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Purification, crystallization and preliminary X-ray crystallographic analysis of a methanol dehydrogenase from the marine bacterium *Methylophaga aminisulfidivorans* MP^T

Jin Myung Choi,[a]‡ Hee Gon Kim,[b]‡ Jeong-Sun Kim,[c] Hyung-Seop Youn,[d] Soo Hyun Eom,[d] Sung-Lim Yu,[e] Si Wouk Kim[b] and Sung Haeng Lee[a]*

[a]Department of Cellular and Molecular Medicine, Chosun University School of Medicine, Gwangju 501-759, Republic of Korea, [b]Department of Environmental Engineering, Chosun University, Gwangju 501-759, Republic of Korea, [c]Department of Chemistry, Chonnam National University, Gwangju 500-757, Republic of Korea, [d]School of Life Sciences, Gwangju Institute of Science and Technology, Gwangju 500-712, Republic of Korea, and [e]Inha Research Institute for Medical Sciences, Inha University School of Medicine, Incheon 400-712, Republic of Korea

‡ These authors contributed equally to this work.

Correspondence e-mail: sunglee@chosun.ac.kr

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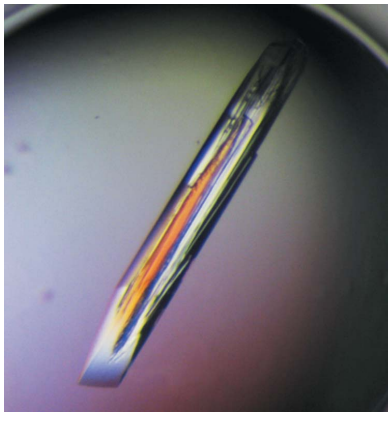

Methylophaga aminisulfidivorans MP^T is a marine methylotrophic bacterium that utilizes C_1 compounds such as methanol as a carbon and energy source. The released electron from oxidation flows through a methanol-oxidizing system (MOX) consisting of a series of electron-transfer proteins encoded by the *mox* operon. One of the key enzymes in the pathway is methanol dehydrogenase (MDH), which contains the prosthetic group pyrroloquinoline quinone (PQQ) and converts methanol to formaldehyde in the periplasm by transferring two electrons from the oxidation of one methanol molecule to the electron acceptor cytochrome c_L. In order to obtain molecular insights into the oxidation mechanism, a native heterotetrameric $\alpha_2\beta_2$ MDH complex was directly purified from *M. aminisulfidivorans* MP^T grown in the presence of methanol and crystallized. The crystal diffracted to 1.7 Å resolution and belonged to the monoclinic space group $P2_1$ (unit-cell parameters a = 63.9, b = 109.5, c = 95.6 Å, β = 100.5°). The asymmetric unit of the crystal contained one heterotetrameric complex, with a calculated Matthews coefficient of 2.24 Å^3 Da^{-1} and a solvent content of 45.0%.

1. Introduction

Gram-negative methylotrophic bacteria utilize one-carbon organic compounds such as methanol to generate energy and participate in the carbon-recycling chain in nature. One of the critical enzymes in the process is methanol dehydrogenase (MDH; EC 1.1.99.8), which resides in the periplasmic region of the bacterial membrane (Anthony, 1986; Williams *et al.*, 2005).

MDH is a pyrroloquinoline quinone (PQQ) containing protein that catalyzes the conversion of methanol to formaldehyde (Miyazaki *et al.*, 1987; Read *et al.*, 1999; Anthony, 1992). The electrons released from PQQ during the oxidation successively reduce cytochrome c_L, cytochrome c_H and finally the membrane oxidase cytochrome aa_3 (Dijkstra *et al.*, 1989; Read *et al.*, 1999). As a result of the oxido-reduction pathway, a proton electrochemical gradient is produced around the membrane, which in turn drives the generation of one ATP molecule per molecule of methanol.

To date, the X-ray structures of MDH from four different bacteria, *Methylobacterium extorquens* AM1 (Ghosh *et al.*, 1995; Afolabi *et al.*, 2001; Williams *et al.*, 2005), *Hyphomicrobium denitrificans* (Nojiri *et al.*, 2006), *Paracoccus denitrificans* (Xia *et al.*, 2003) and *Methylophilus methylotrophus* W3A1 (Xia *et al.*, 1996), have been determined and they show high structural similarity. Indeed, alignment of the sequences shows 66–81% sequence identity between the enzymes and their structures superimpose with relatively small r.m.s. deviations of between 0.5 Å (between *P. denitrificans* and *H. denitrificans*) and 1.1 Å (between *M. extorquens* AM1 and *M. methylotrophus*). They comprise a heterotetrameric ($\alpha_2\beta_2$) complex with only minor differences in the length of the C-terminus in each subunit and inserted regions. Half of the β-subunit folds mainly as α-helix, while the other half remains unstructured. Both of these secondary elements wrap the α-subunit with extensive interactions, although the biological function of the β-subunit remains unknown (Williams *et al.*, 2005). The α-subunit is known to function as the active site for the oxidoreduction reaction, which includes the PQQ group as a redox

Table 1
Data-collection statistics for MDH from *M. aminisulfidivorans* MP^T.

