Purification, crystallization and X-ray crystallographic analysis of methylmalonyl-CoA epimerase from *Metallosphaera sedula*

Seongjoon Joo, Seul Hoo Lee, Donghoon Lee and Kyung-Jin Kim*

School of Life Sciences, BK21 FOUR KNU Creative BioResearch Group, KNU Institute for Microorganisms, Kyungpook National University, Daegu 41566, Republic of Korea

*Correspondence: kkim@knu.ac.kr

Methylmalonyl-CoA epimerase (MMCE) is an enzyme involved in the carbon capturing 3-HP/4-HB cycle, by catalyzing the reaction of converting (S)-methylmalonyl-CoA to (R)-methylmalonyl-CoA. In this study, we overexpressed MMCE from *Metallosphaera sedula* (MsMMCE) and purified the protein to homogeneity by Ni-NTA affinity and size-exclusion chromatography. The MsMMCE protein was crystallized using the hanging-drop vapor-diffusion method in the presence of 20% (w/v) polyethylene glycol 3350, and 0.2 M ammonium nitrate at 293 K. X-ray diffraction data were collected to a maximum resolution of 2.1 Å. The MsMMCE crystals belong to the space group $P2_1$ with unit cell parameters $a = 51.87$ Å, $b = 79.00$ Å, $c = 106.09$, $\alpha = \gamma = 90.0^\circ$, $\beta = 95.80^\circ$. With six molecules of MsMMCE per an asymmetric unit, the crystal volume per unit of protein mass is $2.33 \text{ Å}^3 \text{ Da}^{-1}$, which corresponds to a solvent content of approximately 47.13%.

INTRODUCTION

Accumulation of carbon dioxide (CO_2) in the atmosphere due to continued use of fossil fuels after the Industrial Revolution has caused environmental problems such as global climate change. Many countries are working towards carbon neutrality by reducing carbon emissions, such as reducing the use of fossil fuels (Arctic Climate Impact Assessment, 2004; McGuire et al., 2006; McGuire et al., 2009). There is also an increasing interest in microbes that can capture C1 carbon. In prokaryotes, a total of six distinct autotrophic CO_2 fixation pathway have been identified, such as the reductive pentose phosphate cycle (Pirson, 1960), the reductive citric acid cycle (Antranikian et al., 1982; Shiba et al., 1985), the reductive acetyl-CoA pathway (Pezacka and Wood, 1986), the 3-hydroxypropionate/methylmalonyl-CoA cycle (Strauss and Fuchs, 1993), the dicarboxylate/4-hydroxybutyrate cycle (Huber et al., 2008), and the 3-hydroxypropionate/4-hydroxybutyrate (3-HP/4HB) cycle (Berg et al., 2007).

The *Metallosphaera sedula* identified in the volcanic zone of Pisciarelli Solfatara in Naples, Italy is the first strain known to be capable of carbon fixation through the 3-HP/4-HB cycle (Huber et al., 1989; Berg et al., 2007; Berg et al., 2010; Estelmann et al., 2011). The 3-HP/4-HB cycle can be largely divided into two sub-pathways, a 3-HP sub-pathway and a 4-HB sub-pathway. The 3-HP sub-pathway begins when acetyl-CoA is converted to malonyl-CoA by capturing carbonate enzymatically (Chuakrut et al., 2003; Hügler et al., 2003; Berg et al., 2007; Teufel et al., 2009; Estelmann et al., 2011). This malonyl-CoA is converted to propionyl-CoA through 5 enzymatic reactions (Chuakrut et al., 2003; Hügler et al., 2003; Berg et al., 2007; Teufel et al., 2009; Estelmann et al., 2011). Then, propionyl-CoA is converted

to (S)-methylmalonyl-CoA through an enzymatic reaction that captures carbonate once again (Chuakrut et al., 2003; Hügler et al., 2003; Berg et al., 2007; Teufel et al., 2009; Estelmann et al., 2011). Thereafter, (S)-methylmalonyl-CoA is converted to (R)-methylmalonyl-CoA by methylmalonyl-CoA epimerase (MMCE), and finally becomes succinyl-CoA by methylmalonyl-CoA mutase (Chuakrut et al., 2003; Hügler et al., 2003; Berg et al., 2007; Teufel et al., 2009; Estelmann et al., 2011). In this way, acetyl-CoA was converted to succinyl-CoA with two more carbons by receiving two molecules of carbonate through the 3-HP sub-pathway (Chuakrut et al., 2003; Hügler et al., 2003; Berg et al., 2007; Teufel et al., 2009; Estelmann et al., 2011). Subsequently, succinyl-CoA is divided into two acetyl-CoA through seven enzymatic reactions in the 4-HB sub-pathway (Chuakrut et al., 2003; Hügler et al., 2003; Berg et al., 2007; Teufel et al., 2009; Estelmann et al., 2011).

