VITAMIN B_{12} DEPENDENT GLUTAMATE MUTASE ACTIVITY IN PHOTOSYNTHETIC BACTERIA

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SUMMARY

Cell-free extracts of Rhodopseudomonas spheroides and Rhodospirillum rubrum grown photosynthetically on Co^{2+}-supplemented media converted glutamate to β-methylaspartate and further deaminated the latter to form mesaconate. The conversion was inhibited by hog intrinsic factor and the inhibition was restored by coenzyme B_{12}. The catalytic activity of cell-free extracts from the cells grown on Co^{2+}-deficient media was extremely low and enhanced markedly by the addition of coenzyme B_{12} to the reaction system.

Rhodopseudomonas spheroides and Rhodospirillum rubrum can grow anaerobically in the light utilizing glutamate as a carbon source, and produce significant amounts of vitamin B_{12} (1). In Rsp. rubrum, we found that the intracellular level of B_{12} elevated to an appreciable extent when glutamate was used as the sole carbon source. The major B_{12} produced behaved identically with B_{12} coenzyme (DBCC) in paper chromatography, paper ionophoresis and spectrophotometry, and was transformed to aquo-B_{12} by illumination (2). Recently Hayashi and Kamikubo have confirmed the identity of B_{12} produced by Rps. spheroides with DBCC (3).

This communication presents experimental evidence for the involvement of B_{12}-dependent glutamate mutase in the first step of glutamate dissimilation in these photosynthetic bacteria, as in the case of Clostridium tetanomorphum (4) and Acetobacter suboxydans (5,6,7).

MATERIALS AND METHODS

Materials Rsp. rubrum was kindly supplied by Dr. Katsuki, Kyoto Univ. and Rps. spheroides, by Dr. Kamikubo, Hiroshima Univ. Hog intrinsic

factor (8) was the kind gift of Dr. Okuda, Kurume Univ. Uniformly labeled glutamate-^{14}C was purchased from Daiichi Chemical Co., Tokyo, Japan. Other chemicals were obtained from commercial sources.

Growth of organisms The basal medium used for Rps. spheroides was the medium S supplemented with 0.1 % yeast extracts described by Lascelles (1), and that for Rsp. rubrum was the defined medium by Ormerod et al. (9) in which malate was replaced by 0.35 % glutamate and 0.26 % succinate. To obtain B_{12} deficient cells, $CoCl_2$ was omitted from the original media. The microorganisms were cultured anaerobically in the light at 28°. Cells harvested at the earlier logarithmic growth phase were disrupted in a small amount of 0.02 M K-phosphate buffer (pH 7.8) containing 0.07 M mercaptoethanol. The supernatant obtained by centrifugation at 12,000 X g for 30 min. was used as a crude enzyme preparation. Since glutamate mutase activity in Rsp. rubrum was much lower than those of glutamic-oxaloacetic transaminase and glutamate dehydrogenase as compared with that in Rps. spheroides, Rsp. rubrum extracts were applied to a Sephadex G-25 column and the fractions having the activity to convert glutamate to mesaconate were collected by elution with 0.02 M K-phosphate buffer (pH 7.6) containing 0.07 M mercaptoethanol.

Reaction and Analytical Methods The reaction mixtures for the assay of mesaconate formation were as follows. In the case of Rsp. rubrum, an essentially similar composition was employed as reported by Barker et al. (10) except that Tris-Cl buffer (pH 8.5) and mercaptoethanol in the original method were replaced by K-phosphate buffer (pH 8.3) and dithiothreitol, respectively. In the case of Rps. spheroides, the reaction mixture contained Na-glutamate-^{14}C (U) 500 mμmoles (0.3 mCi), dithiothreitol 500 mμmoles, $MgCl_2$ 500 mμmoles, DBCC 10 mμmoles, K-phosphate buffer (pH 7.8) 15 μmoles and cell-free extracts 0.5 ml (15-20 mg protein) in the total volume of 0.85 ml. α,α'-Dipyridyl, an inhibitor of the further decomposition of mesaconate, was not added in this case. The reactions were carried out

in the dark for 2 hrs. at 37° under nitrogen atmosphere. After separated from glutamate-^{14}C and other compounds retained on a small column of Dowex 50 (H^+) (10,11), mesaconate and other organic acids in the effluents were extracted with ether. The ether extract was concentrated to dryness in vacuo and redissolved in a small amount of aqueous solution of authentic unlabeled mesaconate. An appropriate aliquot, e. g. 0.025 ml, was subjected to paper chromatography using a solvent system composed of ether, benzene, formic acid and water (21:9:7:2). The radioactive products were located by Nuclear Chicago automatic scanner, and the radioactivity of the zone corresponding to mesaconate near the front was measured in Bray's solution with Nuclear Chicago liquid scintillation spectrometer.

