LEVEL OF METHIONINE SYNTHASE ACTIVITY AND INTERCONVERSION OF METHYLCOBALAMIN AND ADENOSYLCOBALAMIN IN A FACULTATIVE METHYLOTROPH, *PROTAMINOBACTER RUBER*

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Summary *Protaminobacter ruber* was cultured in a medium containing [⁵⁷Co]cyanocobalamin with a "two-step cultivation method" and the forms of vitamin B₁₂ compounds in the cells were examined. Methylcobalamin was detected in the early phases of growth and reached a maximum of about 40% of all cobalamins extracted from the cells. In the stationary phase of growth, almost all cobalamins consisted of adenosylcobalamin. Recultivation of the cells of the stationary phase in a fresh medium resulted in the conversion of adenosylcobalamin into methylcobalamin. Interconversion of methylcobalamin and adenosylcobalamin was presumed from these facts.

The formation of adenosylcobalamin from methylcobalamin was demonstrated with a cell-free extract system from *P. ruber*. The rate of conversion of methylcobalamin into adenosylcobalamin was highest among several cobalamin analogs tested. Propylation of 5-methyltetrahydrofolate: homocysteine methyltransferase with 1-iodopropane did not affect this conversion reaction, which was probably catalyzed by methyltransferase and adenosyltransferase.

Keywords *Protaminobacter ruber*, facultative methylotroph, methionine synthase, methylcobalamin, adenosylcobalamin, vitamin B₁₂ interconversion

A facultative methylotroph, *Protaminobacter ruber* NR-1, produces methylcobalamin and adenosylcobalamin (1). We demonstrated that methylcobalamin participates in cobalamin-dependent methionine synthase [5-methyltetrahydropteroyl-L-glutamate: L-homocysteine S-methyltransferase, EC 2.1.1.13], while adeno-

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Abbreviations: B₁₂ is used to denote various cobalamins. CN-Cbl, cyanocobalamin; MeCbl, methylcobalamin; OH-Cbl, hydroxocobalamin; AdoCbl, adenosylcobalamin; FMNH₂, reduced riboflavin 5'-phosphate; SAM, S-adenosylmethionine; DTT, dithiothreitol.

sylcobalamin participates in methylmalonyl-CoA mutase [methylmalonyl-CoA CoA carbonylmutase, EC 5.4.99.2] (2, 3) and glutamate mutase[D-threo-3-methyl-aspartate carboxyaminoethylmutase, EC 5.4.99.1] in the microorganism. The time course of the changes of the B₁₂-forms in the cells during cultivation revealed that more methylcobalamin was produced at earlier phases of the bacterial growth, but after the late logarithmic phase almost all of the B₁₂ compounds were present in the form of adenosylcobalamin (1).

On the other hand, sulfitocobalamin was isolated from partially purified methionine synthase from *Escherichia coli* (4, 5), and Taylor and Weissbach (6) proposed the existence of an inactive (-S-Co)B₁₂ enzyme in the schematic reaction mechanism for B₁₂-dependent methionine synthesis. If such an inactive enzyme exists *in vivo*, one of the B₁₂ compounds extracted from the cells should be sulfitocobalamin or hydroxocobalamin. However, the forms of B₁₂ compounds obtained from the cells of *P. ruber* were only methylcobalamin and adenosylcobalamin (2). Since other vitamin B₁₂ compounds might not have been extracted from the cells under the experimental conditions used, a more precise investigation would be required to definitely arrive at this conclusion. In addition, adenosylcobalamin was the predominant form in the stationary phase cells (2). This may represent the conversion from methylcobalamin into adenosylcobalamin. Thus, it is interesting to examine whether there are interconversions between methylcobalamin and adenosylcobalamin in the cells during the cultivation. The present study was designed to clarify these points.

