Note

Purification and Characterization of Methylmalonyl-CoA Mutase from a Methanol-Utilizing Bacterium, Methylobacterium extorquens NR-1

Emi MIYAMOTO1, Fumio WATANABE1,*, Ryoichi YAMAJI2, Hiroshi INUI2, Kazuyoshi SATO3 and Yoshihisa NAKANO2

1 Department of Health Science, Kochi Women's University, Kochi 780-8515, Japan 2 Division of Applied Biochemistry, Graduate School of Agriculture and Biological Sciences, Osaka Prefecture University, Sakai 599-8531, Japan 3 Graduate School of Education, Hiroshima University, Higashi-Hiroshima 739-8524, Japan

(Received January 11, 2002)

Summary High activity (about 50 mU · mg protein⁻¹) of methylmalonyl-CoA mutase (82–95% apo-enzyme) was constantly found during the cell growth of a methanol-utilizing bacterium, Methylobacterium extorquens NR-1. The apo-enzyme was purified to homogeneity and characterized. The purified enzyme was colorless. An apparent Mr of M. extorquens NR-1 enzyme was calculated to be 150,000±5,000 by Superdex 200 HR gel filtration. SDS-polyacrylamide gel electrophoresis of the purified enzyme gave two protein bands with an apparent Mr of 85,000±2,000 and 70,000±2,000, indicating that the M. extorquens NR-1 enzyme is composed of two nonidentical subunits. NH₂-terminal amino acid sequences of the small and large subunits of M. extorquens NR-1 enzyme showed no significant homology to those of the enzyme from other species. Some enzymological properties of the M. extorquens NR-1 enzyme were studied.

Key Words cobalamin, methylmalonyl-CoA, Methylobacterium extorquens, methylotroph

Methylobacterium extorquens NR-1 (formerly Protaminobacter ruber NR-1) is a pink-pigmented facultative methylotroph that synthesizes a large amount of vitamin B₁₂ (or cobalamin) (Cbl), mainly in a form of AdoCbl and slightly in a form of MeCbl (1). The bacterium contains two Cbl-enzymes, MeCbl-dependent methionine synthase (EC 2.1.1.13) catalyzing synthesis of methionine from N⁵-methyltetrahydrofolate and homocysteine (2), and AdoCbl-dependent methylmalonyl-CoA mutase (MCM) (EC 5.4.99.2), which catalyzes the isomerization of (R)-methylmalonyl-CoA to succinyl-CoA in the methanol-assimilation (3). The MCM activity in heterotrophically- and methylotrophically-grown M. extorquens NR-1 cells suggests that the enzyme is involved in growth on both C₁ and non-C₁ compounds as a sole carbon source (4).

MCM has been purified and characterized from mammalian tissues (5), intestinal worm (6), and Propionibacterium shermanii (7). To elucidate the mechanisms for the enzyme reaction, the anaerobic bacterium P. shermanii MCM has been studied extensively (8, 9); the reverse reaction of the enzyme appears to be physiologically important in the anaerobic bacterium (10). However, little information has been available on the enzymological properties of MCM in aerobic bacteria.

Here we describe the purification and characterization of MCM from the aerobic methylotroph M. extorquens NR-1 in the viewpoint of comparative biochemistry.

Materials and Methods

Culture and organism. M. extorquens NR-1 was aerobically cultured for 48 h at 26°C as described previously (3).