In order to detect β-methylaspartate, magnesium salts in the above-mentioned reaction mixtures were replaced by the same amount of $CaCl_2$ for the inhibition of β-methylaspartase as described by Barker et al. (12) The radioactive β-methylaspartate formed was adsorbed on a Dowex 50 (H^+) column, and eluted with 3N NH_4OH. The eluate was treated analogously to the case of mesaconate and the resulting solution was applied to paper electrophoresis in 0.05 N acetate buffer (pH 3.75) at 30 V/cm for 1.5 hrs. The paper was cut into 0.5 cm strips and the radioactivity of each strip was measured as described above. In parallel to this experiment, the positions of organic acids as well as amino acids were detected by spraying bromocresol blue and ninhydrin, respectively. In order to confirm the dependence of the reaction upon coenzyme B_{12}, the effect of hog intrinsic factor (8) was also studied.

RESULTS AND DISCUSSION

Formation of mesaconate from glutamate Figure 1 shows the paper chromatogram of the organic acids formed in the incubation mixture of glutamate-^{14}C (U) with cell-free extracts of Rsp. rubrum. Mesaconate was clearly observed near the solvent front as a sharp peak a with Rf value 0.95. This zone was extracted with water and cochromatographed with authentic mesaconate

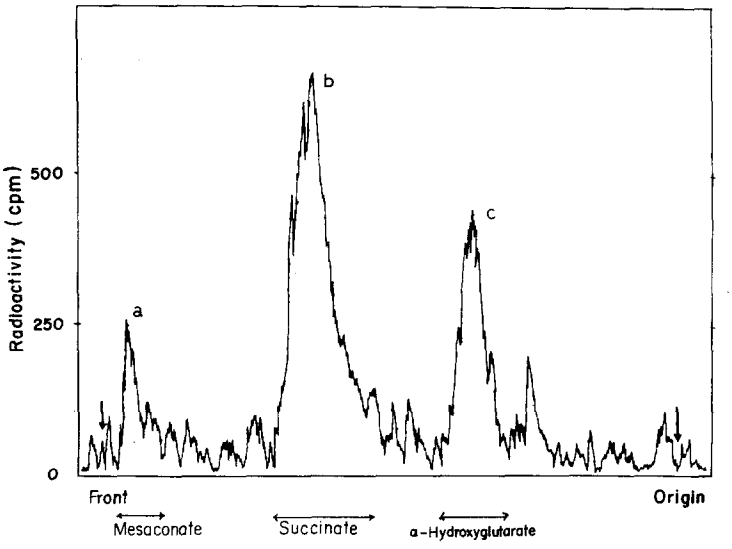

Fig. 1 Paper chromatogram of the organic acids formed in the incubation mixture of glutamate-^{14}C (U) with cell-free extracts of Rsp. rubrum. The solvent system used was a mixture of ether, benzene, formic acid and water (21:9:7:2 (v/v)).

using a mixture of n-amyl alcohol, water and formic acid (20:12:1) as solvent system. The radioactivity also moved identically with authentic mesaconic acid in this solvent system (Rf 0.77). Peaks b and c were identified as succinate and α-hydroxyglutarate, respectively. In the case of Rps. spheroides, an essentially identical result was obtained.

Formation of β-methylaspartate In order to trap the direct product of glutamate mutase reaction, β-methylaspartate, the enzymatic reaction was carried out in the presence of $CaCl_2$ to block β-methylaspartase activity. The amino acid thus formed was separated from organic acids using a Dowex 50 (H^+) column, then applied to paper electrophoresis at 3.75. Figure 2 shows the result obtained by the cell-free extracts of Rps. spheroides. On the anode side of glutamate, a small but obvious peak was detected which coincided with the area of authentic β-methylaspartate. Although the reaction was carried out in the presence of $CaCl_2$, the amount of the acid accumulated was not enough and a considerable amount of mesaconate was still formed under our experimental

