VITAMIN B_{12}- DEPENDENT METHIONINE SYNTHESIS IN RHIZOBIUM MELILOTI

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Summary: Methionine, among the various additions to the medium, could only replace cobalt ion or vitamin B_{12} required for the growth of Rhizobium meliloti. It was demonstrated that there exists a vitamin B_{12}-dependent terminal step in the methionine synthesis, that is, N^5-CH_3-tetrahydrofolate-homocysteine transmethylase, which can also catalyze the methyl transfer from CH_3-B_{12} to homocysteine, in the cell-free extracts of Rhizobium meliloti. These facts seem to indicate that the vitamin B_{12}-dependent pathway to methionine functions mainly among the B_{12}-dependent enzymatic systems in the wild-type symbionts and this is the chief nutritional significance of cobalt.

It was found by Ahmed and Evans that cobalt was required for the bacteria within the nodules of inoculated legumes, but was not required for the leguminous plants per se (1). Evans et al. clearly showed in the subsequent papers that cobalt is necessary for normal growth of Rhizobium species (2) and that cobalt deficiency causes the limit of growth as well as the limit of the synthesis of cobamide coenzyme in Rhizobium japonicum and Rhizobium meliloti(3).

Since cobalt was assumed to play a role as a constituent of vitamin B_{12} in these microorganisms, the significance of vitamin B_{12} in the metabolism has been investigated. Until now, two of B_{12}-dependent enzyme systems, methylmalonyl-CoA mutase (4) and ribonucleotide reductase (5)(6) have been found to participate in the metabolism of Rhizobium. Ribonucleotide reductase system was found by analogy with the case of Lactobacillus leichmannii from the fact that cobalt deficiency in R. meliloti caused a morphological change characterized by the production of many abnormally elongated cells (5). However, in contrast with L. leichmannii, additions of deoxyribonucleosides or the related compounds did not substitute for cobalt as a growth factor for R. meliloti. Cowles et al. discussed the possibility of no incorporation of the additions in the cells and the necessity of investigation for other biochemical sites where vitamin B_{12}

compounds function in the metabolism of Rhizobium species (6).

On the basis of these reports, experiments were carried out to find whether there exists any compound to replace cobalt ion for the growth of R. meliloti. If there is such a compound, it will be possible to presume what reaction is main among the B_{12}-dependent enzymatic systems in the bacteria. This paper reports that methionine does substitute for cobalt ion and that methionine is formed by the catalytic action of B_{12}-dependent transmethylase.

MATERIALS AND METHODS

R. meliloti F-28 was a kind gift from Prof. H. J. Evans of Oregon State University and R. meliloti 1480 was kindly supplied by Dr. Y. Yamamoto in Nagoya University. The basic cobalt-deficient medium contained the following constituents per liter: KH_2PO_4, 1.0 g; K_2HPO_4, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.36 g; $Ca(NO_3)_2 \cdot 4H_2O$, 0.06 g; $(NH_4)_2SO_4$, 0.66 g; KNO_3, 0.40 g; $Na_2MoO_4 \cdot 2H_2O$, 0.23 mg; H_3BO_3, 0.28 mg; $MnSO_4 \cdot 4H_2O$, 0.23 mg; $ZnSO_4 \cdot 7H_2O$, 0.18 mg; $CuSO_4 \cdot 5H_2O$, 0.016 mg; $FeCl_3$, 1.0 mg; thiamine hydrochloride, 0.02 mg; pyridoxine hydrochloride, 0.02 mg; riboflavin, 0.02 mg; p-aminobenzoic acid, 0.02 mg; nicotinic acid, 0.02 mg; calcium pantothenate, 0.02 mg; biotin, 0.02 mg, and mannitol, 3.0 g.

