Occurrence of 5'-Deoxyadenosylcobalamin and Its Physiological Function as the Coenzyme of Methylmalonyl-CoA Mutase in a Marine Eukaryotic Microorganism, *Schizochytrium limacinum* SR21

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Summary A marine eukaryotic microorganism, *Schizochytrium limacinum* SR21, had the ability to absorb and accumulate exogenous cobalamin, which was converted to the cobalamin coenzymes 5'-deoxyadenosylcobalamin (20.1%) and methylcobalamin (29.6%). A considerably high activity (about 38 mU/mg protein) of 5'-deoxyadenosylcobalamin-dependent methylmalonyl-CoA mutase (EC 5.4.99.2) involved in amino acid and odd-chain fatty acid metabolism was found in the cell homogenate of *S. limacinum* SR21. The enzyme was purified to homogeneity and characterized.

Key Words cobalamin, 5'-deoxyadenosylcobalamin, vitamin B₁₂, methylmalonyl-CoA mutase, *Schizochytrium limacinum* SR21

Schizochytrium limacinum SR21, a thraustochytrid (Labyrinthomycota) is an unusual marine eukaryotic microorganism that is closely related to heterokont algae (1) because it contains substantial amounts of docosahexaenoic acid (DHA). It is suitable for use as an excellent source of DHA (2, 3). Indeed, Franklin et al. (4) have demonstrated that feeding *Schizochytrium* cells to dairy cows increases the concentration of DHA in their milk.

Recently, addition of vitamin B₁₂ or cyanocobalamin (CN-Cbl) to the cultivation medium of *S. limacinum* SR21 has been reported to decrease the ratio of odd-numbered to even-numbered fatty acids in cultured cells (5, 6). The result suggested that the cells had the ability to take up CN-Cbl and then synthesize 5'-deoxyadenosylcobalamin (AdoCbl), which is the coenzyme of methylmalonyl-CoA mutase (MCM) (EC 5.4.99.2). The enzyme catalyzes isomerization of (*R*)-methylmalonyl-CoA to succinyl-CoA, an intermediate in the Krebs cycle, and thus is involved in the metabolism of odd-numbered fatty acids and branched-chain amino acids.

Although, our preliminary experiments indicated that *S. limacinum* SR21 requires Cbl for growth, there is no detailed information about whether the CN-Cbl taken up by the cells is indeed converted into the Cbl coenzymes and what kind of Cbl-dependent enzymes occur in the cells. To clarify the physiological roles of Cbl in this organism, we demonstrate occurrence of the

Cbl coenzymes and their related enzymes in CN-Cbl-supplemented cells. The AdoCbl-dependent MCM involved in the metabolism of odd-numbered fatty acids was characterized.

MATERIALS AND METHODS

Organism and culture. *Schizochytrium limacinum* SR21 (IFO032693) was grown in 500 mL shaking flasks at 28°C for 4 d. The basal medium consisted of 3% (w/v) glucose and 1% (w/v) yeast extract in half strength artificial seawater (Marine Art High, Senju Seiyaku Co., Ltd., Osaka, Japan) (pH 6.0). CN-Cbl (50 µg/mL medium) was added to the basal medium. After cultivation for 4 d, cells were harvested by centrifugation (1,500 ×g, 10 min) and washed thrice with distilled water and stored at –80°C.

Enzyme assay. MCM was assayed at 37°C by a modified high-performance liquid chromatography (HPLC) method described previously (7), using Shimadzu apparatus (Kyoto, Japan). The MCM activity was calculated from the amount of succinyl-CoA formed. One unit of enzyme activity was defined as the amount of enzyme that catalyzed the formation of succinyl-CoA at the rate of 1 µmol/min.

