Kinetic Studies on \( \alpha \)-Aminoisobutyrate Decomposing Enzyme

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An enzyme which catalyzes a decomposition of \( \alpha \)-aminoisobutyrate (AIB) was purified and its kinetic properties were investigated. Michaelis constants for AIB decomposing reaction are able to be calculated by Ping Pong initial velocity equation. This enzyme catalyzes also \( \alpha \)-ketobutyrate transamination as well as AIB decomposing reaction. Approximately equal values of Michaelis constants were obtained for \( \alpha \)-ketobutyrate and pyridoxal 5'-phosphate (PLP), which are common substrates of both reactions.

In higher concentration of the enzyme, transamination between PLP and AIB or L-alanine was detected, whereas the reaction between pyridoxamine 5'-phosphate and pyruvate was not observed. These results are probably ascribed to a difference in affinity of two coenzymes for the enzyme.

Several studies\(^{1-3} \) on the enzyme which decomposes \( \alpha \)-aminoisobutyrate (AIB) to acetone, carbon dioxide and an amino compound suggest that transamination and decarboxylation of AIB are catalyzed by a single enzyme. Bailey and his coworkers\(^{1,4} \) named this reaction a decarboxylation-dependent transamination and discussed the difference between the mechanism of this transamination and that of ordinary transamination. In addition, it was found by the authors\(^{5} \) that L-alanine: \( \alpha \)-ketobutyrate transamination was catalyzed by the AIB decomposing enzyme. To confirm the early conclusion that this transamination and decomposition of AIB are catalyzed by a single enzyme, its kinetic properties will be described in this paper.

METHODS

Enzyme. Purification of AIB decomposing enzyme from \textit{Pseudomonas} sp. was performed by a modification of the method described previously.\(^{6} \)

Step 1. To each 100 ml of crude extract from cells (240 g) grown in AIB medium, 34 g of ammonium sulfate was added. The precipitate obtained by centrifugation was suspended in 25% (w/w) ammonium sulfate solution. Then, active fractions were collected by ammonium sulfate gradient solubilization.\(^{7} \) The pH of the enzyme solution was always kept at 7.5.

Step 2. Protein was precipitated by addition of ammonium sulfate (34 g/100 ml) from the active fractions, centrifuged and dissolved in 0.1M triethanolamine-HCl buffer, pH 7.5. After dialysis against the same buffer and centrifugation to remove a precipitate, the enzyme solution was applied to a DEAE-cellulose column (4.6 × 40 cm) equilibrated with 0.1 M triethanolamine-HCl buffer, pH 7.5. A linear gradient elution from the column was carried out with the same buffer in the range of 0.15 to 0.35 M. After the active fractions were treated with ammonium sulfate (34 g/100 ml), the resulting precipitate was collected by centrifugation and dissolved in 0.1 M triethanolamine-HCl buffer, pH 7.5, being followed by dialysis against the same buffer.

Step 3. The dialyzed solution was fractionated by DEAE-Sephadex A-50 column (3.0 × 50 cm) chromatography. For equilibration of the ion exchanger, 0.1 M triethanolamine-HCl buffer, pH 7.5 was used, and for gradient elution, 0.2 M and 0.5 M the same buffer was employed. To the fractions containing the enzyme, ammonium sulfate was added (34 g/100 ml), and the resulting precipitate was dissolved in 0.1 M potassium phosphate, pH 7.5.

Step 4. The enzyme solution was passed through a Bio-Gel A-0.5 m column (3.0 × 58 cm) equilibrated with 0.1 M potassium phosphate, pH 7.5, and the most active fractions were pooled.

A highly purified enzyme preparation was obtained by a repeated operation of steps 3 and 4. No activity was detected in this preparation without addition of PLP. One unit of the enzyme activity represents the amount of enzyme forming 1 \( \mu \)mole of acetone in 0.2 ml of 0.1 M potassium phosphate buffer, pH 7.5, containing 0.1 \( \mu \)M PLP, 75 \( \mu \)M AIB and 10 \( \mu \)M pyruvate per min at 30°C.
TABLE I. PURIFICATION OF AIB DECOMPOSING ENZYME

