Mechanism of Proline Production by *Kurthia catenaforma*

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The fate of aspartic acid used for proline fermentation by *Kurthia catenaforma* was traced by using aspartic acid-\( ^{14}C \). The radioactivities of proline and glutamic acid increased with the disappearance of aspartic acid. After 40 hr, aspartic acid disappeared from the medium and radioactive \( \alpha \)-ketoglutaric acid was detected. The radioactivity of proline reached 44% of aspartic acid radioactivity at 40 hr. The specific radioactivities of these amino acids and of \( \alpha \)-ketoglutaric acid supported the notion that proline is produced mainly from aspartic acid via \( \alpha \)-ketoglutaric acid and glutamic acid. Since the levels of glutamic acid dehydrogenases (EC 1.4.1.2 and EC 1.4.1.4) were low in this organism, it appears that the nitrogen atom of aspartic acid enters proline by the action of aspartate aminotransferase (EC 2.6.1.1). The mechanism of proline production is discussed on the basis of the role of aspartic acid in this fermentation.

It was previously shown that growth-promoting effect of aspartic acid results from its rapid uptake into *Kurthia catenaforma* (3). However, a very high concentration of aspartic acid was required for proline fermentation, the amount being typical of compounds serving as carbon and nitrogen sources. In microorganisms, proline ordinarily is derived from glutamic acid, but we considered that aspartic acid could act as a precursor of proline if aspartic acid is converted into \( \alpha \)-ketoglutaric acid via oxaloacetic acid and citric acid in this fermentation.

Fermentation using L-aspartic acid-\( ^{14}C \) has been performed to confirm a role of aspartic acid in this fermentation. Furthermore, the activities of glutamic acid dehydrogenases and of aspartate aminotransferase have been assayed to determine the origin of the nitrogen atom in proline.

MATERIALS AND METHODS

**Organism and growth conditions.** The organism used in this study was a serine-requiring mutant, strain 45, of *K. catenaforma* IAM 1996. Unless otherwise stated, a medium containing 6% glucose, 0.5% urea, 0.3% \( \text{NH}_4\text{Cl} \), 0.7% corn steep liquor, 1.1% casein hydrolysate, 2% \( \text{K}_2\text{HPO}_4 \), 0.1% \( \text{MgSO}_4 \cdot 7\text{H}_2\text{O} \), and 2% L-aspartic acid-\( ^{14}C \) (2.63 \( \times \) 10\(^4\) counts per min per \( \mu \) mole) was employed. Glucose was autoclaved separately and added aseptically. The organism was grown in 500-ml shaking flasks containing 30 ml of the medium at 30°C with reciprocal shaking (140 rev/min, 8-cm stroke). The fermentation was carried out by passing sterile air through the flask. Carbon dioxide formed was collected in Hyamine hydroxide and 0.1 N \( \text{NaOH} \) solution.

**Analytical methods.** The respective culture broth withdrawn at varying times was fractionated as shown in Fig. 1. Paper chromatography of amino acids and organic acids was carried out with n-butanol-acetic acid-water (4:1:1) and with ethanol-28% ammonium hydroxide-water (76:1:3), respectively. For chromatography of 2,4-dinitrophenylhydrzone, n-butanol-ethanol-water (7:1:2) was used. Radioactive compounds on chromatograms were located with radioautography. After the compounds were extracted from paper with water, the radioactivities of each fraction were determined by the usual techniques of liquid scintillation counting. A suitable portion of the fraction dissolved in Hyamine hydroxide was added to a toluene solution containing phenylbiphenylloxadiazole (4 g/liter) and 1,4-di(2,5-phenyloxazole)benzene (100 mg/liter). Counting was done in a Packard Tri-Carb liquid scintillation spectrometer. Appropriate corrections for quenching were made by using an internal standard. Specific radioactivity was expressed as counts per minute per micromole.

Proline and aspartic acid in culture broth were assayed microbiologically by using *Leuconostoc mesenteroides* P-60. Glutamic acid in culture broth was determined by glutamic decarboxylase (L-glutamate 1-carboxy-lyase; EC 4.1.1.13) from *Escherichia coli* Crooks (9). Glucose was estimated by using the
Shaking culture

Culture broth → CO₂

| Supernatant | Sediment(Cells) |
|-------------|-----------------|
| Retained elution with 2,5N-NH₄OH | Effluent Amberlite IR-120(H) |
| Amino acids | Organic acids |
| paper chromatography | addition of 2,4-dinitrophenylhydrazine |
| Aspartic acid | 2,4-Dinitrophenylhydrazone paper chromatography |
| Glutamic acid | α-Ketoglutaric acid |
| Proline | |

**FIG. 1. Outline of fractionation of the culture.**

The method of Somogyi (8). α-Ketoglutaric acid was measured by using the method of Friedman and Haugen (1). After being separated by paper chromatography, proline was determined colorimetrically by the acidic ninhydrin reaction (6), and aspartic acid and glutamic acid were determined by the ninhydrin reaction (10).