Cbl assay. Cbl was extracted and assayed microbiologically with *Lactobacillus delbrueckii* subsp. *lactis* (formerly *Lactobacillus leichmannii*) ATCC7830 as described previously (8). A Cbl assay medium for *L. delbrueckii* ATCC7830 was obtained from Nissui (Tokyo, Japan). The turbidity (%T) of the test culture of *L. delbrueckii*

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ATCC7830 grown at 37°C for 16–21 h was measured at 660 nm with a UV-1600 UV-Visible spectrophotometer.

Analysis of Cbl coenzymes from *S. limacinum* SR21. Analysis of Cbl coenzymes was done according to the method of reference (9). An aliquot (20 μ L) of the sample was loaded into a reversed-phase HPLC column (Wakosil-II 5C18 RS, ϕ 4.6 $\times$ 150 mm; particle size, 5 μ m) that had been equilibrated with 5% (v/v) methanol solution containing 0.1% (v/v) acetic acid at 40°C. The flow rate was 1 mL/min. Cbl compounds were eluted with a linear gradient of methanol [from 0% to 90% of 50% (v/v) methanol solution containing 0.1% (v/v) acetic acid] for 30 min. Fractions (1 mL) were collected from the HPLC column, evaporated to dryness under reduced pressure, and dissolved in 1 mL of 10 mM acetate buffer, pH 4.5, containing 0.2 g/L KCN. These fractions were boiled for 30 min at 98°C and centrifuged at 3,000 $\times g$ for 10 min. Each supernatant fraction was used for Cbl assay. The retention times of authentic hydroxocobalamin (OH-Cbl), sulfitecobalamin (SO₃-Cbl), CN-Cbl, AdoCbl, and methylcobalamin (MeCbl) were 12.4, 16.0, 20.0, 23.5, and 27.8 min, respectively.

Purification of MCM from *S. limacinum* SR21. The stored *Schizochytrium* cells (about 55 g wet weight) were suspended in 110 mL of 10 mM potassium phosphate buffer, pH 7.0, containing 10% (w/v) sucrose. The cells were disrupted by sonic oscillation (10 kHz, 10 s $\times$ 6) and centrifuged at 10,000 $\times g$ for 10 min. AdoCbl was added to the supernatant fraction at a final concentration of 10 μ M and left overnight in the dark at 4°C to form the holo-enzyme. The treated solution was precipitated with (NH₄)₂SO₄, and the 40–60% (w/w) (NH₄)₂SO₄ saturation was collected and dissolved in 10 mL of 10 mM potassium phosphate buffer, pH 7.0, containing 10% (w/v) sucrose. The solution was dialyzed overnight against 1.5 L of 10 mM potassium phosphate buffer, pH 7.0, containing 10% (w/v) sucrose. The dialyzed solution was put on a column (2.4 cm $\times$ 20 cm) of TSKgel QAE-Toyopearl HW55C equilibrated with 10 mM potassium phosphate buffer, pH 7.0, containing 10% (w/v) sucrose, and eluted with 300 mL of a linear gradient (0–0.5 M) of KCl in the same buffer. The active fractions were collected and concentrated in Centriprep-30 centrifugal concentrator (Millipore Co., Bedford, MA, USA) up to a final volume of 1.0 mL. The concentrated solution (1.0 mL) was put on a HiLoad 16/60 Superdex 200 pg column (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA) equilibrated with 10 mM potassium phosphate buffer, pH 7.0, containing 10% (w/v) sucrose and 200 mM KCl, and eluted with the same buffer. The active fractions were combined and dialyzed overnight against 1 L of 10 mM potassium phosphate buffer, pH 7.0, containing 10% (w/v) sucrose. The dialyzed solution was concentrated in a Centricon-30 microconcentrator (Millipore) up to a final volume of 1.0 mL. The concentrated solution (1.0 mL) was put on a Mono Q column HR 5/5 (GE Healthcare Bio-Sciences Corp.) equilibrated with 10

mM potassium phosphate buffer, pH 7.0, containing 10% (w/v) sucrose, and eluted with 40 mL of a linear gradient (0–0.5 M) of KCl in the same buffer. The active fractions were combined and desalted in a Centricon-30 microconcentrator (Millipore). The solution was put on a Mono Q column HR 5/5 and eluted with 40 mL of a linear gradient (0.1–0.4 M) of KCl in the same buffer. The peak fraction of the enzyme activity was concentrated to a final volume of 0.5 mL in the Centricon-30 and stored at –80°C.