<table>
<thead>
<tr>
<th>Step</th>
<th>Protein (mg)</th>
<th>Activity (u)</th>
<th>Specific activity (u/mg)</th>
<th>Yield (%)</th>
</tr>
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<tr>
<td>Crude extract</td>
<td>22300</td>
<td>ADE 2070</td>
<td>ABT 2040</td>
<td>ADE 0.093 ABT 0.091</td>
</tr>
<tr>
<td>1. Ammonium sulfate</td>
<td>6140</td>
<td>2020</td>
<td>2300</td>
<td>0.33 0.37</td>
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<tr>
<td>1. DEAE-cellulose</td>
<td>910</td>
<td>1480</td>
<td>1270</td>
<td>1.6 1.4</td>
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<tr>
<td>3. DEAE-Sephadex</td>
<td>246</td>
<td>988</td>
<td>870</td>
<td>4.0 3.5</td>
</tr>
<tr>
<td>4. Bio-Gel A-0.5 m</td>
<td>163</td>
<td>840</td>
<td>667</td>
<td>5.2 4.1</td>
</tr>
<tr>
<td>5. DEAE-Sephadex</td>
<td>57.4</td>
<td>428</td>
<td>366</td>
<td>7.5 6.3</td>
</tr>
<tr>
<td>6. Bio-Gel A-0.5 m</td>
<td>25.4</td>
<td>254</td>
<td></td>
<td>10</td>
</tr>
</tbody>
</table>

ADE: AIB decomposing enzyme activity.
ABT: L-Alanine: α-ketobutyrate aminotransferase activity.

Estimation of initial velocity. Initial velocity of the AIB decomposing reaction was estimated by determination of acetone formed in 0.1 or 0.05 M potassium phosphate buffer, pH 7.5, containing PLP (0.1 mM in final concentration, unless otherwise noted), α-keto acid and AIB. A final volume of 0.2, 2.0 or 4.0 ml was used in each experiment, depending on the amount of acetone formed and pyruvate added. After the enzyme was preincubated with PLP and α-keto acid for 10 min at 30°C, the reaction was started by the addition of AIB and stopped by acidification with hydrochloric acid. Then the reaction mixtures which were diluted to 5 ml with water were mixed with 5 ml of 32% sodium hydroxide solution and 0.5 ml of 20% salicylaldehyde solution in ethanol. After being kept for 2 hr at 30°C, optical density at 500 nm was measured. Formation of 1 μmole of acetone caused an increase of 1.21 in optical density. The more amount of pyruvate than 2 μmole disturbed the acetone determination owing to the high blank value. In order to avoid this interference, degradation of pyruvate by enough amount of 3% hydrogen peroxide solution and removal of excess peroxide by catalase were required before acetone determination.

Initial velocity of L-alanine: α-ketobutyrate transamination was estimated by determination of pyruvate formed in 2.5 ml of 0.1 M potassium phosphate buffer, pH 7.5, containing 0.1 mM PLP, α-ketobutyrate and L-alanine at 30°C. Enzyme, PLP and α-ketobutyrate were preincubated for 10 min at 30°C. The reaction was started by the addition of L-alanine and stopped by the addition of 0.2 ml of 3 N HCl. Then, the mixture was mixed with 1.5 ml of 62.5% potassium hydroxide and with 0.3 ml of 3% salicylaldehyde solution in ethanol, and kept at 30°C. Just after 30 min optical density at 470 nm was measured. Formation of 1 μmole of pyruvate caused an increase of 0.790 in optical density.

RESULTS

α-Aminoisobutyrate: pyridoxal 5′-phosphate aminotransferase activity of α-aminoisobutyrate decomposing enzyme

It is generally shown that enzymatic transamination consists of two half reactions. When AIB decomposing enzyme was incubated with AIB and PLP in the absence of pyruvate, a change in an absorption spectrum and acetone formation were observed (Fig. 1). In the

Extinction coefficients. For estimation of PLP and pyridoxamine 5′-phosphate (PMP), molar extinction coefficients of 4900 at 388 nm for PLP and of 8300 at 325 nm for PMP, were used.

![Fig. 1. Reaction of Pyridoxal 5′-Phosphate with α-Aminoisobutyrate.](attachment:image)

AIB decomposing enzyme: 0.65 u/ml. AIB: 25 mM, PLP: 0.26 mM.
1: optical density at 330 nm.
2: optical density at 390 nm.
3: acetone.
absence of the enzyme, the change in the spectrum was nearly negligible under the same conditions. The reaction mixture showed purple fluorescence on exposure to ultraviolet light (365 nm). This fluorescent substance was isolated by Sephadex G-10 gel filtration and identified as PMP by absorption and fluorescence spectra (Fig. 2). Conversion of

![Absorption and Fluorescence Spectra of a Reaction Product of Pyridoxal 5'-Phosphate and α-Aminoisobutyrate.](image1)

**FIG. 2.** Absorption and Fluorescence Spectra of a Reaction Product of Pyridoxal 5'-Phosphate and α-Aminoisobutyrate.

Upper line: reaction product. Lower line: pyridoxamine 5'-phosphate (4.5 × 10^-5 M).