The cells cultured for 16, 24, and 40 hr were harvested by centrifugation. The preparation of cell-free extracts and the assay of aspartate aminotransferase (L-aspartate:2-oxoglutarate aminotransferase, EC 2.6.1.1) have been described elsewhere (3). For glutamate dehydrogenases (L-glutamate: NAD oxidoreductase, EC 1.4.1.2, and L-glutamate: NADP oxidoreductase, EC 1.4.1.4), the reaction mixture contained: tris(hydroxymethyl)aminomethane-hydrochloride (pH 7.6), 100 μmoles; l-glutamic acid, 30 μmoles; nicotinamide adenine dinucleotide (NAD) or nicotinamide adenine dinucleotide phosphate (NADP), 3 μmoles; and crude extracts (2 to 6 mg of protein) in a final volume of 3 ml. The initial velocity was assayed by increase in optical density at 340 nm caused by the formation of NADH or NADPH under anaerobic conditions. Protein concentrations were determined by using the method of Lowry et al. (5). Specific activities are expressed as nanomoles of products formed per minute per milligram of extract protein.

For the estimation of growth, the fermentation broth was diluted 1:10 with saline, and the optical density was measured at 660 nm with a Hitachi photoelectric photometer (EPO-B type).

L-Aspartic acid-U-¹⁴C was purchased from the Daiichi Pure Chemicals Co. (Tokyo). Other chemicals used were reagent grade from several sources.

**RESULTS**

Changes during fermentation. The fermentation using L-aspartic acid-U-¹⁴C showed a pattern identical with that of the normal fermentation previously reported (2), although the aeration was carried out by passing sterile air through the flask (Fig. 2). Production of L-proline increased until 48 hr, at which time the amount of L-proline reached 26.7 mg/ml.

**FIG. 2. Changes during fermentation of L-proline.**

Symbols: O, L-proline; □, L-aspartic acid; ■, L-glutamic acid; ○, α-ketoglutaric acid; Δ, glucose; ●, growth; ▲, pH.
Throughout the experiment, residual aspartic acid and glucose decreased as usual. Growth and pH pattern were also normal. Therefore, the behavior of aspartic acid in this isotopic experiment was considered to be the same as that in the previous fermentation (2) employing unlabeled aspartic acid.

**Incorporation of L-aspartic acid-U-\(^{14}\)C into L-proline.** The radioactivities of the respective fractions in the culture using L-aspartic acid-U-\(^{14}\)C are shown in Fig. 3. We found that 57% of the total radioactivity remained in the amino acid fraction, even at the later stage of fermentation. The observation that aspartic acid disappeared from the medium at 40 hr (Fig. 2) suggests that other amino acids are derived from aspartic acid. The radioactivity of carbon dioxide was high and reached about one-fourth that of aspartic acid. Several per cent of the activity was found in the organic acid fraction and in the cells, but no activity was observed in the sugar fraction.

On the paper chromatogram, aspartic acid, glutamic acid, proline, and \(\alpha\)-ketoglutaric acid were separated from the amino acid fraction and from the organic acid fraction, and changes in their radioactivities were assayed (Fig. 4). The increase in the radioactivities of proline and glutamic acid proceeded in response to the decrease in aspartic acid. The radioactivity of proline paralleled production of proline and occupied the major part of the activity of the amino acid fraction after 40 hr. These results suggest that the carbon skeleton of proline is derived chiefly from aspartic acid. On the other hand, radioactivity of glutamic acid was temporarily observed while aspartic acid was still present in the medium and was reduced after 40 hr when aspartic acid disappeared from the medium. The radioactivity of \(\alpha\)-ketoglutaric acid increased while that of glutamic acid decreased.

The specific radioactivities of the amino acids and the keto acid as calculated from these data are shown in Table 1. Aspartic acid showed constant specific radioactivity throughout the culture period. This fact indicates that aspartic acid is not derived from glucose in this fermentation. The specific radioactivity of \(\alpha\)-ketoglutaric acid was less than half that of aspartic acid and was slightly reduced with culture time. Consequently, \(\alpha\)-ketoglutaric acid was considered to be formed mainly from aspartic acid through the tricarboxylic acid cycle. Since the specific radioac-

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**Fig. 3.** Changes, during fermentation, in the radioactivities of the respective fractions. Symbols: ○, total; ●, broth; ●, amino acid; □, organic acid; ■, CO\(_2\); ▲, cells.