Polyacrylamide gel electrophoresis (PAGE) in the presence or absence of SDS. A precasted slab gel (READY GELS J, Bio-Rad) was used for electrophoresis with 5–20% linear gradient polyacrylamide in the presence or absence of SDS. A purified enzyme (1 μ g of protein) was run on the precasted gel in the presence or absence of SDS at constant current (12 mA per gel) with bromophenol blue as a migration marker. After electrophoresis, proteins in the gel were stained with Simply Blue SafeStain (Invitrogen, Carlsbad, CA, USA) and destained in distilled water according to the manufacturer's instruction. Standard proteins (recombinant protein; 10; 15; 20; 25; 37; 50; 75; 100; 150; 250 kDa) in a Bio-Rad kit were used for the calibration of molecular mass of the subunit of the *S. limacinum* SR21 MCM.

Gel filtration experiments. The molecular mass of the *Schizochytrium* MCM was determined with a HiLoad 16/60 Superdex 200 pg gel filtration column (GE Healthcare Bio-Sciences Corp.) using the BioLogic HR chromatography system (Bio-Rad). The column was equilibrated with 10 mM potassium phosphate buffer, pH 7.0, containing 10% (w/v) sucrose and 200 mM KCl, and eluted with the same buffer. The molecular mass of the MCM was calibrated with blue dextran (average 2,000.0 kDa), apoferritin from horse spleen (480.0 kDa), alcohol dehydrogenase from yeast (150.0 kDa), albumin from bovine serum (66.0 kDa), and cytochrome *c* from horse heart (12.4 kDa). The proteins were monitored by measuring the absorbance at 280 nm.

Optimum temperature and pH. The optimum temperature of the enzyme was determined by incubating at temperatures (10, 15, 20, 25, 30, 35, and 40°C) for 5 min at pH 7.0.

The optimum pH of the enzyme was determined by incubation at various pH values (5.1, 5.8, 7.1, 7.5, 8.0, and 8.8) in 10 mM Tris-acetate buffer for 5 min at 37°C.

Effect of SH-inhibitors. The enzyme activity was assayed at 37°C for 5 min at pH 7.0 using the purified enzyme treated with 3 mM of SH-inhibitors [*N*-ethylmaleimide, 5,5'-dithiobis-(2-nitrobenzoic acid), and iodoacetamide] at 37°C for 10 min at pH 7.0.

Effect of monovalent and divalent cations. The purified MCM was dialyzed overnight against 1 L of 10 mM Tris-HCl buffer, pH 7.0, containing 10% (w/v) sucrose. Using the dialyzed enzyme, the enzyme activity was assayed at 37°C for 5 min at 10 mM Tris-HCl buffer (pH 7.0) in the presence of each monovalent and divalent cation (NaCl, KCl, NH₄Cl, RbCl, CsCl, LiCl, CoCl₂, MnCl₂,

FeCl₂, and MgCl₂, all at 3 mM).

Effect of substrate concentrations. In the case of determination of *K_m* values for methylmalonyl-CoA and succinyl-CoA (reverse reaction), the enzyme activity was assayed at various concentrations of (R, S)-methylmalonyl-CoA (0, 0.005, 0.0063, 0.0084, 0.0125, 0.025, 0.050, 0.10, 0.20, 0.4, 0.8, 1.6, 3.2 mM) or succinyl-CoA (0, 0.5, 0.625, 0.835, 1.25, 2.5, 5.0 mM) under standard assay conditions.