In general, MMCE is involved in the process of converting 3-carbon propionyl-CoA into 4-carbon succinyl-CoA in the lipolysis pathway of an odd numbered carbon chain lipid (Chalmers and Lawson, 1982). When propionyl-CoA is converted to methylmalonyl-CoA by propionyl-CoA carboxylase, it takes the S-form (Chalmers and Lawson, 1982). Since methylmalonyl-CoA mutase recognizes only R-form methylmalonyl-CoA as a substrate and converts it to succinyl-CoA, chiral conversion of S-form methylmalonyl-CoA to R-form is essential (Chalmers and Lawson, 1982). MMCE is an enzyme that catalyzes this chiral conversion of (S)-methylmalonyl-CoA to (R)-methylmalonyl-CoA (Figure 1) (Chalmers and Lawson, 1982).

In *M. sedula*, the same reaction from propionyl-CoA to succinyl-CoA occurs in the 3-HP sub-pathway during the

3-HP/4-HB carbon fixation cycle (Teufel et al., 2009). Therefore, MMCE from *M. sedula* (MsMMCE) is an essential enzyme for carbon fixation of *M. sedula*. Despite this importance, research on MsMMCE is insufficient. Thus, in this study, we described the cloning, expression, purification, crystallization, and X-ray crystallographic analysis of the MsMMCE protein.

MATERIALS AND METHODS

Production of MsMMCE

We designed the forward and reverse primers as 5'-GCGC CATATGGAAACCTT AGATATAGATCACGTG-3' and 5'-GCGC CTCGAGATGGGTTTCTTGAACGAGTTCCA G-3' with introduced *Nde*I and *Xho*I restriction sites, respectively (underlined). The MsMMCE gene was amplified by polymerase chain reaction (PCR) using genomic DNA from *M. sedula* as a template. The PCR product was then subcloned into pET30a expression vector (Novagen) with a six-His tag at the C-terminus. The pET30a:MsMMCE was transformed into the *E. coli* strain BL21(DE3)-T1^R, which was grown in 1 L of Luria-Bertani medium containing kanamycin at 37°C. At an OD₆₀₀ of 0.6, MsMMCE protein expression was induced by addition of 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and the culture medium was maintained for a further 16 h at 18°C. The culture was then harvested by centrifugation at 4,000 × *g* for 15 min at 20°C. The cell pellet was resuspended in buffer A (40 mM Tris-HCl, pH 8.0) and then disrupted by ultrasonication. The cell debris was removed by centrifugation at 13,500 × *g* for 30 min. And supernatant was applied to an Ni-NTA agarose column (Thermo Fisher Scientific, USA). After washing with buffer A containing 30 mM imidazole, the bound proteins were eluted with 300 mM imidazole in buffer A. Finally, a trace number of contaminants

were removed by size-exclusion chromatography by using a HiPrep 26/60 Sephacryl S-300 HR column (GE Healthcare Life Sciences) equilibrated with buffer A. We performed purification experiments at 4°C. SDS-PAGE analysis of the purified proteins shows a single polypeptide of 15.5 kDa corresponding to the molecular weight of the MsMMCE monomer. The purified protein was concentrated to 135 mg/ml in 40 mM Tris-HCl, pH 8.0. Macromolecule-production information is given in Table 1.

Crystallization of MsMMCE

Crystallization of the purified MsMMCE was initially performed with commercially available spares-matrix screens, including Index, PEG ion I and II (Hampton Research), and Wizard Classic I and II (Rigaku Reagents), using the hanging-drop vapor-diffusion method at 20°C. Each experiment consisted of mixing 1.0 μL protein solution (135 mg/ml in 40 mM Tris-HCl, pH 8.0) with 1.0 μL reservoir solution and then equilibrating this against 500 μL reservoir solution. MsMMCE crystals were observed from several crystallization conditions. After several steps for crystal improvement, the best quality crystals appeared in 20% (w/v) polyethylene glycol 3350, and 0.2 M ammonium nitrate. Crystallization information is summarized in Table 2.

X-ray diffraction analysis of MsMMCE

Diffraction data from MsMMCE crystals were collected at the 7A beamline of the Pohang Accelerator Laboratory (PAL, Pohang, Korea) (Park et al., 2017), using a Quantum 270 CCD detector (ADSC, USA). The crystals of MsMMCE diffracted to a resolution of 2.1 Å. All data were indexed, integrated, and scaled using the HKL-2000 software package (Otwinowski and Minor, 1997). The data statistics are summarized in Table 3.

RESULTS AND DISCUSSION

MsMMCE protein was purified by Ni-NTA affinity chromatography and then further purified through size-exclusion chromatography to obtain purified single protein. The eluted protein from size-

FIGURE 1 | Enzyme reaction of MsMMCE.

TABLE 1 | Macromolecule-production information

Source organism	<i>Metallosphaera sedula</i>
DNA source	Chromosomal DNA
Forward primer	GCGCCATATGGAACCTTAGATATAGATCACGTG
Reverse primer	GCGCCTCGAGATGGGTTTCTTGAACGAGTTCCAG
Cloning vector	pET-30a
Expression vector	pET-30a
Expression host	<i>E. coli</i> BL21 (DE3)-T1 ^R
Complete amino-acid sequence of the construct produced	METLDIDHVGVAVENLEEAIKLYTEKMGMKLVHREDLPDR GIKVAFLTGNEGTTAVELMEPMNHEDPNNTVAKFLKTRGQ GMHHLAVKVKDINSSLRDLEGKGLTLIDKNGRKGGARHLVAFVHPKSVMLGLLELVQETH LEHHHHHHH

The underlined characters in the primers indicate restriction-enzyme sites. The bold-face characters in the complete amino-acid sequence of the construct produced indicate C-terminal tag.

