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Crystallization and preliminary X-ray crystallographic analysis of the methionine sulfoxide reductase A domain of MsrAB from *Haemophilus influenzae*

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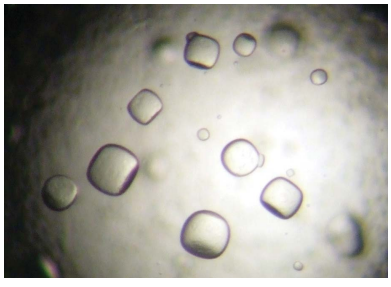
Methionine sulfoxide reductase (Msr) is a repair enzyme that reduces oxidized methionine to methionine. The Msr enzyme is divided into MsrA and MsrB, which reduce the *S* and *R* configurations of the substrate, respectively. In some pathogenic bacteria MsrA and MsrB exist in a fusion-protein form, MsrAB. In this study, the recombinant MsrA part of MsrAB from *Haemophilus influenzae* (HIMsrA) was overexpressed, purified and crystallized using the hanging-drop vapour-diffusion method. A diffraction data set was collected to 1.6 Å resolution. The crystal of HIMsrA was found to belong to space group $P4_12_12$, with unit-cell parameters $a = b = 57.29$, $c = 186.28$ Å, a calculated Matthews coefficient of 1.82 Å^3 Da^{-1} and two molecules per asymmetric unit. A preliminary solution was determined by molecular replacement. Refinement of the structure is currently in progress.

1. Introduction

Reactive oxygen species (ROS) are associated with various diseases and problems such as aging, cardiovascular diseases and cancer. Oxidative stress by ROS results in protein damage by inducing the oxidation of protein residues (Vogt, 1995). Methionine is an easily oxidized amino acid and the oxidation of methionine to methionine sulfoxide can lead to protein damage or dysfunction (Hoshi & Heinemann, 2001; Kim & Gladyshev, 2007). Methionine sulfoxide reductase (Msr) enzymes represent cellular repair systems for such protein oxidation and reduce methionine sulfoxide back to methionine (Skaar *et al.*, 2002; Kantorow *et al.*, 2004). Methionine sulfoxide has two stereoisomeric forms because of the prochiral nature of the S atom: methionine-(*S*)-sulfoxide and methionine-(*R*)-sulfoxide. For the reduction of the *R* and *S* forms, two types of Msr exist: MsrA is specific for the *S* form, while MsrB only reduces the *R* form (Sharov & Schöneich, 2000; Grimaud *et al.*, 2001). MsrA and MsrB are typically separate in most organisms and there is no sequence homology between MsrA and MsrB. However, enzymatic studies have demonstrated that both enzymes have the same mechanism (Boschi-Muller *et al.*, 2000; Kumar *et al.*, 2002; Antoine *et al.*, 2003; Olry *et al.*, 2004; Kauffmann *et al.*, 2005). It has been reported that a single-enzyme combined form of MsrA and MsrB exists in some pathogenic bacteria such as *Neisseria gonorrhoeae*, *Haemophilus influenzae* and *Streptococcus pneumoniae* (Kryukov *et al.*, 2002; Delaye *et al.*, 2007). To date, structural information has only been reported for a fusion MsrAB enzyme from *S. pneumoniae* (Kim *et al.*, 2009; Lowther *et al.*, 2002; Tête-Favier *et al.*, 2000). This crystal structure, consisting of MsrA, MsrB and a linker, explained the potential role of the linker region in structural stability (Kim *et al.*, 2009). However, there are many other questions as to why MsrA and MsrB exist in a fused form in these bacteria. To better understand the structure and biochemical function of each part of the MsrAB fusion protein and to compare them with those of the whole fusion form, we cloned the MsrA and MsrB domains and the full MsrAB gene from *H. influenzae*. Here, we report the protein preparation, crystallization and preliminary X-ray crystallographic analysis of the MsrA domain.



2. Materials and methods

2.1. Cloning

The MsrA gene coding for 166 amino acids (31–196) of MsrAB from *H. influenzae* (hereafter referred to as HIMsrA) was amplified by the polymerase chain reaction (PCR); the primers used were 5′-GGAATTC**CATATG**GAACAAAAAATGGCAATGGAAAATACA-3′ as the forward primer and 5′-CCG**CTCGAG**TTAGGTAATTAGGTAATATCAATATGGCAGTAGCCATT-3′ as the reverse primer (*Nde*I and *Xho*I restriction-enzyme sites are shown in bold). The amplified DNA was digested with *Nde*I and *Xho*I and cloned into pET28a (Novagen). The recombinant protein has an N-terminal histidine tag and a thrombin cleavage site (MGSSHHHHHHSSGLVPR/GSH, where / represents the thrombin cleavage site). The construct containing HIMsrA was transformed into *Escherichia coli* Rosetta (DE3) cells (Novagen) for protein expression. The transformed cells were grown in Luria–Bertani broth (LB) containing 50 µg ml^{-1} kanamycin to an OD_{600} of 0.6 at 310 K. Overexpression of HIMsrA was induced by the addition of 0.5 m*M* isopropyl *β*-D-1-thiogalactopyranoside (IPTG) at 310 K. 2 h later, the cells were harvested using a centrifugal device (Hanil, Republic of Korea) at 1260*g* and 277 K and were stored at 257 K.

