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Crystallization and preliminary X-ray crystallographic analysis of MxaJ, a component of the methanol-oxidizing system operon from the marine bacterium *Methylophaga aminisulfidivorans* MP^T

The methanol-oxidizing system (*mox*) is essential for methylotrophic bacteria to extract energy during the oxidoreduction reaction and consists of a series of electron-transfer proteins encoded by the *mox* operon. One of the key enzymes is the $\alpha_2\beta_2$ methanol dehydrogenase complex (type I MDH), which converts methanol to formaldehyde during the $2e^-$ transfer through the prosthetic group pyrroloquinoline quinone. MxaJ, a product of *mxj* of the *mox* operon, is a component of the MDH complex and enhances the methanol-converting activity of the MDH complex. However, the exact functional mechanism of MxaJ in the complex is not clearly known. To investigate the functional role of MxaJ in MDH activity, an attempt was made to determine its crystal structure. Diffraction data were collected from a selenomethionine-substituted crystal to 1.92 Å resolution at the peak wavelength. The crystal belonged to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters $a = 37.127$, $b = 63.761$, $c = 99.246$ Å. The asymmetric unit contained one MxaJ molecule with a calculated Matthews coefficient of $2.11 \text{ Å}^3 \text{ Da}^{-1}$ and a solvent content of 41.7%. Three-dimensional structure determination of the MxaJ protein is currently in progress by the single-wavelength anomalous dispersion technique and model building.

1. Introduction

Gram-negative methylotrophic bacteria extract energy when they convert methanol to formaldehyde (Anthony, 1986). This reaction is catalysed by the $\alpha_2\beta_2$ methanol dehydrogenase (MDH; EC 1.1.99.8) complex in the periplasmic region of the bacterial membrane, where the enzyme carries out redox reactions of cytochromes such as Cyt_c_L, Cyt_c_H and Cyt_a₃ (Williams *et al.*, 2005; Cox *et al.*, 1992). During these reactions, the prosthetic group pyrroloquinoline quinone (PQQ) in MDH mediates the release of proton molecules into the periplasmic region and the resulting proton gradient across the membrane drives the generation of one ATP molecule per methanol molecule (Dijkstra *et al.*, 1989; Read *et al.*, 1999; Afolabi *et al.*, 2001).

The *mox* operon of *Methylophaga aminisulfidivorans* MP^T consists of *mxjFJGIRS* (Kim *et al.*, 2012). Among the genes in this cluster, three (*mxjF*, *mxjI* and *mxjG*) encoding the α and β subunits and cytochrome c_L in the MDH complex have been characterized in detail. The roles of MxaJ, MxaR and MxaS remain unclear, although in other methylotrophs MxaJ is likely to be involved in the functional assembly of the MDH–PQQ complex (Van Spanning *et al.*, 1991; Tanaka *et al.*, 1997). Functional MDHs are divided into two types depending on the presence of MxaJ in the MDH complex. The type I MDH complex is composed of only α and β subunits, whereas the type II complex forms when an additional MxaJ is added to the type I MDH complex ($\alpha_2\beta_2$ -MxaJ; Tanaka *et al.*, 1997; Matsushita *et al.*, 1993).

MxaJ, a component of the type II MDH complex, was first found as an additional protein to type I MDH from *Acetobacter methanolicus* (Matsushita *et al.*, 1993). However, its exact role in methanol oxidation by MDH is not fully understood. Kim *et al.* (2012) reported that type II MDH in *M. aminisulfidivorans* MP^T was successfully copurified with MxaJ, which enhanced the affinity for methanol fourfold

compared with type I MDH, indicating the important role of MxaJ in the original MDH activity in the presence of methanol.

X-ray structures of heterotetrameric type I MDH have been determined from other methylotrophic bacteria, including *Methylobacterium extorquens* (Ghosh *et al.*, 1995; Afolabi *et al.*, 2001; Williams *et al.*, 2005) and *Methylophilus methylotrophus* W3A1 (Xia *et al.*, 1996). In addition, a preliminary crystallographic analysis of MDH from the marine bacterium *M. aminisulfidivorans* MP^T has recently been reported (Choi *et al.*, 2011). MxaJ has no recognizable similarity to proteins of known structure in the Protein Data Bank, indicating that the protein may have a novel function. Thus, not only detailed biochemical analyses but also structural studies of $\alpha_2\beta_2$ MDH MxaJ (type II MDH) are required to demonstrate the exact role of MxaJ in the methanol-oxidation pathway. We initiated crystallographic studies of MxaJ in order to obtain structural insight into the MxaJ-mediated MDH regulatory mechanism. Here, we report the purification, crystallization and preliminary crystallographic analysis of MxaJ.