The methods used for purification of cultivation medium and cultural techniques were almost similar to those reported by Kliewer et al. (7) with following modifications. $FeCl_3$ was purified by Dowex 50W column chromatography as follows: 0.2 g of $FeCl_3$ in 20 ml of H_2O was applied on the Dowex 50W-X2(H^+) 200-400 mesh, 1.6 x 9.0 cm column. The column was first washed with 50 ml of H_2O and then with 30 ml of 1 N HCl to remove cobalt ion. Finally ferric ion was eluted with 3 N HCl. The eluate was concentrated to dryness with rotary evaporator. To the residue small amount of distilled water was added and it was evaporated completely with rotary evaporator in order to remove HCl. This operation was repeated 2 times. The residue was dissolved in 30 ml of distilled water and the $FeCl_3$ content was determined colorimetrically by using the absorption at 343 mμ.

All constituents of the final medium except biotin and trace elements were further purified by 1-nitroso-2-naphthol extraction. Additions such as

amino acids, succinate, vitamin B_{12} and bases except guanine were purified with P-cellulose column chromatography. In advance, P-cellulose column was washed with 0.1 M potassium phosphate buffer (pH 7.2) and then distilled water. The materials were applied on the column and eluted almost quantitatively with distilled water. The compounds except vitamin B_{12} were also purified with 1-nitroso-2-naphthol extraction methods. There was basically no difference between the two purification methods.

In order to obtain severely cobalt deficient R. meliloti, at least two transfers of the bacteria were made before the final inoculation. The bacteria were grown in a test tube containing 5 ml of the purified medium. Cultures used for inoculation usually had an optical density reading between 0.1 and 0.3 at a wavelength of 600 mμ in a Shimadzu spectronic 20.

For preparation of cell-free extracts, the bacteria were grown in the medium containing the following components per liter: KH_2PO_4, 1.0 g; K_2HPO_4, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.36 g; $CaSO_4 \cdot 2H_2O$, 0.13 g; $FeCl_3$, 4.0 mg; $CoCl_2 \cdot 6H_2O$, 0.06 mg; yeast extract, 1.0 g, and D-mannitol, 3.0 g, The cells were harvested at the end of logarithmic phase of growth by centrifugation at 4,500 x g for 30 min. One liter culture yielded about 2.5 g of fresh cells. Cell-free extracts were obtained by grinding the cells from 1 liter culture with 7 g of alumina and centrifuging the slurry at 15,000 x g for 20 min. Ammonium sulfate fraction up to 70 % of the supernatant was dialyzed against 0.01 M potassium phosphate buffer (pH 7.2) overnight at 4 °C and used for the enzyme assay.

Methyltransferase activity was determined by measurement of the formation of [^{14}C]methionine from [^{14}C]CH_3-B_{12} or N^5-[^{14}C]CH_3-tetrahydrofolate (8)(9). The reaction mixture and incubation conditions were described in the legend of table.

[^{14}C]CH_3-B_{12} was synthesized by the method of Smith et al. (10) and N^5-[^{14}C]CH_3-tetrahydrofolate was obtained from [^{14}C]CH_2O and tetrahydrofolate according to the procedure of Keresztesy and Donaldson (11). S-adenosyl methionine (SAM) was isolated from yeast by the method of Schlenk et al. (12)

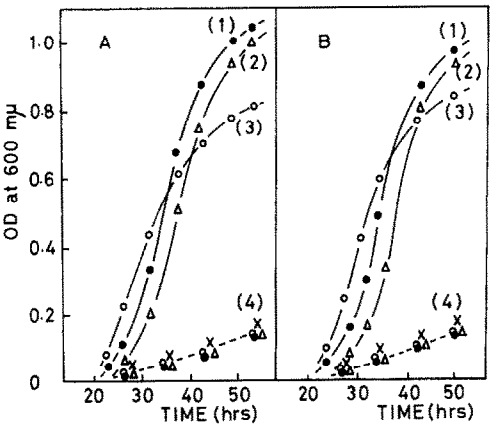

Fig. 1 Time course of R. meliloti 1480 (A) and F-28 (B) cultured in cobalt-deficient media supplemented with various compounds. The concentrations of supplements are as follows: (1) $CoCl_2$ 4.2 mμM (●—●); (2) CN-B_{12} 740 mμM (Δ—Δ); (3) methionine 20 μM (o—o); (4) none (●--●); deoxyribose 50 μM and adenine, guanine, uracil and cytosine, each at 12.5 μM (Δ--Δ); succinate 370 μM (o--o); amino acids except methionine (serine 95 μM; glycine 130 μM; aspartate 75 μM; glutamate 68 μM) (×--×).

