curves were obtained. Such findings suggest that the racemizations of leucine and $\alpha$-aminobutyrate may be catalyzed by a single racemase or at least the enzymes with very close
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resemblance. Further purification will throw light on the individuality of the enzyme.

The partially purified enzyme was activated about 20% with pyridoxal phosphate. The enzyme was inhibited by d-cycloserine and hydroxylamine, which are regarded as the specific inhibitors for vitamin B6 enzymes. Such inhibition was released with pyridoxal phosphate. The racemase was resolved to the inactive form of enzyme by incubation with hydroxylamine, followed by dialysis. In the absence of added pyridoxal phosphate, the resolved enzyme has substantially no activity and regains the almost full activity by addition of the coenzyme. FAD, FMN and riboflavin have no influence on the activity as reported in the previous paper, although glutamate racemase and alanine racemase require FAD, and both FAD and pyridoxal phosphate as the coenzyme, respectively. It is suggested that only pyridoxal phosphate functions as the cofactor for this enzyme racemizing leucine and α-aminobutyrate. The binding of pyridoxal phosphate is under investigation.

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