2. Materials and methods

2.1. Expression and purification of selenomethionine-substituted MxaJ

The *mxaj* gene encoding residues 12–281 of MxaJ was amplified by polymerase chain reaction (PCR) using the cloned *mox* operon of *M. aminisulfidivorans* MP^T as the template. The PCR forward primer was designed by omitting the putative disordered region consisting of 11 amino acids at the N-terminus which resulted from secondary-structure analysis using the *Hydrophobic Cluster Analysis* (HCA) program (Callebaut *et al.*, 1997). The PCR product was then cloned into pET28a(+) expression vector with *Nde*I and *Xho*I restriction-enzyme sites, resulting in the production of soluble 6×His-tagged MxaJ.

pET28a(+)-*mxaj* was transferred to *Escherichia coli* BL21 (DE3) for recombinant protein expression. The transformed cells were grown in LB medium at 310 K with 100 µg ml⁻¹ kanamycin until the OD₆₀₀ reached 0.8 and were harvested at 5000g. The methionine-

pathway inhibition technique was employed using the heterotrophic *E. coli* strain to prepare selenomethionine-substituted (SeMet) MxaJ protein for phase determination (Doublié, 1997). The cell pellets were resuspended in dH₂O to wash the cells. This washing step with water was repeated twice to completely remove residual LB medium. Finally, the cells were resuspended in 1 l selenomethionine-containing minimal medium supplemented with 50 mg selenomethionine and were induced with 1 mM IPTG overnight at 291 K.

Cells expressing SeMet MxaJ were harvested and resuspended in lysis buffer (20 mM Tris pH 7.5, 500 mM NaCl, 1 mM PMSF, 5 mM DTT, 1 mM MgCl₂) containing a protease-inhibitor cocktail tablet (Roche, Germany) and disrupted by sonication on ice. The crude lysate was centrifuged at 20 000g for 30 min at 277 K and the soluble fraction was subjected to Ni-NTA affinity column chromatography. However, to our surprise, the initial affinity-chromatography trial using crude lysate resulted in the expressed MxaJ passing through without binding to the Ni-NTA column, suggesting cleavage of the N-terminal part of the protein including the His tag. This explanation was supported by the observation that the molecular weight (~28 kDa) was smaller than the expected molecular weight (~32 kDa) including the extra amino-acid residues from pET28a(+) (Fig. 1). Therefore, we decided to bypass affinity chromatography and start the purification with ion-exchange chromatography.

We dialysed the crude extract for 2 d at 277 K in low-salt buffer (20 mM Tris pH 8.0, 20 mM NaCl) and subsequently subjected it to ion-exchange chromatography on a Q Sepharose packing column (GE Healthcare, USA). The bound proteins were eluted with a linear gradient of 20 mM–1 M NaCl and the fractions from the major peaks containing MxaJ were collected for further purification. MxaJ was subjected to size-exclusion chromatography on a HiLoad 16/60 Superdex 200 column (GE Healthcare, USA) pre-equilibrated with 20 mM Tris pH 8.0, 50 mM NaCl, 1 mM DTT.

The peak fractions were concentrated to 10 mg ml⁻¹ for crystallization. We were typically able to obtain ~8 mg of SeMet MxaJ from 1 l of culture medium.

2.2. Crystallization of MxaJ

Preliminary crystallization was performed by the hanging-drop vapour-diffusion method in a drop consisting of concentrated SeMet MxaJ protein (~10 mg ml⁻¹ in 20 mM Tris pH 8.0, 50 mM NaCl, 1 mM DTT) and well solution; 1 µl protein solution was mixed with 1 µl well solution at 293 and 277 K, respectively. Crystallization conditions were extensively examined using commercial screens from Hampton Research and Emerald BioStructures, including Index, SaltRx, PEG/Ion, PEG/Ion 2, Crystal Screen, Crystal Screen 2, Crystal Screen Lite, Wizard Screens I, II, III and IV, and 500 additional customized solutions. SeMet MxaJ initially crystallized within 3–4 d using Hampton Research Index condition Nos. 95 [0.1 M potassium thiocyanate, 30%(w/v) polyethylene glycol monomethyl ether 2000] and 96 [0.15 M potassium bromide, 20%(w/v) polyethylene glycol monomethyl ether 2000] and PEG/Ion 2 condition No. 46 [0.2 M sodium bromide, 20%(w/v) polyethylene glycol 3350]. Further improvements in crystal size and quality from the three conditions were carried out by combining various salts, precipitant concentrations, pH values and protein concentrations. However, among these three conditions only the PEG/Ion 2 condition produced a diffracting crystal in an initial diffraction test at the synchrotron facility (the Pohang Light Source; PLS). Therefore, we decided to focus on this condition, which yielded a better diffraction data set.