Fresh solution of $FMNH_2$ was prepared each day by the catalytic hydrogenation of FMN with PtO_2 (13).

RESULTS AND DISCUSSION

As R. meliloti little grows in the cobalt-deficient medium, it is possible to investigate whether any additions to the medium will promote the growth of the bacteria to a similar degree to cobalt ion. Fig. (A) and (B) show that methionine could substitute for cobalt ion as a growth factor for R. meliloti. The compounds related to B_{12}-dependent ribonucleotide reductase did not replace for cobalt ion (6). Amino acids such as glutamate and aspartate except methionine, succinate, or bases had almost negligible effect for the growth. In the case of vitamin B_{12}, a little longer lag period was observed, probably because some difficulties exist for its incorporation into the cell, or for the transformation of B_{12} into the active form. However, the effect of vitamin B_{12} on the growth of R. meliloti was essentially similar to that of cobalt ion or methionine. Vitamin B_{12} was incorporated intactly into the cell judging from radioassay using [^{57}Co]CN-B_{12} and bioassay with B_{12} auxotroph E. coli 215 and this will be

Table 1. Requirements for methyl group transfer from [^{14}C]CH_3-B_{12} to homocysteine.

Reaction mixture	Methionine formed
Complete	7,730 cpm
- homocysteine	60
- enzyme, + boiled enzyme	0

The complete system contained [^{14}C]CH_3-B_{12} 10 mμmoles (89,000 cpm), homocysteine 300 mμmoles, potassium phosphate buffer 15 μmoles, crude extract 1.0 ml (protein 1.8 mg) in a total volume of 1.4 ml. The incubation was carried out for 1 hr at 30 °C in the dark.

Table 2. Requirements for methyl group transfer from N^5-[^{14}C]CH_3-tetrahydrofolate to homocysteine.

Reaction mixture	Methionine formed
Complete	2.9 mμmoles
- homocysteine	0.16
- ATP, - $MgSO_4$	1.8
- SAM	0.34
- ATP, - $MgSO_4$	0.05
- DTT, - $FMNH_2$	0.17
- ATP, - $MgSO_4$, - SAM, - DTT	0.03

The complete system contained N^5-[^{14}C]CH_3-tetrahydrofolate 67 mμmoles (132,000 cpm), homocysteine 100 mμmoles, SAM 25 mμmoles, ATP 50 mμmoles, $MgSO_4$ 50 mμ moles, DTT 50 mμmoles, $FMNH_2$ 50 mμmoles, potassium phosphate buffer 25 μmoles, 0 to 70 % ammonium sulfate fraction 0.1 ml (protein 1.5 mg) in a total volume of 0.5 ml. The incubation was carried out for 1 hr at 30 °C under an atmosphere of H_2 and in the dark.

described in detail elsewhere. These data suggest that cobalt ion functions as the constituent of vitamin B_{12} and that B_{12}-dependent methionine synthetase may have a very important role in the metabolic sites where vitamin B_{12} participates in the bacteria.

Evidences for existence of B_{12}-dependent transmethylase were shown in Table 1 and 2. In the transmethylation from [^{14}C]CH_3-B_{12} to homocysteine

(Table 1), the activity was lost by boiling the extracts. This shows that the reaction proceeds enzymatically, although the significance of the existence of this reaction is unknown. Table 2 demonstrates the occurrence of the methyl transfer from N^5-[^{14}C]CH_3-tetrahydrofolate to homocysteine. The reaction was strictly dependent on SAM, reducing system and dithiothreitol (DTT). These cofactor requirements are well-known in the B_{12}-dependent methionine synthetase system in various organisms.

Addition of methionine to the medium also caused a very similar morphological change to the one caused by the addition of cobalt ion according to the microscopic observation. These data will be published elsewhere.

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