Values in parentheses are for the highest resolution shell.

Beamline	PAL-4A
Wavelength (Å)	1.0000
Temperature (K)	100
Space group	$P2_1$
Unit-cell parameters (Å, °)	a = 63.9, b = 109.5, c = 95.6, α = 90.0, β = 100.5, γ = 90.0
Data resolution (Å)	50.0–1.7 (1.73–1.70)
Completeness (%)	99.7 (98.7)
Multiplicity	5.3 (3.9)
Total reflections	742859
Unique reflections	140540 (6897)
R_{merge}† (%)	11.2 (47.8)
Average $I/\sigma(I)$	8.8 (2.8)
Matthews coefficient (Å^3 Da^{-1})	2.24
Solvent content (%)	45.0
No. of molecules in asymmetric unit	1

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ and $\langle I(hkl)\rangle$ are the intensity of an individual reflection and the mean value of all measurements of an individual reflection, respectively.

cofactor and a calcium ion coordinated to vicinal charged and hydrophobic amino-acid residues (Anthony, 2001; Afolabi *et al.*, 2001).

Similar to the operons from terrestrial methylotrophs, the gene structure of the methanol-oxidizing system (*mox*) of *M. aminisulfidivorans* MP^T consists of *mxaFJGIRS* (GenBank accession No. EF378629). Of these genes, *mxaF*, *mxaI* and *mxaG* encode the α- and β-subunits of MDH and cytochrome c_L, respectively. Although an additional 32 kDa MxaJ binds to the $\alpha_2\beta_2$ MDH complex from *Acetobacter methanolicus* (Matsushita *et al.*, 1993; Tanaka *et al.*, 1997), substantial evidence for direct interaction and functional involvement of MxaJ and MDH has not been reported. Our preliminary results, however, indicate that MxaJ (γ-subunit) appears to be a component of a ternary complex ($\alpha_2\beta_2\gamma$) that enhances methanol oxidation followed by careful reconstitution in comparison with that by $\alpha_2\beta_2$ MDH (data not shown). Furthermore, the MDH complex from the marine bacterium *M. aminisulfidivorans* MP^T seems to be more resistant to NaCl concentration, whereas MDHs from other terrestrial methylotrophic bacteria become completely inactive (data not shown), and its isoelectric point is acidic (pI = 5.4) in comparison with those (pI = ∼8.0) of MDHs from terrestrial bacteria even though they share high sequence identity (65–78% for the α-subunit and 58–75% for the β-subunit between MDH from *M. aminisulfidivorans* MP^T and the other members). Therefore, it was expected that determination of the MDH structure from a marine bacterium could help to explain these observations. In addition, the precise molecular mechanism of the electron-transfer pathway mediated by MDH has not been elucidated in conjunction with the role of the other components coded by the operon. Thus, the accumulation of three-dimensional structural data for the proteins of the operon might provide molecular insights into the intermolecular and intramolecular electron-transfer pathway and/or substrate interaction. In this paper, the MDH complex from a marine bacterium, *M. aminisulfidivorans* MP^T, was purified and crystallized for the first time and preliminary X-ray crystallographic analysis was carried out.

2. Materials and methods

2.1. Expression and purification of MDH

The overproduction of genome-coded MDH from *M. aminisulfidivorans* MP^T was carried out using the protocol described by Ghosh *et al.* (1992) and Kim *et al.* (2007). Briefly, cells were harvested after growth at 303 K in seawater medium supplemented with 1%(*v*/*v*) methanol. The cell pellet was then resuspended in standard buffer (25 m*M* Tris–HCl pH 8.0) containing lysozyme and disrupted by several freeze–thaw cycles. Cell debris and the membrane part were then removed by centrifugation (15 000*g*). In order to purify the active $\alpha_2\beta_2$ MDH complex, which consisted of 662 amino acids after cleavage of the N-terminal signal sequences (31 amino acids for the α-subunit and 24 amino acids for the β-subunit), the cell-free extract was centrifuged at 100 000*g* for 90 min and the soluble fraction was loaded onto a MonoQ anion-exchange column (GE Healthcare, USA) pre-equilibrated with standard buffer. The bound $\alpha_2\beta_2$ MDH complex eluted at between 100 and 150 m*M* NaCl in an active form. The protein was further purified by gel filtration on a HiLoad 16/60 Superdex 200 column (GE Healthcare, USA) equilibrated with standard buffer containing 150 m*M* NaCl. The purity and size of the purified protein was examined by 15% SDS–PAGE.