Through further optimization of the crystallization condition, we finally obtained separated long crystals from a solution consisting

Figure 1
Purification of SeMet MxaJ from *M. aminisulfidivorans* MP^T. SeMet MxaJ was purified by Q-Sepharose and size-exclusion column chromatography and was analysed by SDS-PAGE. Lane 1, molecular-weight markers (labelled in kDa); lane 2, 3 µg purified SeMet MxaJ (M_r = ~28 kDa).

Table 1

Data-collection statistics for SeMet MxaJ.

Values in parentheses are for the outermost resolution shell.

Beamline	5C, PLS
Space group	$P2_12_12_1$
Wavelength (Å)	0.97952
Unit-cell parameters (Å, °)	$a = 37.127, b = 63.761, c = 99.246,$ $\alpha = \beta = \gamma = 90$
Resolution (Å)	50–1.92 (1.95–1.92)
Total reflections	260073
Unique reflections	18666 (926)
Completeness (%)	99.9 (100)
Multiplicity	13.9 (13.3)
$R_{\text{merge}}^{\dagger}$ (%)	7.1 (25.4)
Average $I/\sigma(I)$	20.5 (16.049)
R_{anom} (50–1.92 Å)	0.058
No. of molecules in asymmetric unit	1
Solvent content (%)	41.7
Matthews coefficient (Å ³ Da ^{−1})	2.11

 $\dagger R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the observed intensity and $\langle I(hkl) \rangle$ is the average intensity of symmetry-related observations.

of 0.2 M sodium bromide, 20% (w/v) polyethylene glycol 3350, 10% (w/v) glycerol, 100 mM Tris–HCl pH 8.5 (Fig. 2). After screening various kinds of cryoprotectants including glycerol, Paratone-N oil, 2-methyl-2,4-pentanediol (MPD) and polyethylene glycol, the crystals appeared to be stable in a cryoprotectant containing 35% (w/v) polyethylene glycol 3350. The crystals were then soaked for 30 s in a cryosolution consisting of 0.2 M sodium bromide, 10% (w/v) glycerol, 35% (w/v) polyethylene glycol 3350, 100 mM Tris–HCl pH 8.5 and were cooled in liquid nitrogen for synchrotron-radiation diffraction.

2.3. Diffraction experiment and initial model building

The cryoprotected crystals were mounted in a cryogenic N₂-gas stream (100 K) during diffraction. X-ray diffraction data were collected from a SeMet MxaJ crystal on beamline 5C at the PLS using an ADSC Quantum 315 CCD detector with an oscillation of 1.0° and 3 s exposure per frame over a 360° range at a peak wavelength of 0.97952 Å. The crystals from the above conditions containing 35% (w/v) polyethylene glycol 3350 diffracted to a maximum resolution of 1.92 Å. The diffraction data were processed and scaled with HKL-2000 (Otwinowski & Minor, 1997) and the diffraction statistics are listed in Table 1.

Figure 2

Crystal image of SeMet MxaJ. Crystals were grown in a drop consisting of 0.2 M sodium bromide, 10% (w/v) glycerol, 20% (w/v) polyethylene glycol 3350, 100 mM Tris–HCl pH 8.5 at 293 K. The crystal dimensions were 0.1 × 0.1 × 1.7 mm in the drop. The crystals were dipped into a cryoprotectant containing 35% polyethylene glycol before diffraction.

The initial solution of the MxaJ structure was obtained using the single-wavelength anomalous dispersion (SAD) method. The absorption peak wavelength of Se atoms was used for SAD. A heavy-atom search and initial phasing were carried out with *AutoSol* in the *PHENIX* suite (Adams *et al.*, 2002). Subsequently, an initial MxaJ model was automatically built with the *AutoBuild* program in the *PHENIX* suite.

3. Results and discussion

Although the *mxaJ* genes encoding MxaJ in the *mox* operon from many methylotrophs have previously been cloned (Matsushita *et al.*, 1993; Kim *et al.*, 2007), functional characterization of the protein was not successful. A recent elaborately controlled purification of type II *M. aminisulfidivorans* MP^T MDH revealed that MxaJ might modulate the methanol-conversion activity of the MDH complex (Kim *et al.*, 2012). However, the precise mechanism at the molecular level is not available. As one way of demonstrating that the MxaJ molecular mechanism on MDH mediates oxidation, we attempted to determine the three-dimensional structure of MxaJ from *M. aminisulfidivorans* MP^T.

