The Presence of Glutamate Mutase in a Methanol-Utilizing Bacterium, *Protaminobacter ruber*

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(Received October 1, 1981)

Summary Cell-free extracts of a facultative methylotroph and strict aerobe, *Protaminobacter ruber*, could catalyze formation of β -methylaspartate from glutamate. β -Methylaspartate formed was further converted to mesaconate. From these results, it was found that the cells of *P. ruber* contained a sequential reaction system of glutamate mutase and β -methylaspartase. The level of glutamate mutase activity was almost constant throughout the period of cultivation. When *P. ruber* was grown on several non-one-carbon compounds in addition to methanol as a sole carbon source, the activity of glutamate mutase was not markedly affected by the kinds of carbon sources.

Key Words glutamate mutase, methanol-utilizing bacterium, *Protaminobacter ruber*, vitamin B₁₂-dependent glutamate mutase, β -methylaspartate, methylotroph, facultative

In the previous papers (1-3), we showed that a facultative methylotroph, *Protaminobacter ruber*, produced a considerable amount of vitamin B₁₂ in the forms of methyl-B₁₂ and adenosyl-B₁₂, and contained methionine synthase and methylmalonyl-CoA mutase as B₁₂-dependent enzymes. Although *P. ruber* is a strict aerobe and classified as a non-photosynthetic bacterium, it has some characteristics very similar to those of photosynthetic bacteria such as *Rhodopseudomonas spheroides* and *Rhodospirillum rubrum*. Namely, *P. ruber* forms bacteriochlorophyll under certain conditions (4) and lacks isocitrate lyase (*icl*) which seems to be essential for one-carbon assimilation (5). Therefore, we presumed that *P. ruber* might have some metabolic pathway resembling that of the photosynthetic bacteria and found that it contained glutamate mutase which catalyzed adenosyl-B₁₂-dependent isomerization of glutamate to β -methylaspartate. To date, this mutase has been found in several anaerobically and heterotrophically grown microorganisms such as *Clostridium tetanomorphum* (6), and *R. spheroides* and *R. rubrum* (7,8). In these microorganisms, it has been also found that β -methyl-

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aspartate derived from glutamate by glutamate mutase is converted to mesaconate by β -methylaspartase.

This paper describes the findings on sequential reactions of glutamate mutase and β -methylaspartase in the cells of *P. ruber*.

MATERIALS AND METHODS

Materials. [U- ^{14}C]Glutamic acid was purchased from the Japan Radioisotope Association. All other materials were of analytical grade.

Microorganism. *P. ruber* was grown aerobically on a mineral medium containing methanol (1%, v/v) as a sole carbon source unless especially specified. Detailed methods for the cultivation have been previously described (3). The cells were harvested at the middle logarithmic growth phase.

Enzymatic reactions. Cell-free extracts were prepared as described previously (3). Glutamate mutase was assayed by measuring ^{14}C - β -methylaspartate formed from ^{14}C -glutamate according to the method described by Ohmori *et al.* (8) with some modifications. The reaction mixture contained potassium phosphate buffer (pH 7.8), 15 μmol ; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.5 μmol ; dithiothreitol, 0.5 μmol ; [U- ^{14}C]sodium glutamate, 0.5 μmol (0.5 μCi); adenosyl- B_{12} , 10 nmol; and cell-free extracts (protein 8 mg) in a total volume of 1.5 ml. The reaction was performed at 30°C for 1.5 hr under N_2 in the dark and stopped with 0.5 ml of 1 N HCl. After centrifugation at $21,000 \times g$ for 15 min, authentic β -methylaspartic acid (15 μg) was added to 1.5 ml of the supernatant. The mixture was then passed through a Dowex 50W-X12 (H^+ form) column (1 $\times$ 2 cm). The column was first washed with 10 ml of distilled water to elute organic acids and the acidic amino acids adsorbed on the resin were eluted with 10 ml of 3 N NH_4OH . The acidic amino acid fraction was evaporated to dryness and the residue was dissolved in 0.1 ml of distilled water. Aliquots of this solution (10 to 20 μl) were subjected to paper electrophoresis on Toyo filter paper No. 50. Electrophoresis was allowed to proceed for 3 hr at a potential of 28 V/cm in 0.05 N acetate buffer (pH 3.8). After completion of electrophoresis, the positions of authentic samples were visualized by spraying ninhydrin reagent.

In order to detect ^{14}C -mesaconate from ^{14}C -glutamate, the conditions for the enzymatic reaction were made the same as those described above except that $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ was replaced by the same amount of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ in the reaction mixture. To the organic acid fraction eluted with distilled water from the Dowex column, authentic organic acids shown in Fig. 2 were added. The mixture was continuously extracted with ether for 12 hr and subjected to paper chromatography on Toyo filter paper No. 50 using ether-benzene-formic acid-water (21:9:7:2, v/v) as the developing solvent. The authentic organic acids were visualized by spraying 0.2% neutral bromocresol green in *n*-butanol.

Measurement of radioactivity. The papers after paper electrophoresis or paper chromatography were cut at 0.5 cm intervals and the radioactivity on each piece was

measured in the scintillation mixture consisting of 2,5-diphenyloxazole, 5 g; 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene, 0.3 g; and toluene to a total volume of 1 liter; with a liquid scintillation counter (Packard Tri-Carb 3320-515 type).