2.2. Crystallization and data collection

Prior to crystallization trials, the purified protein was concentrated to ∼8 mg ml^{-1} in 25 m*M* Tris–HCl pH 8.0. Crystallization conditions were screened using commercial screens from Hampton Research, including Index, SaltRx, PEG/Ion, PEG/Ion 2, Crystal Screen, Crystal Screen 2 and Crystal Screen Lite, at 293 K. Methanol dehydrogenase crystals were initially obtained from Hampton Research Crystal Screen Lite condition No. 18, consisting of 0.1 *M* sodium cacodylate pH 6.5, 0.2 *M* magnesium acetate tetrahydrate and 10%(*v*/*v*) polyethylene glycol 8000 using the hanging-drop vapour-diffusion method at 293 K. Further improvements in crystal size and quality were obtained by a combination of varying the salt and precipitant concentrations and the pH. The single large crystals were soaked for 30 s in cryosolution consisting of 0.1 *M* sodium cacodylate pH 6.5, 0.2 *M* magnesium acetate tetrahydrate, 10%(*v*/*v*) polyethylene glycol 8000 and 20%(*v*/*v*) glycerol and flash-cooled in liquid nitrogen.

Data collection was carried out by mounting a crystal in a stream of cold nitrogen at 100 K. Complete X-ray diffraction data were

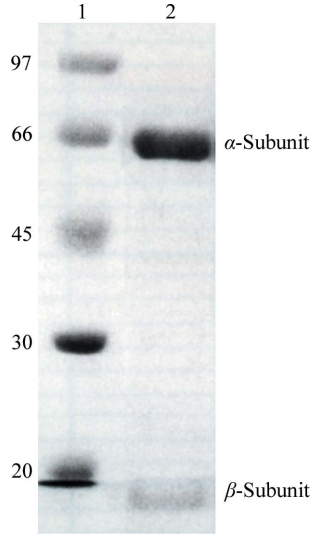

Figure 1
Purification of methanol dehydrogenase (MDH) from *M. aminisulfidivorans* MP^T. The purity and size of MDH were analyzed on SDS–PAGE by loading 4 µg purified protein. The molar ratio between the α-subunit and the β-subunit appears to be 1:1 after considering the molecular-weight difference between the subunits. Lane 1, molecular-weight markers (kDa); lane 2, separated α-subunit (M_r = 65 000) and β-subunit (M_r = 7500) of purified MDH.

collected to 1.7 Å resolution from a native MDH complex crystal on beamline 4A (PAL-4A) at Pohang Accelerator Laboratory (Pohang, Republic of Korea) using an ADSC Quantum 315r CCD detector with an oscillation of 1.0° and 5 s exposure per frame over a 320° range. The diffraction data were processed with the program *HKL*-2000 (HKL Research Inc.); the data-collection statistics are shown in Table 1.

2.3. Molecular replacement and refinement

A molecular-replacement solution was obtained with the program *MOLREP* (Vagin & Teplyakov, 2010) using the 1.2 Å resolution structure of methanol dehydrogenase from *Methylobacterium extorquens* AM1 (PDB entry 1w6s; Williams *et al.*, 2005), which shares 78 and 75% sequence identity for the α- and β-subunits, respectively, as a search model. To build the search model, the PQQ prosthetic group and the calcium ion in the MDH structure were removed from the original coordinates.