Protein assay. Protein was assayed using a Bio-Rad protein assay kit, with ovalbumin as standard according to the manufacturer's instructions.

RESULTS AND DISCUSSION

Occurrence of Cbl coenzymes in *S. limacinum* SR21

S. limacinum SR21 contained 1.4 mg of Cbl per 100 g dry cell weight. To elucidate whether Cbl coenzymes, AdoCbl, and MeCbl occur in the cells, Cbl-compounds were extracted, separated by a reversed-phase HPLC, and determined by the microbiological assay method. The *Schizochytrium* cells contained five known types of biologically active Cbl-compounds with different upper-

ligands [approximately OH-Cbl, 5.8%; SO₃-Cbl, 18.2%; CN-Cbl, 27.3%; AdoCbl, 20.1%; and MeCbl, 29.6%] (Fig. 1). The results indicate that *S. limacinum* SR21 has the ability to take up and accumulate exogenous CN-Cbl; half of the CN-Cbl taken up by the cells was converted to the Cbl coenzymes. Considerable amounts (18.2%) of SO₃-Cbl were also found in the cell homogenate. SO₃-Cbl has been reported to be formed by the decomposition of glutathionylcobalamin, an intermediate in the conversion from CN-Cbl to AdoCbl (10). These results suggest that the enzymatic system for synthesis of the Cbl coenzymes is present in *S. limacinum* SR21.

MCM activity in a cell homogenate of *S. limacinum* SR21

Our preliminary experiments indicated that *S. limacinum* SR21 had the highest MCM (holo-form) activity (about 19.4 mU/mg protein) in a cell homogenate among the twelve species of thraustochytrids (about 0.7–14.8 mU/mg protein) tested.

Schizochytrium MCM activity increased significantly up to about 38 mU/mg protein in the presence of AdoCbl. The results indicate that about 50% of MCM found in the *Schizochytrium* cells occurred as the apo-enzyme.

Purification of MCM from *S. limacinum* SR21

A cell homogenate of *S. limacinum* SR21 was incubated with 10 μ M AdoCbl at 4°C overnight in the dark so that the apo-enzyme could be converted to the holo-enzyme. This treatment significantly increased the MCM activity (76 mU/mg protein). The holo-MCM was purified to homogeneity. Figure 2 shows the elution profile of the *Schizochytrium* MCM during Mono-Q HR 5/5 column chromatography (the final purification step). The enzyme activity gave a single peak at 0.3 M KCl. The purification procedures for MCM from a homo-

Fig. 1. Occurrence of Cbl coenzymes in the *S. limacinum* SR21. Cbl-compounds were extracted from the *Schizochytrium* cells by boiling with 80% (v/v) ethanol solution. The extract was concentrated and treated with HPLC. Cbl was assayed in the eluate from HPLC by the microbiological method. Detailed procedures are described in the text. The data are the typical pattern of Cbl-compounds found in *Schizochytrium* cells from three independent experiments.

Fig. 2. Elution profile of the *S. limacinum* SR21 MCM activity during final Mono-Q column chromatography. (—), absorbance at 280 nm; (●), MCM activity; and (---), KCl concentration. A typical elution pattern from three independent experiments is shown.

Fig. 3. PAGE of MCM from the final purification step. A, purified enzyme (1 μ g protein) in the absence of SDS. B 1, molecular mass standard proteins; 2, the purified enzyme (1 μ g protein) in the presence of SDS. Typical migration patterns of the purified enzyme and preparations obtained in the purification steps are shown.

Table 1. Purification of methylmalonyl-CoA mutase from *Schizochytrium limacinum* SR21.