Enzyme assay. MCM was assayed by a modification of the HPLC method described by Gaire et al. (11). Briefly, the assay mixture (0.15 mL) for total MCM activity contained 100 mM KPb, pH 7.5, 30 mM AdoCbl (Sigma, St. Louis, MO, USA), 0.15 mM (R,S)-methylmalonyl-CoA (Sigma), and enzyme. AdoCbl was replaced by the same volume of distilled water for measuring apo-MCM activity. The reaction mixture was preincubated for 5 min, started by the addition of (R,S)-methylmalonyl-CoA, and left for 5 min. The enzyme reaction was stopped by the addition of 50 µL of 10% (w/v) trichloroacetic acid. We filtered the reaction mixture...
through a 0.45 µm membrane filter (Millex Syringe Driven Filter Unit, LH-type, Millipore, Bedford, MA, USA). We then analyzed the aliquot (20 µL) of the filtrate by HPLC, using a Shimadzu HPLC apparatus (two LC-10ADVP pumps, DVG-12A degaser, SCL-10Amp system controller, SPD-10AVp UV-VIS detector, CTO-10Avp column oven, 100 µL sample loop, and C-R6A chromatopac integrator). The sample (20 µL) was put on a reversed-phase HPLC column (Cosmosil 5C18-AR-II, φ 3.0×150 mm) equilibrated with 50% (v/v) solvent A (100 mM acetic acid in 100 mM KPB, pH 7.0) and 50% (v/v) solvent B [18% (v/v) methanol in solvent A], (R,S)-methylmalonyl-CoA and succinyl-CoA were eluted with a linear gradient of methanol [50–100% (v/v) solvent B] for 7.0 min at 40°C and assayed by measurement of absorbance at 254 nm. The flow rate was 1.0 mL·min⁻¹. The enzyme activity was defined as the amount of enzyme that catalyzed the formation of succinyl-CoA at the rate of 1 µmol·min⁻¹.

Purification of the Methylobacterium MCM. Purification was performed at 0–4°C with a BioLogic HR chromatography system (Bio-Rad Laboratories, Hercules, CA, USA). The stored M. extorquens NR-1 cells (about 5 g wet weight) were suspended in 50 mL of 10 mM KPB, pH 7.0, containing 10% (w/v) sucrose. The cells were ground with aluminium oxide by using a mortar and pestle, disrupted by sonic oscillation (10 kHz, 20×8), and centrifuged at 10,000×g for 10 min. The supernatant fraction was fractionated with (NH₄)₂SO₄. The precipitate between 40% and 60% (w/w) saturation was collected and dissolved in 200 mL of 10 mM KPB, pH 7.0, containing 10% (w/v) sucrose and 200 mM KCl, and eluted with the same buffer. The dialyzed solution was put on a column (2.4×7.0 cm) of TSKgel QAE-Toyopearl HW55C equilibrated with the same buffer and eluted with 300 mL of a linear gradient (0–5.0 m) of KCl in the same buffer. The peak fractions of the enzyme activity were combined, concentrated to a final volume of 0.1 mL in the Centricon-30, and stored at −80°C.

Polyacrylamide gel electrophoresis (PAGE) in the presence or absence of SDS. A precasted gel (READY GELS J, Bio-Rad) was used in electrophoresis on slab gel of 5–20% linear gradient polyacrylamide in the presence or absence of SDS.

Gel filtration experiments. The Mr of the M. extorquens NR-1 MCM was determined with Superdex 200 HR 10/30 (Amersham Pharmacia Biotech Ltd.) gel filtration column (1.0×30.0 cm) by the use of BioLogic HR chromatography system (Bio-Rad). The column was equilibrated with 10 mM KPB, pH 7.0, containing 10% (w/v) sucrose and 200 mM KCl, and eluted with the same buffer.

Sequencing of NH₂-terminal amino acid residues. The purified enzyme (50 µg) was treated by SDS-polyacrylamide gel electrophoresis, as described, then transferred electrophoretically to a PVDF membrane (Sequiblot, Bio-Rad) in a transfer-blotting apparatus ETB-15 (Advantec, Tokyo, Japan) following the manufacturer’s suggested protocol. The protein was visualized by Coomassie staining, and the band was excised and stored at −20°C until sequenced. The NH₂-terminal amino acid sequence of each subunit of the enzyme was determined by Edman degradation with a Shimadzu (Kyoto, Japan) PPSQ-21 gas-phase protein sequencer.