RESULTS AND DISCUSSION

Figure 1 shows that β -methylaspartate was definitely identified as the radioactive compound formed from ^{14}C -glutamate by the cell-free extracts of *P. ruber*. This

Fig. 1. Formation of β -methylaspartate from glutamate by cell-free extracts of *P. ruber*. The acidic amino acid fraction obtained as described in MATERIALS AND METHODS was subjected to paper electrophoresis at 28 V/cm for 3 hr in 0.05 N acetate buffer (pH 3.8). Authentic samples were colored with ninhydrin. Abbreviations used are: Glu, glutamic acid; $\beta\text{-CH}_3\text{-Asp}$, β -methylaspartic acid.

Table 1. Influence of adenosyl- B_{12} on the formation of ^{14}C - β -methylaspartate from ^{14}C -glutamate by cell-free extracts of *P. ruber*.

In this experiment, cell-free extracts were treated with charcoal for 20 min on ice with the mild stirring. The complete reaction mixture contained potassium phosphate buffer (pH 7.8), 15 μmol ; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.5 μmol ; dethiothreitol, 0.5 μmol ; $[\text{U-}^{14}\text{C}]$ sodium glutamate, 0.5 μmol (0.5 μCi); adenosyl- B_{12} , 10 nmol; and charcoal-treated cell-free extracts (protein 15 mg) in a total volume of 1.5 ml. Incubation was carried out for 2 hr at 30°C under N_2 in the dark.

Reaction conditions	β -Methylaspartate formed (cpm)
Complete system	1,016
– Adenosyl- B_{12}	852
– Enzyme + Boiled enzyme	38

reaction was allowed to proceed in the presence of CaCl_2 in order to block β -methylaspartase activity and prevent further progress of the reaction. Table 1 shows the effect of adenosyl- B_{12} on formation of β -methylaspartate from glutamate. The addition of adenosyl- B_{12} to the reaction mixture resulted in the increase in the amount of β -methylaspartate formed. In the present experiment, some β -methylaspartate was formed even when adenosyl- B_{12} was not added to the reaction mixture. This β -methylaspartate formation would be due to the presence of endogenous adenosyl- B_{12} which could not be removed from the cell-free extracts even by treatment with charcoal. The experimental results described here indicate that glutamate mutase is the third B_{12} -dependent enzyme found in the cells of *P. ruber*.

The paper chromatogram of the organic acid fraction is shown in Fig. 2. It was found that four different radioactive compounds were formed from ^{14}C -glutamate, and one of them was identified as mesaconate. R_f values of the other three radioactive components coincided with those of authentic succinic, fumaric and malic acids. This datum implies that mesaconate is formed from glutamate through sequential reactions by glutamate mutase and β -methylaspartase, and that the other organic acids were formed through several reactions included in the tricarboxylic acid cycle after conversion of glutamate to α -ketoglutarate by glutamate dehydrogenase.

The relationship between the growth of *P. ruber* and the activity of glutamate mutase was then investigated. As shown in Fig. 3, the enzymatic activity was

Fig. 2. Paper chromatogram of radioactive products derived from ^{14}C -glutamate by cell-free extracts of *P. ruber*. The organic acid fraction obtained as described in MATERIALS AND METHODS was subjected to paper chromatography with ether-benzene-formic acid-water (21:9:7:2, v/v) as the developing solvent. Authentic acids were colored with 0.2% neutral bromocresol green in *n*-butanol. Abbreviations used are: Mal, malic acid; αKG , α -ketoglutaric acid; Suc, succinic acid; Fum, fumaric acid; Mes, mesaconic acid.

Fig. 3. Time course of the growth of *P. ruber* and the activity of glutamate mutase. Glutamate mutase was assayed as described in MATERIALS AND METHODS except that the enzymatic reaction was run for 20 min and 5 mg of protein were used per assay.

Table 2. Formation of ^{14}C - β -methylaspartate from ^{14}C -glutamate by cell-free extracts of *P. ruber* grown on various carbon sources.

The cells were harvested at the middle logarithmic phase. In the enzymatic reaction, incubation was carried out for 20 min at 30°C , and 5 mg of protein were used. Other conditions were as described in MATERIALS AND METHODS.

Carbon source (1%, v/v)	β -Methylaspartate formed (cpm)
Methanol	290
1,2-Propanediol	250
Fructose	230

detected at an approximately constant level throughout the period of cultivation.

P. ruber can grow not only on methanol but also on non-one-carbon compounds such as 1,2-propanediol and fructose as sole carbon sources. Further, this bacterium is also able to produce vitamin B_{12} when grown on these non-one-carbon compounds. Table 2 shows formation of ^{14}C - β -methylaspartate from ^{14}C -glutamate by the extracts of *P. ruber* grown on several carbon sources. The amount of β -methylaspartate formed was not markedly affected by the kinds of carbon sources.

The occurrence of glutamate mutase as one of the B_{12} -dependent enzymes in the cells of *P. ruber* was the first such case in methanol-utilizing microorganisms. In particular, it is of great interest that glutamate mutase could be found in *P. ruber*,

because this bacterium is an obligate aerobe whereas the mutase has been up to now mainly found in microorganisms grown anaerobically and recognized to be important in the anaerobic metabolism of glutamate. Very recently, we proposed an enzymatic pathway for giving glyoxylate from mesaconyl-CoA through several reactions in *icl⁻* *P. ruber* (5). The reactions of glutamate mutase and β -methylaspartase in *P. ruber* might have the role of generating mesaconyl-CoA from α -ketoglutarate.

This work was supported in part by a grant (No. 456062) from the Ministry of Education, Science and Culture of Japan.

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