---: absorption spectrum, M/10 potassium phosphate, pH 7.5.

-----: fluorescence spectrum, M/500 potassium phosphate, pH 7.5, exciting wavelength; 330 nm.

![Reaction of Pyridoxal 5'-Phosphate with L-Alanine, and Pyridoxamine 5'-Phosphate with Pyruvate.](image2)

**FIG. 3.** Reaction of Pyridoxal 5'-Phosphate with L-Alanine, and Pyridoxamine 5'-Phosphate with Pyruvate.

AIB decomposing enzyme: 0.79 u/ml. M/10 potassium phosphate, pH 7.5, 30°C.

(1): O.D. at 330 nm in solution containing 0.1 mM PLP and 10 mM L-alanine. An arrow indicates the addition of 20 mM pyruvate. (2): O.D. at 390 nm in the same mixture as in (1). (3): O.D. at 330 nm in solution containing 0.1 mM PMP and 10 mM Pyruvate. (4): O.D. at 390 nm in the same mixture as in (3).

![Reciprocal Plots of AIB Decomposing Reaction Rate against Concentration of Pyridoxal 5'-Phosphate.](image3)

**FIG. 4.** Reciprocal Plots of AIB Decomposing Reaction Rate against Concentration of Pyridoxal 5'-Phosphate.

Preincubation condition: AIB decomposing enzyme 0.074 u, pyruvate 2 μmoles and PLP; total volume, 0.15 ml; 30°C and 30 min. Start of the reaction: AIB 10 μmoles; final volume, 0.20 ml.

![Reciprocal Plots of AIB Decomposing Reaction Rate against Concentration of Pyridoxamine 5'-Phosphate.](image4)

**FIG. 5.** Reciprocal Plots of AIB Decomposing Reaction Rate against Concentration of Pyridoxamine 5'-Phosphate.

Preincubation condition: AIB decomposing enzyme 0.074 u, pyruvate 2 μmoles and PMP; total volume, 0.15 ml; 30°C and 30 min. Start of the reaction: AIB 10 μmoles; final volume, 0.20 ml.
PLP to PMP occurred also in the mixture containing L-alanine instead of AIB (Fig. 3). However, as indicated in Fig. 3, the reverse of this reaction, transamination between PMP and pyruvate, was not detected. These observations may possibly come from difference between the affinity of PLP for the enzyme protein and that of PMP. Figures 4 and 5 show the effect of PLP and PMP concentrations on overall reaction of AIB decomposing enzyme. Each figure shows the cross of two straight lines. These findings can be possibly interpreted from the point of view that equilibrium between apoenzyme and each coenzyme was almost attained in the range of higher concentration of coenzymes, but not in the range of lower concentration within the preincubation period. Preincubation at 0.13 mM PLP for 10 min or at 0.0026 mM PLP for 5 hr was required for the maximum activity of the enzyme. From straight lines in the range of higher concentration, Michaelis constants were calculated to be $3.1 \times 10^{-9}$ M for PLP and $4.8 \times 10^{-5}$ M for PMP. Thus, equilibrium between the apoenzyme and the holoenzyme was investigated by determination of enzyme activity when preincubation was performed at lower concentration of PLP. There was no detectable loss of activity during the preincubation period. From the results in Fig. 6 a dissociation constant, $[\text{apoenzyme}][\text{PLP}]/[\text{holoenzyme}]$, was calculated to be $2.4 \times 10^{-6}$ M. Apparent Michaelis constant for PMP changed according to pyruvate concentration in the preincubation, e.g., $4.8 \times 10^{-5}$ M at 13.3 mM pyruvate, $4 \times 10^{-4}$ M at 1.1 mM pyruvate and $7.8 \times 10^{-4}$ M in the absence of pyruvate. Therefore, it is possible to assume that the dissociation constant for PMP bound on the enzyme is close to $7.8 \times 10^{-4}$ M.

**Effect of substrate concentration on AIB decomposing enzyme activity**

The higher concentrations than 2 mM of pyruvate inhibited AIB decomposing enzyme (Fig. 7), although optimal pyruvate concentration was slightly affected by AIB concentration. Figure 8 indicates that the substrate inhibition is competitive with AIB and inhibition constant, $K_i$, is about $1.3 \times 10^{-2}$ M. At lower concentrations of pyruvate, plots of reciprocal velocities against the reciprocal concentration of one substrate at various fixed concentrations of the second substrate give parallel lines (Fig. 9). Similar results were obtained in the experiments for L-alanine: $\alpha$-ketobutyrate aminotransferase activity of AIB decomposing enzyme. $\alpha$-Ketobutyrate pro-

![Fig. 6. Equilibrium between Holoenzyme and Apoenzyme of AIB Decomposing Enzyme.](image)

Preincubation condition: in dark and at room temperature, (1) AIB decomposing enzyme 0.073 u, PLP 0.0026 mM and pyruvate 1.05 mM; total volume, 1.9 ml. (2) the enzyme, 0.073 u. The enzyme was activated in the presence of 0.05 mM PLP and 20 mM pyruvate, and diluted with eighteen volumes of buffer. Determination of activity: AIB 10 mM; final volume, 2.0 ml. Maximum activity was obtained in the similar experiment to that given in Fig. 4.

