Note

Occurrence of Pseudovitamin B₁₂ and Its Possible Function as the Cofactor of Cobalamin-Dependent Methionine Synthase in a Cyanobacterium *Synechocystis* sp. PCC6803

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Summary To clarify the physiological function of pseudovitamin B₁₂ in cyanobacteria, we determined pseudovitamin B₁₂ contents and cobalamin-dependent methionine synthase activity in *Synechocystis* sp. PCC6803 grown under CoSO₄-sufficient and -limited conditions. Pseudovitamin B₁₂ and cobalamin-dependent methionine synthase activity (0.8 nmol/min/mg protein) were found in a homogenate of the *Synechocystis* cells grown for 10 d in the CoSO₄-sufficient medium. The cellular pseudovitamin B₁₂ contents increased significantly at the early logarithmic growth phase and thereafter decreased rapidly at the stationary phase; a similar fluctuation pattern was shown in the cobalamin-dependent methionine synthase activity. Although the CoSO₄-limited conditions did not reduce the cell growth, pseudovitamin B₁₂ contents and methionine synthase activity decreased significantly in the limited cells relative to the sufficient cells. These results indicate that the cyanobacterium *Synechocystis* sp. PCC6803 can synthesize pseudovitamin B₁₂ de novo and utilize it as the cofactor for cobalamin-dependent methionine synthase.

Key Words pseudovitamin B₁₂, methionine synthase, cyanobacteria, *Synechocystis* sp. PCC6803

Vitamin B₁₂ or cyanocobalamin (B₁₂) has a corrin ring in which the central cobalt atom is coordinated by tetra-pyrroles. Two axial (upper and lower) ligands also coordinate the cobalt atom. The lower ligand of B₁₂ molecule contains a cobalt-coordinated nucleotide with 5,6-dimethylbenzimidazole as a base. The upper ligand consists of either 5'-deoxyadenosine (adenosylcobalamin) or a methyl group (methylcobalamin), which functions as the cofactor for methylmalonyl-CoA mutase (MCM, EC 5.4.99.2) and methionine synthase (MS, EC 2.1.1.13), respectively, in mammals (1).

Certain bacteria can synthesize B₁₂ and/or other B₁₂-related compounds with a variety of alternative bases (adenine, pseudovitamin B₁₂; 2-methyladenine, Factor A; and so on) in the lower ligand (2, 3). We have previously reported that edible cyanobacteria (*Spirulina platensis*, *Aphanothece sacrum*, *Aphanizomenon fros-aquae*, *Nostoc commune*, and *Nostoc flagelliforme*) contain a substantial amount of pseudovitamin B₁₂ (4–8). However,

there is little information available on whether these cyanobacteria have the ability to synthesize pseudovitamin B₁₂ de novo, and whether pseudovitamin B₁₂ functions as a cofactor for certain cobalamin-dependent enzymes. Although most of these edible cyanobacteria are difficult to use as experimental organisms because of their low growth rates and little biochemical and genomic information, *Synechocystis* sp. PCC6803 has been studied extensively as a model of cyanobacterial species in many scientific fields (biochemistry, molecular biology, and so on).

In this paper, we describe the occurrence of pseudovitamin B₁₂ in *Synechocystis* sp. PCC6803, and the effects of the addition or depletion of cobalt ion as a constituent of pseudovitamin B₁₂ on its cell growth, pseudovitamin B₁₂ contents, and cobalamin-dependent MS activity. We also discuss the physiological function of pseudovitamin B₁₂ in this organism.

Materials and Methods

Materials. B₁₂ and methylcobalamin (CH₃-B₁₂) were

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obtained from Sigma (St. Louis, USA). Pseudovitamin B₁₂ purified and identified previously from *Aphanizomenon flos-aquae* (6) was used as an authentic preparation. A B₁₂ assay medium for *Lactobacillus delbrueckii* was obtained from Nissui (Tokyo, Japan). Silica gel 60 thin-layer chromatography (TLC) aluminium sheets were obtained from Merck (Darmstadt, Germany).

Organisms and culture conditions. *Synechocystis* sp. PCC6803 was grown in Allen medium (9) (5 L) containing 0.18 mM CoSO₄, aseptically bubbled with air (3 mL/min) at 25°C, and illuminated at 240 μ E/m²/s for 10 d. The cells grown under the conditions were used as the CoSO₄-sufficient cells. The CoSO₄-sufficient cells were inoculated into Allen medium (5 L) without CoSO₄, cultured for 10 d, and used as the CoSO₄-limited cells. The CoSO₄-limited cells were further inoculated into the medium (5 L) without CoSO₄, cultured for 10 d, and used as the CoSO₄-deficient cells. The cell growth was determined in each cell culture grown for the indicated time courses by measuring absorbance at 750 nm.