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Yield (%)
Crude homogenate	4,533.4	344.6	0.076	100.0
40–60% (NH ₄) ₂ SO ₄ fractionation	1,500.9	350.1	0.23	101.6
TSKgel QAE-Toyopearl 550	393.2	260.2	0.66	75.5
HiLoad 16/60 Superdex 200 pg	43.21	89.81	2.08	26.1
1st Mono-Q	0.91	9.66	10.62	2.8
2nd Mono-Q	0.45	5.57	12.38	1.6

genate of *S. limacinum* SR21 are summarized in Table 1. The MCM was purified about 163-fold in a yield of 1.6% from the homogenate. Polacrylamide gel electrophoresis (PAGE) of the purified enzyme in the absence of SDS using a precast gel showed a single protein band (Fig. 3A).

Molecular mass

The apparent molecular mass of the enzyme was calculated to be 160 ± 5 kDa by Superdex 200 pg gel filtration. SDS-PAGE of the purified enzyme gave a single protein band with an apparent molecular mass of 80 ± 5 kDa (Fig. 3B), indicating that the *Schizochytrium* enzyme is composed of two identical subunits.

The *Schizochytrium* MCM has a similar subunit structure to the enzymes of human livers (11), an intestinal worm (12), a marine alga (*Pleurochrysis carterae*) (7), and a root-nodule bacterium (*Sinorhizobium meliloti*) (13). The enzymes of an anaerobic bacterium (*Propionibacterium shermanii*) (14) and an aerobic bacterium (*Methylobacterium extorquens* NR-1) (15) consist of two non-identical subunits with molecular masses of 79–85 kDa and 67–70 kDa.

Some properties of the *Schizochytrium* MCM

The optimum temperature of the enzyme was 37°C. The optimum pH of the enzyme was 7.0.

The apparent *K_m* values of the enzyme were 0.68 mM for (*R, S*) methylmalonyl-CoA and 2.4 mM for succinyl-CoA (the reverse reaction).

The purified *Schizochytrium* MCM activity was not affected by the usual SH-reagents [iodoacetamide, *N*-ethylmaleimide, and 5,5'-dithiobis-(2-nitrobenzoic acid)] at 3 mM.

The MCM activities of *P. shermanii* (16), *M. extorquens* NR-1 (15), and an intestinal worm (12) are not affected by the SH-inhibitors, but those of *S. meliloti* (13), *P. carterae* (7), and human livers (11) are considerably inhibited.

Although *S. meliloti* MCM (13) is activated by the addition of some monovalent cations (especially NH₄⁺ and K⁺), the addition of monovalent (Na⁺, K⁺, NH₄⁺, Rb⁺, Cs⁺, and Li⁺) and divalent cations (Co²⁺, Mn²⁺, Fe²⁺, and Mg²⁺) at 3 mM did not affect the enzyme activity of *S. limacinum* SR21 as shown in MCM from the other organisms described previously (7, 11, 15, 16).

These results presented here indicate that *S. limacinum* SR21 cells grown in the Cbl-supplemented medium contain substantial amounts of Cbl, half of which is the Cbl coenzymes, especially AdoCbl that functions as the coenzyme of MCM.

Relatively high amounts of MeCbl (29.6%) were also found in the *Schizochytrium* cells. MeCbl mainly functions as the cofactor of Cbl-dependent methionine synthase (EC 2.1.1.13), which is widely distributed in various organisms (17). In our preliminary experiment, the enzyme activity (0.5 nmol/min/mg protein) could be detected in a cell homogenate of *S. limacinum* SR21 under the anaerobic enzyme assay conditions (18).

Shirasaka et al. (5) have reported that the addition of CN-Cbl significantly decreased the level of odd-numbered fatty acids in *Schizochytrium* cells. This observation suggests that the concentration of Cbl in the usual basal medium (artificial seawater as the Cbl source) would be insufficient to support the normal function of MCM in the methylmalonyl-CoA pathway for various cellular metabolisms. Since the concentration of Cbl in natural seawater appears to be very low, the *Schizochytrium* cells acquire Cbl through a symbiotic relationship with Cbl-synthesizing bacteria in the natural environment, as suggested recently by Croft et al. (19).

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