2.2. Purification

The cell pellets were resuspended in a lysis buffer (buffer *A*) consisting of 50 m*M* Tris pH 8.0, 100 m*M* NaCl, 2 m*M* *β*-mercaptoethanol and were lysed by sonication on ice. The lysate was centrifuged for 45 min at 1260*g* and 277 K and the soluble part was filtered with a membrane filter (0.45 µm, Millipore) and loaded onto a nickel-chelation column (HiTrap Chelating HP colum, GE Healthcare) equilibrated with buffer *A*. After washing in buffer *A* to remove nonspecifically bound bacterial proteins, His-tagged recombinant HIMsrA eluted at between 150 and 200 m*M* imidazole in a linear gradient to buffer *B* (buffer *A* with 500 m*M* imidazole). The N-terminal histidine tag was not removed. The eluted HIMsrA fraction was diluted tenfold with buffer *C* (50 m*M* Tris pH 9.0, 5 m*M* dithiothreitol). Using a HiTrap Q HP column (GE Healthcare), the HIMsrA was purified by anion-exchange chromatography and eluted at between 100 and 150 m*M* NaCl with buffer *D* (buffer *C* with 500 m*M* NaCl). The purified protein was concentrated using an Amicon ultracentrifugal concentrator (10 kDa cutoff, Millipore) at 1260*g* and loaded onto a gel-filtration column (Superdex 75, 120 ml, GE Healthcare) equilibrated with buffer *E* (50 m*M* Tris pH 8.0, 100 m*M* NaCl, 5 m*M* dithiothreitol). The collected purified HIMsrA was concentrated to 48 mg ml^{-1} in final buffer (buffer *E*) and stored at 193 K prior to use.

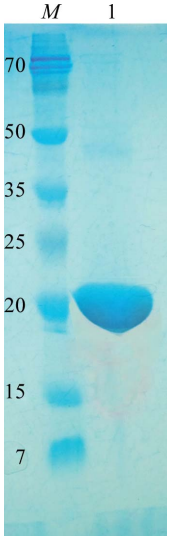


Figure 1
SDS–PAGE analysis of purified HIMsrA (31–196). Lane *M*, markers (labelled in kDa); lane 1, purified HIMsrA after two-step purification.

2.3. Crystallization

Initial crystallization conditions were screened using the sitting-drop vapour-diffusion method by seven initial crystallization screens with a Mosquito automated liquid-handling system (TTP LabTech). The kits used were PEG/Ion, SaltRx, Crystal Screen, Index, Natrix and MembFac from Hampton Research and the Wizard kit from Emerald BioSystems. Each 200 nl drop consisted of 100 nl HIMsrA protein solution and 100 nl reservoir solution and was equilibrated against 60 µl reservoir solution in a 96-well plate at 295 K. Of the various conditions used, square quasi-crystals were observed with a solution consisting of 3.5 *M* sodium formate, and optimization was performed to improve the quality of the crystals using the hanging-drop vapour-diffusion method. Diverse optimization conditions were tested using 3.0–4.0 *M* sodium formate, incubation temperatures of 295 and 297 K (room temperature) and varying the protein concentration and the protein:reservoir solution ratio. The best crystals were obtained when 3.5 *M* sodium formate was used as reservoir solution at a temperature of 297 K and the final protein concentration was 35 mg ml^{-1}. It was observed that the rounded quasi-crystals became square through the formation of corners over 10 d when the temperature was increased to 297 K.

2.4. Data collection and processing

To protect the crystals from radiation damage, the HIMsrA crystals were quickly transferred into a cryoprotectant solution (3.5 *M* sodium formate containing 25% ethylene glycol). The crystals were exposed at 100 K under a nitrogen stream on beamline BL1A of the Photon Factory (Tsukuba, Japan) using a Quantum 270 CCD detector (ADSC, Poway, USA). Data sets were collected to a resolution of 1.6 Å at a wavelength of 1.0 Å with a crystal-to-detector distance of 213.724 mm. Each frame had an exposure time of 3 s and 1° oscillation; the total oscillation range was 360°. All collected images were

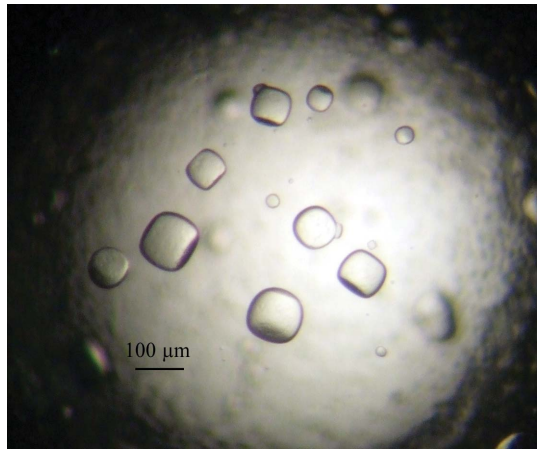


Figure 2
Crystals of *H. influenzae* MsrA grown in 3.5 *M* sodium formate at 297 K using the hanging-drop vapour-diffusion method. The crystal dimensions are approximately 0.1 × 0.1 × 0.05 mm.