TABLE 2 | Crystallization

Method	Hanging-drop vapor diffusion
Plate type	SPL plate (24-well)
Temperature (K)	293
Protein concentration (mg ml ⁻¹)	135
Buffer composition of protein solution	40 mM Tris-HCl pH 8.0
Composition of reservoir solution	20% polyethylene glycol 3350, and 0.2 M Ammonium nitrate
Volume and ratio of drop	2 µL; 1:1 ratio of protein and reservoir solutions
Volume of reservoir (ml)	0.5

TABLE 3 | Data collection and processing

Diffraction source	Beamline 7A, PAL
Wavelength (Å)	0.97934
Temperature (K)	100
Detector	Quantum 270 CCD
Crystal-to-detector distance (mm)	200
Rotation range per image (°)	1
Total rotation range (°)	360
Exposure time per image(s)	1
Space group	<i>P</i> 2 ₁
a, b, c (Å)	51.9, 79.0, 106.1
α, β, γ (°)	90.0, 95.8, 90.0
Resolution range (Å)	50.00-2.05 (2.09-2.05) ^a
Total No. of reflections	54070
Completeness (%)	97.2 (95.7) ^a
Rsym or Rmerge (%)	4.7 (30.4) ^a
I / σI	20.8 (2.5) ^a
Redundancy	3.1 (2.5) ^a

^aThe numbers in parentheses are statistics from the highest resolution shell.

exclusion chromatography column, calibrated with Gel Filtration Calibration Kits (GE Healthcare Life Sciences), had a molecular weight of ~31 kDa which match a dimer form of MsMMCE protein (data not shown). SDS-PAGE of the purified MsMMCE protein showed a single band with a monomeric molecular weight of 15.5 kDa (Figure 2).

The MsMMCE protein was crystallized in 20% (w/v) polyethylene glycol 3350, and 0.2 M ammonium nitrate (Figure 3). The crystals of MsMMCE protein were moved to cryo protectant solution containing 26% (w/v) polyethylene glycol 3350, 0.2 M ammonium nitrate, and 30% (v/v) glycerol. The crystals were fished out with a loop larger than the crystals, and flash-frozen by immersion in liquid nitrogen. The crystals of MsMMCE protein diffracted to resolution of 2.1 Å. The MsMMCE crystals belonged to the space group *P*2₁ with unit cell parameters *a* = 51.87 Å, *b* = 79.00 Å, *c* = 106.09 Å, α = γ = 90.0°, β = 95.80°. Assuming six molecules of MsMMCE protein (15.5 kDa) per asymmetric unit,

FIGURE 2 | Purification of MsMMCE. SDS-PAGE of purification of recombinant MsMMCE protein. Lane 1 shows molecular-weight markers (labelled in kDa). Lanes 2-11 show the purification procedure of MsMMCE using Ni-NTA chromatography. Lane 2, whole cell extract; lanes 3 and 4, pellet and supernatant fraction after centrifugation of whole cell extract, respectively; lane 5, flowthrough from Ni-NTA column; lane 6 to 9, wash with 0, 10, 20, and 30 mM imidazole, respectively; lane 10 and 11, elution with 300 mM imidazole. Purified MsMMCE is indicated with an arrow.

FIGURE 3 | Crystals of MsMMCE. The best quality crystal was produced from the condition with 20% polyethylene glycol 3350, and 0.2 M ammonium nitrate and grew to maximum dimensions of 0.2 × 0.2 × 0.05 mm within 3 days.

FIGURE 4 | Initial 2Fo-Fc maps of MsMMCE structure. The 2Fo-Fc maps (contoured at 1.0 σ) are shown as gray meshes.

the crystal volume per unit of protein mass was $2.33 \text{ \AA}^3 \text{ Da}^{-1}$, meaning that the solvent content was approximately 47.13% (Matthews, 1968).

We determined the structure of MsMMCE protein by molecular replacement with the CCP4 version of MOLREP (Vagin and Teplyakov, 1997) using the structure of hypothetical protein BH1468 from *Bacillus halodurans* C-125 (BhHP) (PDB CODE 3OA4) as a search model. MsMMCE shows 38.64% amino acid sequence identity to BhHP. We performed model building and refinement using the program WinCoot (Emsley and Cowtan, 2004) and CCP4 remlcp5 (Murshudov et al., 1997), respectively. The initial electron density map, which was good quality with backbone atoms well defined by electron density, allowed us to build a three-dimensional structure of MsMMCE (Figure 4). Crystallographic model building and structure refinement to 2.1 Å resolution are in progress.

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CONFLICT OF INTEREST

The authors have declared that no competing interest exist.

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