MATERIALS AND METHODS

Chemicals. [¹⁴C]Methyl iodide, 5-[¹⁴C]methyl-tetrahydrofolate and [⁵⁷Co]-cyanocobalamin were purchased from the Japan Radioisotope Association. S-Adenosylmethionine was obtained from Kyowa Hakko Kogyo Co. Unlabeled 5-methyltetrahydrofolate was prepared by the methods of Keresztesy and Donaldson (7). [¹⁴C]Methyl-cobalamin was synthesized as described by Smith *et al.* (8) and FMNH₂ was prepared each day by the catalytic hydrogenation of FMN with PtO₂ (9). Other reagents were of the best grade commercially available.

Organism and cultivation conditions. *Protaminobacter ruber* NR-1 was grown in a medium containing 2% methanol (v/v) as the sole carbon and energy source (2, 10), except for *in vitro* adenosylation experimentation in which the bacterium was cultured in a medium containing 1,2-propanediol (v/v) as the sole carbon and energy source (2, 10). The bacterium was grown by the following two methods. A) Conventional cultivation method: 0.5 ml aliquots of seed culture grown for about two days (OD: *ca.* 6.0) were inoculated into 500 ml-Sakaguchi flasks containing 100 ml of the medium and the flasks were shaken at 30°C on a reciprocating shaker operating at 150 strokes per min. B) The cultivation method that we call the "two-step cultivation method": The cells cultured by means of the conventional cultivation method were collected aseptically by centrifugation at

5,000 × *g* for 20 min with sterilized, capped centrifuge tubes. The cells thus obtained were suspended in the same volume of the sterilized medium as that of the cultures. Fifty ml aliquots of the suspension were inoculated into Sakaguchi flasks containing 50 ml of the sterilized medium or containing 50 ml of the same sterilized medium except that [⁵⁷Co]cyanocobalamin (50 μg, 4 × 10⁵ cpm) was added instead of cobaltous ion. The flasks were shaken at 30°C on the reciprocating shaker operating at 150 strokes per min. The bacterial growth was followed turbidimetrically at 660 nm (ADS) with an Akiyama D.S. 374 Fuji spectrophotometer. Optical density of the cultures was calculated by using the relationships between ADS and OD values obtained in separate experiments (3).

Preparation of cell-free extracts and assays. All the cells were stored at -15°C until use. Cell-free extracts were prepared as described previously (1).

5-Methyltetrahydrofolate: homocysteine methyltransferase and methylcobalamin: homocysteine methyltransferase were assayed as previously described (11-13). The reaction mixture for propylation of methionine synthase contained 1-iodopropane 1 μmol, homocysteine 20 μmol, SAM 0.05 μmol, FMNH₂ 0.4 μmol, potassium phosphate buffer (pH 7.5) 100 μmol and cell-free extracts (protein, *ca.* 5 mg) in a total volume of 1.5 ml. FMNH₂ and 1-iodopropane (40 μmol per ml of 50% ethanol) in an atmosphere of H₂ were added with syringes through serum rubber stoppers after the gas substitution into H₂. As the control, 50% ethanol solution (25 μl) was added instead of 1-iodopropane. The mixtures were incubated at 37°C for 2 hr and dialyzed 3 times against 2 liters of 0.01 M potassium phosphate buffer for 40 hr at 4°C. Aliquots of the dialysate with or without light exposure (at 10 cm, for 2 min with 200 W tungsten lamp) were used for enzymatic assays of methionine synthesis and adenosylation.

Adenosylating enzyme [ATP: cob(I) alamin Co-β-adenosyltransferase, EC 2.5.1.17] was assayed in the dark as follows. The standard reaction mixtures contained any one cobalamin analog 0.4 μmol, homocysteine 20 μmol (omitted when hydroxocobalamin was used), ATP-Na₂ 5 μmol, MgCl₂ 200 μmol, KCl 200 μmol, DTT 10 μmol, FMNH₂ 0.2 μmol, Tris-HCl buffer (pH 7.5) 300 μmol and cell-free extracts (protein 2-5 mg) in a total volume of 2.0 ml. FMNH₂ was added with a syringe through a serum rubber stopper after gas substitution into H₂. The reaction was terminated by adding 0.5 ml of 1 N HCl to the mixtures incubated for 1.5 hr at 37°C. After centrifugation at 10,000 × *g* for 20 min, the supernatant was applied onto an Amberlite XAD-2 column (0.6 × 3 cm, 48-100 mesh). The column was washed with 5 ml of deionized water and then B₁₂ compounds were eluted with 2 ml of 25% ethanol (v/v). The identification of cobalamins was done with paper electrophoresis.