Extraction and assay of pseudovitamin B₁₂. The cell culture grown for the indicated time courses was centrifuged at 5,000 $\times g$ for 10 min to collect the cells. The precipitated cells were washed twice with distilled water, and then stored at -80°C until use. The stored cells (about 2 g wet weight) were disrupted with sonic treatment (6 kHz, 60 s, three times), and boiled for 30 min at 57 mmol/L acetate buffer (pH 4.5) containing 0.05% (w/v) KCN. The extraction procedure was done in a Dalton (Tokyo, Japan) draft chamber. Pseudovitamin B₁₂ was assayed with B₁₂ as a standard by a bioassay method (10) using *Lactobacillus delbrueckii* subsp. *lactis* ATCC7830.

Bioautography with B₁₂-dependent *Escherichia coli* 215. Pseudovitamin B₁₂ found in the above cell extract was separated with silica gel 60 TLC, and visualized with B₁₂-dependent *Escherichia coli* 215 bioautography as described previously (8).

Enzyme assay. *Synechocystis* cells (0.1 g) were suspended in 0.5 mL of 10 mmol/L potassium phosphate buffer (pH 7.0), containing 10% (w/v) sucrose, disrupted by a bead beater (Biospec Products, USA) (15 s strokes with 45 s intervals, 8 times), and centrifuged at 5,000 $\times g$ for 20 min at 4°C. The supernatant was used as the crude extract.

Methionine synthase was assayed at 37°C under anaerobic conditions according to Banerjee et al. (11). The reaction mixture (0.5 mL) contained 100 mmol/L potassium phosphate buffer (pH 7.2), 500 μ mol/L DL-homocysteine, 19 μ mol/L *S*-adenosyl-L-methionine, 6 mmol/L titanium citrate, 250 μ mol/L (6*R*,6*S*)-5-CH₃-H₄-folate, and the crude enzyme. The reaction was initiated with CH₃-H₄-folate, incubated for 30 min at 37°C, and terminated by heating at 98°C for 2 min. The H₄-folate formed by the enzyme reaction was assayed with HPLC as described in the cited reference (12). Apo-MS activity was calculated by subtracting the holo-MS activity (in the absence of CH₃-B₁₂) from the total MS activity (in the presence of 50 μ mol/L CH₃-B₁₂).

Fig. 1. Bioautogram of pseudovitamin B₁₂ found in the *Synechocystis* cells. Corrinoid compounds were extracted from the *Synechocystis* cells grown for 10 d under the CoSO₄-sufficient conditions, separated with a silica gel 60 TLC, and visualized with B₁₂-dependent *E. coli* 215. 1: authentic B₁₂; 2: authentic pseudovitamin B₁₂; 3: *Synechocystis* cell extract. The data are a typical bioautogram from three independent experiments.

Protein assay. Proteins were assayed with ovalbumin as a standard by the method of Bradford (13).

Results and Discussion

Identification of pseudovitamin B₁₂ found in the *Synechocystis* cells

To clarify whether the *Synechocystis* cells grown in the CoSO₄-sufficient medium contain considerable amounts of pseudovitamin B₁₂, corrinoid compounds were extracted, separated with silica gel 60 TLC, and then visualized with B₁₂-dependent *E. coli* 215 bioautography (Fig. 1). The B₁₂-active compound of the *Synechocystis* cell homogenate was found as only one spot, whose *R_f* value was identical to that of authentic pseudovitamin B₁₂. The genome database for cyanobacteria (CyanoBase; <http://www.kazusa.or.jp>) indicates that the *Synechocystis* cells contain the gene homologs of the enzymes involved in the B₁₂ biosynthesis. These results suggest that the *Synechocystis* cells have the ability to synthesize pseudovitamin B₁₂ de novo.

Occurrence of the cobalamin-dependent MS in the *Synechocystis* cells

The CyanoBase indicates that *Synechocystis* sp. PCC6803 contains a gene similar to *metH* (slr0212) encoding cobalamin-dependent MS, but not to *metE* encoding cobalamin-independent MS. It did not contain any genes related with other cobalamin-dependent enzymes (MCM, ribonucleotide reductase, diol dehydratase, and so on), either.