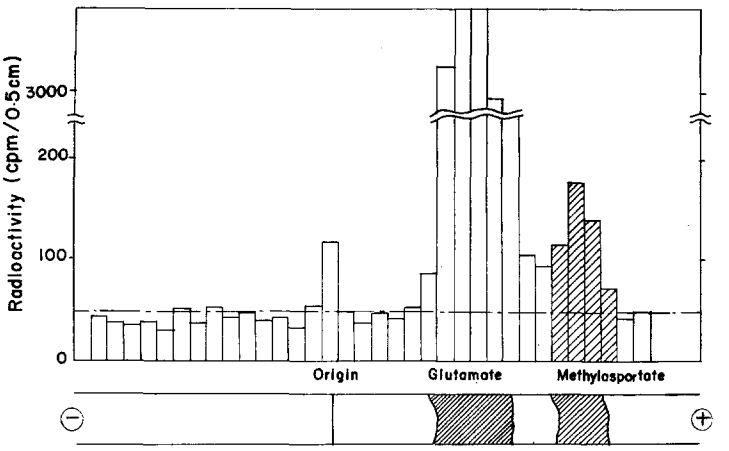

Fig. 2 Detection of β-methylaspartate as a reaction product of glutamate-^{14}C(U) and cell-free extracts of Rps. spheroides performed in the presence of $CaCl_2$. Paper electrophoresis was run at 30 V/cm for 1.5 hrs. in 0.05 N acetate buffer (pH 3.75).

Table 1 Inhibitory effect of hog intrinsic factor (IF) on the conversion of glutamate-^{14}C to mesaconate-^{14}C by a partially purified extracts of Rhodospirillum rubrum.

	Mesaconate formed
	cpm
Complete[1)]	197
minus coenzyme B_{12} (DBCC)	55
minus DBCC, plus IF 0.4 mg[2)]	23

1) The complete system contained 15 μg of DBCC and a partially purified extracts (ca. 5 mg protein) of Rsp. rubrum grown on a Co^{2+}-supplemented medium. For experimental details, see Materials and Methods.

2) This amount was sufficient to inactivate ca. 2 μg of B_{12}.

conditions. Similar results were obtained using Rsp. rubrum extracts.

Inhibitory action of intrinsic factor As shown in Table 1, intrinsic factor exhibited an inhibitory effect on the enzymatic conversion of glutamate to mesaconate mediated by a crude enzyme system of Rsp. rubrum. Since the complete system contained 15 μg of DBCC, the addition of 0.4

mg intrinsic factor(the amount necessary to inactivate ca. 2 μg of B_{12}) resulted in only a weak inhibition. However, the same amount of intrinsic factor added to the system deprived of DBCC exerted a marked inhibitory action. This result support the dependence of the glutamate mutase reaction upon B_{12}.

Effect of coenzyme B_{12} added to the extracts of the cells grown on Co-deficient media The observations mentioned above indicate that glutamate is metabolized, at least to some extent, in Rsp. rubrum and Rps. spheroides according to the following pathway.

Glutamate → β-Methylaspartate → Mesaconate ---→ Pyruvate, Acetyl-CoA

However, the extracts prepared from the cells grown on Co^{2+}-deficient media could hardly catalyze the conversion. In this case, the addition of DBCC caused a remarkable increase in mesaconate formation. Figure 3 shows a typical result of Rps. spheroides. The increment of mesaconate was proportional to the amount of DBCC added. On the other hand, the activity of the extracts of the cells grown on Co^{2+}-sufficient media was

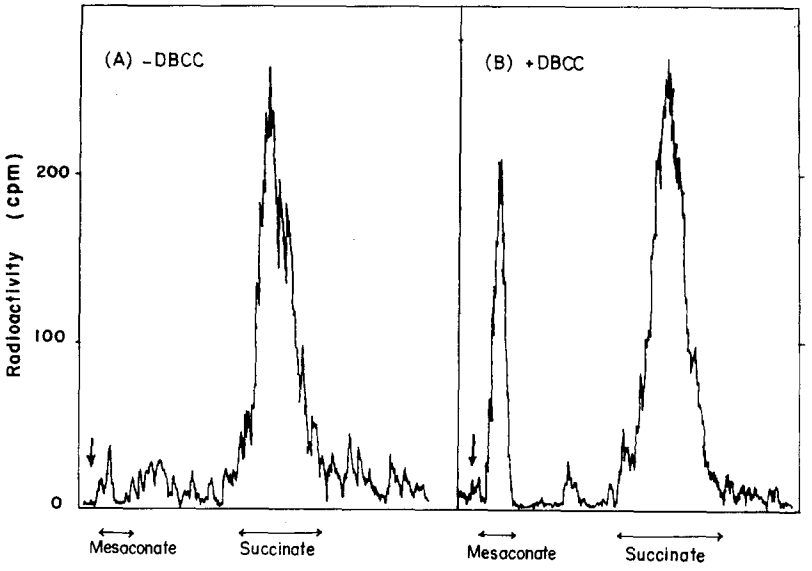

Fig. 3 Effect of coenzyme B_{12} on the formation of mesaconate by the cell-free extracts of Rps. spheroides grown on a Co^{2+}-free medium. The paper chromatography was carried out in a similar way to that in Fig. 1.

not further enhanced. Similar results were obtained using Rsp. rubrum. These facts would imply that glutamate mutase exists as holoenzyme in these photosynthetic bacteria grown in the presence of cobalt ion.

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