When [¹⁴C]ATP was used, the complete reaction mixture contained cobalamin analog 0.1 μmol, homocysteine 5 μmol, [¹⁴C]ATP 0.4 μmol (0.25 μCi), MgCl₂ 12.5 μmol, KCl 12.5 μmol, DTT 1.25 μmol, FMNH₂ 0.1 μmol, Tris-HCl buffer 100 μmol and cell-free extracts (protein, *ca.* 0.4 mg) in a total volume of 0.5 ml. FMNH₂ and [¹⁴C]ATP were added after gas substitution into H₂. After incubation

at 37°C for 1 hr the reaction was terminated by adding 0.5 ml of 1 N HCl. In order to adjust pH and change the color of cobalamins, which influence the efficiency of counting radioactivity, 1 ml of 1 M K_2HPO_4 was added. After Amberlite XAD-2 column chromatography in a way similar to the above experiment with nonlabeled ATP, the eluates were counted in 10 ml of Bray's solution in a Packard Tri-Carb liquid scintillation spectrometer (Model 3320-515). Since quenching of radioactivity by cobalamin was very high, the correction was made by examining the recovery of the radioactivity of known amount of [^{14}C]ATP added after counting.

Extraction and partial purification of [^{57}Co]B $_{12}$ compounds from the cells. The cells containing [^{57}Co]B $_{12}$ were suspended in 80% ethanol (one ml per the cells obtained from one ml of the culture). The suspension was refluxed for 20 min in the dark, all subsequent operations being carried out in the dark. The ethanol extract was centrifuged at $10,000 \times g$ for 20 min. The precipitate was washed with the same volume of 80% ethanol as that of the extract and the suspension was centrifuged. The supernatants were combined and evaporated to dryness with a rotary evaporator. To the residue 2 ml of carrier B $_{12}$ (about 70 μg each of cyano-, hydroxo-, methyl-, adenosyl-cobalamin) was added and the solution was applied to an Amberlite XAD-2 column. The column was washed with 3 ml of deionized water and then B $_{12}$ compounds were eluted with 3 ml of 25% ethanol. The eluate was evaporated to dryness with a rotary evaporator. The residue was dissolved in one drop of deionized water (*ca.* 0.05 ml) and used as the sample (10 μl for each run) for paper electrophoresis. The radioactivity of [^{57}Co]B $_{12}$ compounds was measured using a well-type scintillation counter (Fuji Denki Co., ATS-700).

Extraction of [^{57}Co]B $_{12}$ compounds from cells and cell-free extracts. *P. ruber* was cultivated for 95 hr in 100 ml of the medium containing [^{57}Co]B $_{12}$ instead of cobaltous ion by inoculating the cells cultured for 40 hr in a cobalt-deficient medium. The cells thus obtained were divided into two parts. From the one part of the cells B $_{12}$ compounds were directly extracted with 80% ethanol, while from the other part the cell-free extracts were first prepared and B $_{12}$ compounds then extracted. After measuring the radioactivity of the B $_{12}$ extracts in order to calculate the extraction efficiency, the samples for paper electrophoresis were obtained by the method described above.

Determination of the forms of B $_{12}$ compounds with paper electrophoresis. Paper electrophoresis was carried out at 800 V for 2 hr on Whatman 3MM filter paper (2 $\times$ 40 cm) with a Toyo type C-II paper electrophoretic apparatus in 0.52 N acetic acid or 0.1 N acetate buffer (pH 3.4) in the dark (1). The paper was cut at 0.5 cm intervals. The radioactivity of [^{57}Co]B $_{12}$ of each piece was measured.

RESULTS

Time course of growth and B $_{12}$ -dependent methionine synthase activities

Bacterial growth on 2% methanol in conventional cultivation reached a maximum after about 60 hr (Fig. 1). The time courses of the specific activities of 5-

Fig. 1. Time course of growth and specific activities of 5-methyltetrahydrofolate: homocysteine methyltransferase and methylcobalamin: homocysteine methyltransferase of *P. ruber*.