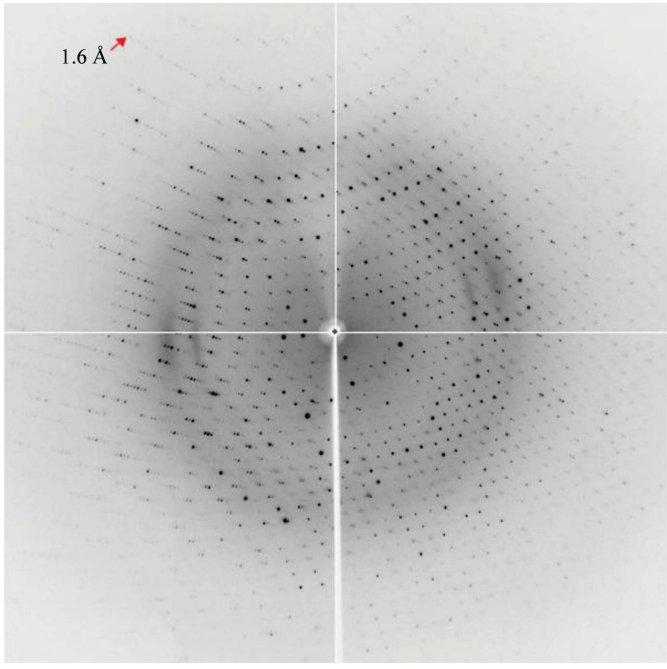


Figure 3
X-ray diffraction image of a crystal of *H. influenzae* MsrA. The resolution limit at the edge is 1.6 Å.

Table 1
Data-collection statistics.

Values in parentheses are for the highest resolution shell.

Beamline	BL1A, Photon Factory
Wavelength (Å)	1.0
Temperature (K)	100
Resolution range (Å)	30.00–1.60 (1.63–1.60)
Space group	$P4_12_12$
Unit-cell parameters (Å)	$a = b = 57.29$, $c = 186.28$
No. of molecules per asymmetric unit	2
V_M (Å^3 Da^{-1})	1.82
Solvent content (%)	32.45
Total reflections	296385
Unique reflections	34925
Completeness (%)	97.2 (83.0)
Average $I/\sigma(I)$	27.32 (1.4)
R_{merge}† (%)	0.088 (0.416)
Multiplicity	7.7 (2.5)

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the measured intensity and $\langle I(hkl)\rangle$ is the average intensity over symmetry-equivalent reflections.

processed and scaled using the *HKL*-2000 package (Otwinowski & Minor, 1997).

3. Results and discussion

Recombinant HIMsrA was expressed in *E. coli* and purified, with a yield of 60 mg per litre of culture. The purity, as obtained by SDS–PAGE, was greater than 95% and the final protein concentration for crystallization was 35 mg ml^{-1} (Fig. 1). Initial crystals were obtained in 3.5 *M* sodium formate (Index condition No. 25). After optimization of the crystallization temperature, the rounded crystals became square in shape through the formation of corners over a period of 10 d. The crystals had overall dimensions of approximately 0.1 × 0.1 × 0.05 mm (Fig. 2). The crystals diffracted to a resolution of 1.6 Å (Fig. 3) and belonged to the tetragonal space group $P4_12_12$ or $P4_32_12$, with unit-cell parameters $a = b = 57.29$, $c = 186.28$ Å. There were two molecules in the asymmetric unit, with a solvent content of 32.45% (based on the calculated unit-cell volume); the protein molecular weight was 21 328 Da and the Matthews coefficient (V_M) was 1.82 Å^3 Da^{-1} (Table 1). Structure determination of HIMsrA was carried out by the molecular-replacement program *Phaser* (McCoy *et al.*, 2007) using the MsrA domain of MsrAB from *S. pneumoniae* (PDB entry 3e0m; Kim *et al.*, 2009) as the search model. A unique solution with a log-likelihood gain of 701.9 and a *Z* score of 26.7 was obtained when searching for two molecules in the asymmetric unit (space group $P4_12_12$, 1σ, reflections in the resolution range 1.6–28.7 Å). In addition to electron-density inspection, initial cycles of rigid-body and restrained refinement improved the primary solution from an *R* factor of 36.5% and an R_{free} of 44.2% to values of 31.7% and 35.2%, respectively, indicating a correct structure solution. Initial model building was performed automatically with *PHENIX* (Adams *et al.*, 2002). Structure refinement is now in progress.

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