Preparation of AdoCbi. AdoCbi was prepared by the method of Hay and Finke (12) and further purified with a reversed-phase HPLC [column, Wakosil-IISCI18RS 4.6×150 mm; column oven, 40°C; flow rate, 1.0 mL·min⁻¹; elution, 5–50% linear gradient of methanol in 0.1% (v/v) acetic acid for 30 min; and detection, 278 nm]. The final purified preparation was given a single peak on the reversed-phase HPLC and shown the identical visible spectrum to that described in the cited reference. The concentration of the prepared AdoCbi was calculated on the basis of ε₄₅₈ = 9.3×10³ M·cm⁻¹ for AdoCbi (12).

Determination of inhibition constants (Ki values). Ki values were determined by the HPLC method in which the effect of varying concentrations of AdoCbl at different fixed concentrations of each Cbl-compound was monitored. The assay mixture (0.15 mL) contained 100 mM KPB, pH 7.5; various concentrations of AdoCbl; various concentrations of CN-Cbl, OH-Cbl, or McCbl (Sigma); and the purified apo-enzyme. The reaction mixture was preincubated for 5 min, started by the addition of (R,S)-methylmalonyl-CoA, and left for 5 min.

Assay of Cbl. A cell homogenate of M. extorquens NR-1 and the purified MCM were incubated with 10 µM AdoCbl for 4 h at 4°C and separated in the dark from the excess of AdoCbl by Sephadex G-25 gel filtration. Aliquot (0.15 mL) of the protein fractions combined was added to 1.35 mL of 0.1 mM acetic acid buffer, pH 4.8, containing 1 mM KCN. The solution was boiled for 30 min for 98°C in the dark, cooled to room temperature, and centrifuged at 10,000×g for 10 min at 4°C. The supernatant fraction was used for the following Cbl
assay. Total Cbl was assayed microbiologically with Lactobacillus delbrueckii subsp. lactis ATCC 7830 and a Cbl assay medium (Nissui, Tokyo, Japan) (13).

Protein assay. Protein was assayed by the use of a Bio-Rad protein assay kit, with ovalbumin as a standard according to the manufacturer’s instructions.

Results and Discussion

The time course of changes in the MCM activity and the amount of Cbl during M. extorquens NR-1 cell growth are shown in Fig. 1. The enzyme activities derived from total (holo- and apo-) and apo-MCM reached a maximum (56.1 and 52.1 mU·mg protein⁻¹, respectively) at the end of logarithmic growth phase and slightly decreased thereafter; the identical pattern was also shown in the cellular Cbl level. Only about 5–13% of the total MCM activity was derived from the holo-enzyme; most of the MCM occurred as the stable apo-enzyme during M. extorquens NR-1 cell growth. The total MCM activity of 48.9 mU·mg protein⁻¹, detected in a cell homogenate of M. extorquens NR-1 grown for 48 h by the HPLC assay method, was similar to the enzyme activity determined by the hydroxamic acid method, described previously (3).

The holo-enzyme converted from the apo-enzyme by incubation with 10 μM AdoCbl at 4°C for 4 h was treated with fluorescent light at a distance of 10 cm at 4°C for several hours. Most of the holo-enzyme activity was lost during 0.5 h-light exposure, indicating that the prosthetic AdoCbl bound to the enzyme is very labile to light exposure. Thus the apo-MCM was purified to homogeneity.

The purification procedures for apo-MCM from a homogenate of M. extorquens NR-1 are summarized in Table 1. The purified MCM was colorless, and the enzyme activity was completely lost in the absence of AdoCbl. PAGE of the purified enzyme in the absence of SDS showed a single protein band (Fig. 2A).