**Fig. 4.** Changes, during fermentation, in the incorporation of aspartic acid-U-\(^{14}\)C into \(\alpha\)-ketoglutaric acid, glutamic acid, and proline. Symbols: ■, aspartic acid; ▲, \(\alpha\)-ketoglutaric acid; □, glutamic acid; ○, proline.
Table 1. Changes in the specific radioactivities of aspartic acid, α-ketoglutaric acid, glutamic acid, and proline

| Culture time (hr) | Specific radioactivitya |
|-------------------|-------------------------|
|                   | Aspartic acid | α-Keto-glutaric acid | Glutamic acid | Proline |
| 0                 | 2.63         | 1.26                 | 1.01          | 1.17    |
| 16                | 2.54         | 1.18                 | 1.04          | 1.06    |
| 24                | 2.55         | 1.00                 | 0.98          | 1.02    |
| 32                | 2.51         | 0.95                 | 0.93          | 0.85    |
| 40                | 2.90         | 0.90                 | 0.89          | 0.80    |

a Specific radioactivity is expressed as counts per minute per micromole $\times 10^{-4}$.

Activities of glutamic acid and proline were almost the same as that of α-ketoglutaric acid, proline seems to be derived from glutamic acid via α-ketoglutaric acid by a known pathway in microorganisms.

Amination of α-ketoglutaric acid. The above observation that aspartic acid was converted to proline suggests that aspartic acid is first deaminated by aspartate aminotransferase. α-Ketoglutaric acid accumulation was also observed in the medium after the disappearance of aspartic acid. From these results, aspartate aminotransferase was considered to play an important role in amination of α-ketoglutaric acid. To examine this, aspartate aminotransferase and glutamate dehydrogenases were measured as the activity concerned in the amination of α-ketoglutaric acid. An appreciable difference was observed between the activities of both enzymes (Table 2). The activity of glutamate dehydrogenase could be detected with either NAD or NADP, but the activity was low—about one-tenth that in Micrococcus glutamicus, which produces large amounts of glutamic acid (4). On the other hand, the activity of aspartate aminotransferase did not differ significantly from the activities in other microorganisms (3). Accordingly, the difference between the two enzyme activities indicates that aspartic acid also plays a role in proline production by supplying its amino group. We conclude that, in this fermentation, the amination of α-ketoglutaric acid depends mainly on the transamination by aspartic acid, and the resulting oxaloacetic acid is converted to α-ketoglutaric acid. Thus, aspartic acid is utilized not only as carbon source for production of proline, but also as nitrogen source.

Table 2. Comparison of aspartate aminotransferase and glutamate dehydrogenasa

| Culture time (hr) | Specific activitya |
|-------------------|-------------------|
|                   | Aspartate aminotransferase | Glutamate dehydrogenase |
|                   | NAD | NADP |
| 16                | 92  | 0.35 | 0.43 |
| 24                | 63  | 0.25 | 0.33 |
| 40                | 61  | 0.27 | 0.43 |

a Cells were grown on medium consisting of nonlabeled aspartic acid.
a Specific activity is expressed as nanomoles per minute per milligram of protein.

Discussion

These results verify the role of aspartic acid in proline production by K. catenaforma. Based on the role of aspartic acid, the mechanism of proline production is shown in Fig. 5. Aspartic acid acts as amino donor in amination of α-ketoglutaric acid and, in so doing, is converted to oxaloacetic acid. The oxaloacetic acid combines with acetyl-coenzyme A (CoA) derived from glucose, and yields α-ketoglutaric acid through the tricarboxylic acid cycle. Glutamic acid is supplied by repetition of this series of reactions and is metabolized to proline without being released from the cells. Thus, large
amounts of proline are accumulated in the medium.

As described above, proline seems to be derived predominantly from aspartic acid, as indicated by the heavy lines in Fig. 5. However, a part of the carbon may be supplemented as oxaloacetic acid, which is formed by carbon dioxide fixation due to phosphoenolpyruvate carboxylase (EC 4.1.1.31). Consideration of this mechanism is based on Sanwal's model (7), in which phosphoenolpyruvate carboxylase is regulated by feedback inhibition of aspartic acid and activation of acetyl-CoA. A part of the nitrogen of proline may be derived from the assimilation of ammonia due to glutamate dehydrogenase. These considerations are supported by the following results: (i) α-ketoglutaric acid showed a considerably low specific radioactivity compared with aspartic acid; (ii) the specific radioactivity of α-ketoglutaric acid fell gradually with time; and (iii) the molar ratio of proline to aspartic acid in the conversion was 130 to 140%.

The radioactivity of the amino acid fraction reached over 50% of the total activity, even during the later stages of fermentation. The activity of carbon dioxide, nevertheless, reached about 25% of the total activity (Fig. 3). The amount of carbon dioxide agrees with the carbon of carboxylate at the alpha position of aspartic acid, assuming a decarboxylation of citric acid derived from aspartic acid. Accordingly, the amount of carbon dioxide supports the possibility that a large portion of aspartic acid is metabolized through the pathway shown in Fig. 5.

α-Ketoglutaric acid, glutamic acid, and proline showed almost identical specific radioactivities throughout the culture period. This fact indicates that proline is produced from aspartic acid via glutamic acid. This mechanism of proline production is also supported by the fact that proline was produced from glutamic acid when permeability was increased by the presence of a detergent. This observation has been reported and discussed elsewhere (3).

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