We assayed the cobalamin-dependent MS activity in the cell homogenates of the *Synechocystis* cells grown in the CoSO₄-sufficient medium and some mammalian cells. The *Synechocystis* cells showed considerably

Fig. 2. Changes in pseudovitamin B₁₂ contents and MS activity during the growth of *Synechocystis* cells under the CoSO₄-sufficient conditions. *Synechocystis* cells were grown in a medium supplemented with CoSO₄ at 0.18 mmol/L for 10 d. Cell density in the culture medium (A), pseudovitamin B₁₂ content (B), and MS (holo-enzyme) activity (C) were assayed at the indicated time courses by the methods described in the text. The data represent means $\pm$ SD from three independent experiments.

higher MS activity (0.80 nmol/min/mg protein) than the Cos-7 monkey kidney cells (0.67 nmol/min/mg protein) and rat liver (0.14 nmol/min/mg protein) did. These results suggest that pseudovitamin B₁₂ can function as the cofactor of the cobalamin-dependent MS because it is the only corrinoid compound found in the *Synechocystis* cells.

Changes in pseudovitamin B₁₂ contents and MS activity during growth of the *Synechocystis* cells under the CoSO₄-sufficient conditions

The cellular pseudovitamin B₁₂ contents increased significantly at the early logarithmic growth phase and thereafter decreased rapidly at the stationary phase; a similar fluctuation pattern was shown in the cobalamin-dependent MS activity (Fig. 2). These results suggest that the MS actively functions in the DNA synthesis at the early logarithmic growth phase.

Effects of the depletion of CoSO₄ as a constituent of pseudovitamin B₁₂ on the cell growth, pseudovitamin B₁₂ contents and MS activity in the *Synechocystis* cells

To determine whether the depletion of CoSO₄ can

Fig. 3. Effects of the depletion of CoSO₄ on the growth, pseudovitamin B₁₂ contents, and MS activity in *Synechocystis* cells. A: *Synechocystis* cells were grown under the CoSO₄-sufficient (●), -limited (○) or -deficient (■) conditions. B: Pseudovitamin B₁₂ contents were extracted from the cell grown for 10 d under the CoSO₄-sufficient (S) and -limited (L) conditions and assayed. C: MS activity was determined in the absence (□) or presence (■) of CH₃-B₁₂ using the homogenates of *Synechocystis* cells grown under the CoSO₄-sufficient (S) and -limited (L) conditions. The data represent means $\pm$ SD from four independent experiments.

affect pseudovitamin B₁₂ contents, MS activity, and cell growth, *Synechocystis* cells were grown under the CoSO₄-limited and -deficient conditions. Hardly any *Synechocystis* cells were grown under the CoSO₄-deficient conditions (Fig. 3A). These results suggest that *Synechocystis* sp. PCC6803 absolutely requires the cobalt ion as a constituent of pseudovitamin B₁₂ for cell growth. Although the CoSO₄-limited conditions did not reduce the cell growth, pseudovitamin B₁₂ contents decreased significantly in the limited cells relative to the CoSO₄-sufficient cells (Fig. 3B). The MS activity of the CoSO₄-limited cells was also decreased as low as about 10% of the activity of the CoSO₄-sufficient cells.

To evaluate whether the CoSO₄-limited cells contain the apo-enzyme of the MS, the MS activity was determined after being converted from the apo-enzyme to the holo-enzyme by pre-incubation with CH₃-B₁₂ (Fig.

3C). There was no change in the MS activity of the CoSO₄-sufficient or -limited cells in the presence or absence of added CH₃-B₁₂, suggesting that MS predominantly exists as the holo-enzyme in both CoSO₄-sufficient and -limited cells. These results indicate that the decrease in the MS activity of the CoSO₄-limited cells may be due to a decrease in the stability of the enzyme protein because the pseudovitamin B₁₂ contents were significantly low in the limited cells. Yamada et al. (14, 15) have demonstrated that MS activity became extremely low in B₁₂-deficient rats and that the rat apo-enzyme is very unstable and stabilized by forming a complex with CH₃-B₁₂.

These results presented here indicated that *Synechocystis* sp. PCC6803 (probably the edible cyanobacteria) has the ability to synthesize pseudovitamin B₁₂ de novo and then to utilize the compound as the cofactor for cobalamin-dependent MS.

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