An apparent Mr of M. extorquens NR-1 MCM was calculated to be 150,000±5,000 by Superdex 200 HR gel filtration. SDS-PAGE of the purified enzyme gave two protein bands with an apparent Mr of 85,000±3,000 and 70,000±2,000 (Fig. 2B), indicating that the M. extorquens NR-1 MCM is composed of two nonidentical subunits. The mammalian enzymes consist of a homodimer (identical subunits with Mr of 72,000–77,500), each of which contains one AdoCbl molecule (5). Although the P. shermanii enzyme consists of two non-

---

**Table 1. Purification of MCM from Methyllobacterium extorquens NR-1.**

<table>
<thead>
<tr>
<th>Step</th>
<th>Total protein (mg)</th>
<th>Total activity (U·min⁻¹)</th>
<th>Specific activity (U·mg protein⁻¹)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude homogenate</td>
<td>576.0</td>
<td>27.5</td>
<td>0.05</td>
<td>100</td>
</tr>
<tr>
<td>40–60% (NH₄)₂SO₄ fractionation</td>
<td>274.3</td>
<td>22.4</td>
<td>0.08</td>
<td>81.5</td>
</tr>
<tr>
<td>TSKgel QAE-Toyopearl 550</td>
<td>2.5</td>
<td>11.1</td>
<td>5.62</td>
<td>50.1</td>
</tr>
<tr>
<td>HiLoad 16/60 Superdex 200 pg</td>
<td>0.8</td>
<td>6.5</td>
<td>7.48</td>
<td>23.7</td>
</tr>
<tr>
<td>1st UNO Q-1</td>
<td>0.5</td>
<td>4.2</td>
<td>8.96</td>
<td>15.2</td>
</tr>
<tr>
<td>2nd UNO Q-1</td>
<td>0.1</td>
<td>1.9</td>
<td>15.86</td>
<td>6.9</td>
</tr>
</tbody>
</table>
identical subunits with Mr of 79,000 and 67,000 (7), only the large one contains one AdoCbl molecule (8). Cbl content was assayed microbiologically in the holo-MCM converted from the purified apo-enzyme by incubation with 10 μM AdoCbl at 4°C for 4 h. The results indicated that the enzyme contains one AdoCbl per heterodimer enzyme as the prosthetic group. The NH₄-terminal amino acid sequences of the small and large subunits of the M. extorquens NR-1 MCM were Met-Glu, Ile-Pro-Asp-Phe- and Gly-Gly-Arg-, respectively. They showed no significant homology to those of MCM from other species.

The optimum temperature of the enzyme, when incubated at various temperatures for 5 min at pH 7.5, was 37°C. The optimum pH of the enzyme, when incubated at various pH for 5 min at 37°C, was 7.5. The apparent Km values of the enzyme were 30.9 μM for (R,S)-methylmalonyl-CoA [(R)-methylmalonyl-CoA 15.4 μM] and 0.29 mM for succinyl-CoA (the reverse reaction).

The apparent Km values of the apo-enzyme were 30.9 nM and 5.6 μM for AdoCbl and AdoCbi, respectively; Vmax in the presence of AdoCbi was about 28% of that in the presence of AdoCbl. The MCM activity was competitively inhibited by some Cbl-compounds (CN-Cbl, Ki=0.12 mM; OH-Cbl, Ki=1.2 μM; and MeCbl, Ki=1.2 μM). The results indicate that the M. extorquens NR-1 MCM specifically binds AdoCbl as a cofactor.

When the apo-MCM purified from M. extorquens NR-1, before being preincubated with AdoCbl for 5 min, was treated with various metal ions for 5 min at 37°C, the enzyme activity was inhibited completely by the addition of 3 mM Hg²⁺. Other metal ions (Na⁺, K⁺, Ca²⁺, Fe²⁺, Mg²⁺, Co²⁺, each at 3 mM) and 3 mM EDTA (a chelator) caused no inhibition of the enzyme activity; the identical results were obtained when the apo-enzyme was preincubated with AdoCbl for 5 min at 37°C, then treated with each metal ion or EDTA for 5 min.

The M. extorquens NR-1 MCM activity was not affected by usually used SH-reagents, such as P. shermanii (10) and intestinal worm (6).

The results presented here indicate that M. extorquens NR-1 is an aerobic bacterium suitable for use as an apo-MCM source in biochemical study of MCM.

REFERENCES