3. Results and discussion

Native methanol dehydrogenase (MDH; $\alpha_2\beta_2$) of *M. aminisulfidivorans* MP^T was overproduced in the presence of methanol and was successfully purified as a complex form with the expected molecular weight (Fig. 1). The post-translational cleavage of a signal peptide from each subunit was verified by N-terminal amino-acid determination, which produced 596 amino acids (residues 32–627, M_r = 65 000) for the α-subunit and 66 amino acids (residues 25–90, M_r = 7500) for the β-subunit. Highly concentrated native protein (~8 mg ml^{-1}) in 25 m*M* Tris–HCl pH 8.0 was used in crystallization using the hanging-drop vapour-diffusion method at 293 K by mixing 1 µl protein solution and 1 µl reservoir solution consisting of 0.1 *M* sodium cacodylate pH 6.5, 0.2 *M* magnesium acetate tetrahydrate, 10%(*v*/*v*) polyethylene glycol 8000. Initial crystals appeared as multiple needle clusters which were not suitable for diffraction trials. Further improvement trials varying the constituents of the reservoir solution resulted in the formation of large single rod-shaped crystals with a smooth-surfaced tapered tip at one end (arrow in Fig. 2). Each drop (2 µl) contained a single large crystal of approximate dimensions 0.2 × 0.2 × 1.5 mm. The cryoprotected crystals diffracted to a maximum resolution of 1.7 Å (Fig. 3). During data collection, the X-ray beam was focused on the tip of the crystal in order to obtain the best data quality and resolution.

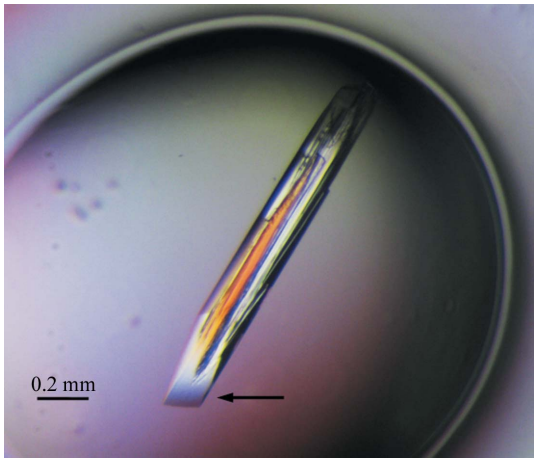

Figure 2
Crystal of MDH. Only one crystal grew in each drop, with dimensions of 0.2 × 0.2 × 1.5 mm. The crystal was stable in cryoprotectant containing 20% glycerol for diffraction.

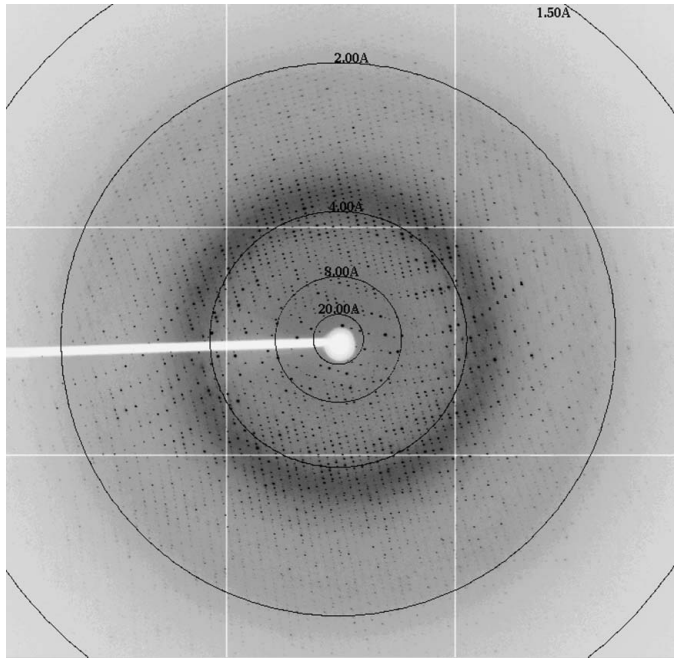

Figure 3
Typical diffraction image of an MDH crystal. The diffraction data were collected by focusing the X-ray beam on one end of the crystal (shown by the arrow in Fig. 1). The oscillation was 1.0° per frame with 5 s exposure over a 320° range and the edge of the detector corresponds to 1.7 Å resolution.

The results of autoindexing and scaling indicated that the crystal belonged to the monoclinic space group $P2_1$, with unit-cell parameters a = 63.9, b = 109.5, c = 95.6 Å, β = 100.5° (Table 1). The asymmetric unit is likely to contain one $\alpha_2\beta_2$ complex, which corresponds to a calculated Matthews coefficient of 2.24 Å^3 Da^{-1} and a solvent content of 45.0% (Matthews, 1968). Initial phases from molecular replacement followed by an initial round of refinement with the *CCP*4 program *REFMAC* converged to *R*-factor and R_{free} values of 27% and 31%, respectively. Two α-subunits and two β-subunits were traced from the initial phasing map and further refinement for completion of the structure is now in progress.

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