Fig. 2. Time course of growth and specific activity of 5-methyltetrahydrofolate: homocysteine methyltransferase of *P. ruber* cultured in "two-step cultivation method."

methyltetrahydrofolate: homocysteine methyltransferase and methylcobalamin: homocysteine methyltransferase are shown in Fig. 1. Both specific activities were high in the early phases of bacterial growth (*i.e.* 13.5 nmol/mg/15 min for the former and 9.0 nmol/mg/30 min for the latter) and decreased with cultivation time, but it is noteworthy that the decrease of 5-methyltetrahydrofolate: homocysteine methyltransferase activity was more drastic than that of methylcobalamin: homocysteine methyltransferase.

Figure 2 shows the time courses of the growth and the specific activity of 5-

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methyltetrahydrofolate: homocysteine methyltransferase by a two-step cultivation method. The lag phase of the bacterial growth was scarcely observed and the logarithmic phase continued until about 20 hr cultivation. The enzyme activity was high in the cells of the early phases of bacterial growth. The maximal specific activity reached *ca.* 15 nmol/mg protein/15 min in the cells after about 10 hr cultivation, which well corresponds to the activity obtained from the cells in conventional cultivation (Fig. 1). Therefore, the two-step cultivation method was employed for the greater part of this study.

Changes of the forms of intracellular cobalamins

The enzyme activity decreased drastically and became very low at the stationary phase of the bacterial growth (Fig. 2). Under these circumstances, methylcobalamin in the cells almost disappeared and most B₁₂ compounds consisted of adenosylcobalamin (I). In order to examine how B₁₂-forms change when such stationary phase cells would multiply by transferring to a fresh medium, *P. ruber* was cultivated in the medium containing [⁵⁷Co]cyanocobalamin until the stationary phase of growth and then transferred to the fresh medium (MATERIALS AND METHODS (B)). B₁₂ compounds extracted from the cells were examined by

Fig. 3. A typical radioactigraph after paper electrophoresis of [⁵⁷Co]cobalamins extracted from the cells in 0.1 N acetate buffer (pH 3.4) (A) and 0.52 N acetic acid (B).

paper electrophoresis. Figure 3 shows a typical radioactigraph of [⁵⁷Co]B₁₂ compounds extracted from the cells. As shown in Fig. 3 (A), no hydroxocobalamin was detected in the sample by paper electrophoresis in 0.1 N acetate buffer (pH 3.4). Paper electrophoresis in 0.52 N acetic acid revealed that the sample contained adenosylcobalamin and a small amount of methylcobalamin (Fig. 3 (B)).

The samples extracted from the cells in each cultivation time were treated similarly to the sample in Fig. 3. From the figures thus obtained, the percentages of the radioactivities of methyl- or adenosyl-cobalamin to those of total cobalamins were calculated for each sample and are summarized in Fig. 4. Almost all cobalamins in the inoculated cells at zero cultivation time were present as adenosylcobalamin, which was gradually converted to methylcobalamin during the logarithmic phase of growth. The conversion reached a level of about 40% around the middle logarithmic phase of the bacterial growth. After the late logarithmic phase, methylcobalamin was again converted into adenosylcobalamin.

Forms of B₁₂ compounds in cells and cell-free extracts

We could not detect any hydroxo- and sulfito-cobalamin in the extracts from the cells. Those cobalamins might be detected in the B₁₂-extracts from the cell-free extracts. To test this possibility, [⁵⁷Co]B₁₂ compounds were extracted from cells and cell-free extracts and the patterns of B₁₂ forms were compared in paper electrophoresis. As shown in Fig. 5 (A) and (B), neither hydroxocobalamin nor sulfitocobalamin was detected in the extracts from the cells and the cell-free extracts. When the samples were exposed to light, all the B₁₂ compounds were converted into hydroxocobalamin (Fig. 5 (C) and (D)).