MxaJ (12–281) was cloned into an expression vector that provided an N-terminal hexahistidine affinity tag. The first 11 amino acids prior to cloning were predicted to be disordered from secondary-structural analysis. The fusion protein was successfully overproduced in soluble form in *E. coli* BL21 (DE3) cells with a ~5 kDa lower molecular weight than the expected molecular weight, resulting in the production of ~28 kDa MxaJ (Fig. 1). This observation suggests that the fused MxaJ was cleaved at a specific sequence by an endogenous bacterial Sec-type signal peptidase (Schallenberg *et al.*, 2012; Kim *et al.*, 2012) and, in turn, spontaneously separated during expression. Furthermore, the putative cleavage target sequence was likely to be

Figure 3

Diffraction image of the SeMet MxaJ crystal. Diffraction data were collected from the crystal at the peak wavelength (Table 1, Fig. 2). The oscillation was 1.0° per frame with 3 s exposure over a 360° range and the detector edge corresponds to 1.92 Å resolution.

located near the N-terminus of the fusion protein after the 6×His tag owing to its lack of binding to Ni-NTA beads. These results strongly suggest the existence of a signal peptide at the N-terminus of MxaJ; the MxaJ signal peptide cleavage sequence of MxaJ in Gram-negative bacteria is predicted to fall between Ala32 and Asn33 based on a *SignalP* 4.0 analysis (Petersen *et al.*, 2011). Thus, it is possible that the expressed MxaJ could be targeted to the cytoplasmic membrane, processed and associated with the type I MDH complex. Indeed, both the α and the β subunits of the MDH I complex also harbour signal peptides (Kim *et al.*, 2012).

Three crystallization conditions yielded crystals as mentioned in §2. Not only were the crystals from two conditions small with poor shape, but their quality did not improve commensurate with diffraction. However, the crystals from the other condition (PEG/Ion 2 condition No. 46) showed improved quality and diffracted to 1.92 Å resolution. Crystals appeared from a drop comprised of 1 µl protein solution and 1 µl reservoir solution consisting of 0.2 M sodium bromide, 20% (w/v) polyethylene glycol 3350 at 293 K. A further improvement trial was performed, resulting in the formation of separated, long rod-shaped crystals in 0.2 M sodium bromide, 10% (w/v) glycerol, 20% (w/v) polyethylene glycol 3350, 100 mM Tris-HCl pH 8.5 at 293 K with dimensions of approximately 0.1 × 0.1 × 1.7 mm in a 2 µl drop (Fig. 2). Although the glycerol in the improved crystallization condition might play a role as a cryoprotectant, the crystal diffracted poorly. Thus, we tested various kinds of cryoprotectant, and crystals were flash-cooled in cryoprotectants containing Paratone-N oil, 30–40% (v/v) MPD or 20–40% (w/v) polyethylene glycol 3350. SeMet-labelled crystals in Paratone-N oil diffracted to 2.7 and 3.0 Å resolution. A complete diffraction data set from an SeMet-incorporated crystal in 35% (w/v) polyethylene glycol 3350 was collected to a maximum resolution of 1.92 Å for SAD phasing (Fig. 3).

Auto-indexing and scaling using peak absorption data assigned the orthorhombic space group $P2_12_12_1$, with unit-cell parameters $a = 37.127$, $b = 63.761$, $c = 99.246$ Å, $\alpha = \beta = \gamma = 90^\circ$ (Table 1). A total of 260 073 reflections were measured in the resolution range 50–1.92 Å. The asymmetric unit appeared to contain one MxaJ molecule, which corresponded to a calculated Matthews coefficient of $2.11 \text{ Å}^3 \text{ Da}^{-1}$ and a solvent content of 41.7% (Matthews, 1968). The positions of seven of the eight Se atoms in the structure were found with a figure of merit of 0.505 using a heavy-atom search in *PHENIX* and the initial phases were obtained with the *AutoSol* program in *PHENIX*. Subsequently, an initial MxaJ model was obtained using *PHENIX*, in which 245 (98%) out of 249 residues (33–281) were automatically built ($R_{\text{free}} = 24\%$, R factor = 20%). Further model

building and refinement are in progress with *Coot* (Emsley & Cowtan, 2004) and *PHENIX*.

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