![Fig. 7. Effect of Pyruvate Concentration on AIB Decomposing Enzyme.](image)

AIB concentration: (1) 50 mM, (2) 10 mM, (3) 3 mM, (4) 2 mM. Enzyme: 0.073 u.
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FIG. 8. Reciprocal Plots of AIB Decomposing Reaction Rate against Concentration of AIB in Higher Concentrations of Pyruvate.
Pyruvate concentration: (1) 50 mM, (2) 10 mM, (3) 1 mM. Enzyme: 0.073 u.

FIG. 9. Reciprocal Plots of AIB Decomposing Reaction Rate against Concentration of AIB in Lower Concentrations of Pyruvate.
Pyruvate concentration: (1) 0.2 mM, (2) 0.15 mM, (3) 0.1 mM. Enzyme: 0.073 u.

Inhibition by L-alanine, a product of AIB decomposing reaction, was investigated with AIB or pyruvate as a varied substrate (Figs. 10 and 11). L-Alanine produced competitive inhibition with AIB and noncompetitive one with pyruvate.

DISCUSSION

It is suggested that enzymatic decomposition of AIB with pyruvate is composed of two half reactions, as follows:

AIB + Enzyme-PLP → acetone + CO₂ + Enzyme-PMP
Enzyme-PMP + pyruvate →
Enzyme-PLP + L-Alanine

If enzyme-PMP is dissociated into protein and PMP more rapidly than enzyme-PLP into protein and PLP in the first half reaction, a reaction, AIB + PLP → acetone + CO$_2$ + PMP, may proceed. AIB decomposing enzyme catalyzed this reaction (Fig. 1) and ratio of rate of this reaction to that of AIB decomposition in the presence of pyruvate was about 0.001. Transamination between PMP and pyruvate, which corresponds to the second half reaction was not observed, though the enzyme-bound PMP is converted to the bound PLP, because the apoenzyme was activated by PMP in the presence of pyruvate with the same maximum velocity as that with PLP (intercepts of ordinate in Figs. 4 and 5).

It has been reported$^9$ that aspartate $\beta$-decarboxylase catalyses decarboxylation of aspartate and transamination of various $\alpha$-keto acids, that ratio of rate of the latter reaction to the former was 0.002, and that PMP was formed in the half reaction of the PLP enzyme with various amino acids including aspartate. In this reaction the transamination functions to restore the decarboxylase activity by regenerating PLP.

Michaelis constants were calculated according to Ping Pong initial velocity equation$^{10}$ (Table II). Almost equal values were obtained for $\alpha$-ketobutyrate and PLP which are the common substrates in AIB decomposition and L-alanine: $\alpha$-ketobutyrate transamination. These results indicate that both the reactions were catalyzed by a single enzyme. The $K_m$ values for AIB and pyruvate obtained here are different from those obtained with Pseudomonas fluorescens enzyme$^{11}$ and P. cepacia enzyme.$^4$ Patterns of those kinetic data are similar to that for L-aspartate: $\alpha$-ketoglutarate aminotransferase.$^{10}$

These results seem to support a postulation on decarboxylation-dependent transamination by Bailey,$^4$ that AIB decomposition includes removal of $\alpha$-carboxylate in place of removal of $\alpha$-hydrogen in the usual transamination.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>for AIB decomposing activity</th>
<th>for L-Alanine: $\alpha$-ketobutyrate aminotransferase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIB</td>
<td>$2.1 \times 10^{-3}$ M</td>
<td>$7.2 \times 10^{-4}$ M</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>$1.2 \times 10^{-4}$</td>
<td>$4.8 \times 10^{-5}$</td>
</tr>
<tr>
<td>$\alpha$-Ketobutyrate</td>
<td>$7.2 \times 10^{-4}$</td>
<td>$7.2 \times 10^{-4}$ M</td>
</tr>
<tr>
<td>L-Alanine</td>
<td></td>
<td>$4.8 \times 10^{-5}$</td>
</tr>
<tr>
<td>PLP</td>
<td>$3.1 \times 10^{-6}$</td>
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### REFERENCES