Conversion of methylcobalamin into adenosylcobalamin in vitro

Since the interconversion of methylcobalamin and adenosylcobalamin is probable from the results of Fig. 4, we examined the possibility of the

Fig. 4. Time course of growth and changes of cobalamin-forms of *P. ruber*.

Fig. 5. Actigraphs after paper electrophoresis of the samples obtained from cells (A) and cell-free extracts (B) of *P. ruber* in 0.1 N acetate buffer (pH 3.4). (C) and (D) were obtained by exposing (A) and (B), respectively, to light.

Table 1. Requirements for the conversion of methylcobalamin into adenosylcobalamin.

Reaction mixture	[¹⁴ C]Adenosylcobalamin formed (nmol/mg protein/min)
Complete	0.20
—Methylcobalamin	0
—Homocysteine	0.07
—FMNH ₂	0

interconversion by the *in vitro* enzymatic reaction. Table 1 shows that adenosylcobalamin was enzymatically formed from methylcobalamin. For the reaction, homocysteine and FMNH₂ were required.

The rates of conversion of various cobalamin analogs into adenosylcobalamin are shown in Table 2. Methylcobalamin was most effective for this reaction. Hydroxo- and cyano-cobalamin were also converted well into adenosylcobalamin. Propyl- and adenosyl-cobalamins had low but significant conversion rates. This adenosylation was also confirmed by paper electrophoresis (Fig. 6, Sample 1). Adenosylcobalamin could be formed in the absence of homocysteine, when hydroxocobalamin was used.

Effect of propylation on the activities of methyltransferase and adenosyltransferase

When methylcobalamin is used for adenosylation, it is plausible that the methyl group of the compound is transferred to homocysteine by methyltransferase. In order to examine whether this reaction is catalyzed by 5-methyltetrahy-

Table 2. Rates of conversion of various cobalamin analogs to adenosylcobalamin.

Substrates	[¹⁴ C]Adenosylcobalamin formed (nmol/mg protein/min)
Methylcobalamin	0.18
Hydroxocobalamin	0.16
Cyanocobalamin	0.15
Adenosylcobalamin	0.05
Propylcobalamin	0.10

Fig. 6. Paper electrophoretic properties of samples obtained by the adenosylation. The compounds used as substrates were ATP and methylcobalamin in sample 1, ATP and hydroxocobalamin in sample 2.

Table 3. Effect of chemical propylation on methyl group transfer and adenosylation reaction.

Conditions	[¹⁴ C]Methionine formed		[¹⁴ C]Adenosylcobalamin formed from [¹⁴ C]ATP
	from 5-[¹⁴ C]methyl- tetrahydrofolate	from [¹⁴ C]methyl- cobalamin	
		(nmol/mg protein/min)	
Propylated enzyme	0.04	0.30	0.16
Propylated enzyme + light	0.26	0.27	0.13
Non-propylated enzyme	0.41	0.32	0.17
Non-propylated enzyme + light	0.41	0.28	0.12

drofolate: homocysteine methyltransferase or by methylcobalamin: homocysteine methyltransferase, the enzyme was propylated with 1-iodopropane. The propylated and non-propylated (control) enzymes with or without exposure to light were assayed for methyltransferase and the adenosylation reaction. Table 3 summarizes the

results. Only the reaction with 5-methyltetrahydrofolate: homocysteine methyltransferase was inhibited by propylation and the activity was considerably restored by exposure to light. In contrast, no such effect of light was observed with the non-propylated enzyme of the control. The activity change of methylcobalamin: homocysteine methyltransferase coincided very well with that of the enzyme systems catalyzing adenosylation.

DISCUSSION

The high content of methylcobalamin in younger tissues of human brain or liver has been reported by Linnell (14). The content of methylcobalamin in *P. ruber* or *Streptomyces olivaceus* was also high in the early phases of bacterial growth (1, 15). The high specific activity of 5-methyltetrahydrofolate: homocysteine methyltransferase during logarithmic phases of the growth (Fig. 1) well corresponds to the high content of methylcobalamin during these phases. Similar results, such as the maximal specific activity 15 nmol/mg protein/15 min, were obtained by the "two-step cultivation method" (Fig. 2) (10). This "two-step cultivation method" seems to be very useful for obtaining sufficient numbers of cells in the case where an enzyme has a high specific activity in an early phase of microbial growth, while in conventional culture the early phase cells would be obtained only by a large-scale cultivation.

By the two-step cultivation method, we could find changes of B₁₂-forms in the cells during the cultivations (Fig. 4). The formation of methylcobalamin by the conversion of adenosylcobalamin was confirmed, since [⁵⁷Co]methylcobalamin was formed in an early phase of the growth under the conditions of little *de novo* B₁₂ formation by cultivating the cells that contained B₁₂ compounds mostly in the form of [⁵⁷Co]adenosylcobalamin in a fresh medium for the second culture. The reverse conversion from methylcobalamin to adenosylcobalamin was also observed in the later stages of the growth. B₁₂ compounds were extracted almost quantitatively from the cells and the cell-free extracts, and no hydroxocobalamin was detected in both B₁₂ extracts. Therefore, amounts of B₁₂ compounds other than methyl- and adenosyl-cobalamin were negligible, if any. The stable existence of sulfitocobalamin in the cells is not likely, at least in *P. ruber*, although sulfitocobalamin was isolated from partially purified methionine synthase in *E. coli* (4, 5).

The demonstration of conversion of methylcobalamin into adenosylcobalamin *in vitro* (Table 1, Fig. 6) suggests that the reaction is catalyzed by methyltransferase (4) and adenosyltransferase (16). It is noteworthy that methylcobalamin was the best compound among various B₁₂ analogs for the enzymatic conversion to adenosylcobalamin (Table 2). All the B₁₂ compounds tested were effective in different degrees for adenosylation, and adenosylcobalamin was also converted to labeled compounds by the exchange reaction. These findings seem to show that there are differences in the reductive cleavage or cleavage mode depending upon

Scheme 1. Formation of adenosylcobalamin from methylcobalamin.

the nature of the upper ligand of cobalamins.

It is known that cobalamin-dependent methyltransferase from *E. coli* gives an inhibited propylcobalamin-enzyme by propylation with 1-iodopropane and that the inactive propylcobalamin-enzyme can be reactivated by exposure to visible light (4, 17, 18). On the other hand, methyl group transfer from methylcobalamin to homocysteine is not affected by propylation (17, 19).

As shown in Scheme 1, the first step of adenosylation in the case of methylcobalamin is considered to be catalyzed by methyltransferase and the second step by adenosyltransferase (16). The first step of the reaction was not inhibited at all by propylation with 1-iodopropane (Table 3). The highly purified methionine synthase from *P. ruber* catalyzed methyl group transfer from 5-methyltetrahydrofolate as well as methylcobalamin to homocysteine, and it was thus assumed that both reactions were catalyzed at separate catalytic sites on the same protein (12, 20). This was confirmed by the fact that only methyl group transfer from 5-methyltetrahydrofolate to homocysteine was inhibited by the propylation (Table 3). Since *in vitro* adenosylcobalamin formation from methylcobalamin was not influenced by propylation, it is plausible that the first step of the reaction is catalyzed by methylcobalamin: homocysteine methyltransferase (Scheme 1- ①). Both specific activities of 5-methyltetrahydrofolate: homocysteine methyltransferase and methylcobalamin: homocysteine methyltransferase showed similar patterns, but the latter was inactivated more slowly than the former (Fig. 1). This slower inactivation of the latter enzyme, *i.e.* methylcobalamin: homocysteine methyltransferase might facilitate the decomposition of methylcobalamin released from the active site of the former enzyme which would be inactivated faster with the cultivation time. The accumulation of adenosylcobalamin in the cells thus might be promoted in collaboration with adenosyltransferase whose activity is relatively high in comparison with other sources (21, 22). The reverse reaction from adenosylcobalamin into methylcobalamin *in vitro* might be catalyzed by an enzyme resembling holoenzyme synthetase obtained from *E. coli* (23), but this remains to be demonstrated.

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