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Overexpression, Purification, and Preliminary X-Ray Crystallographic Studies of Methionine Sulfoxide Reductase B from *Bacillus subtilis*

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The peptide methionine sulfoxide reductases (Msrs) are enzymes that catalyze the reduction of methionine sulfoxide back to methionine. Because of two enantiomers of methionine sulfoxide (*S* and *R* forms), this reduction reaction is carried out by two structurally unrelated classes of enzymes, MsrA (E.C. 1.8.4.11) and MsrB (E.C. 1.8.4.12). Whereas MsrA has been well characterized structurally and functionally, little information on MsrB is available. The recombinant MsrB from *Bacillus subtilis* has been purified and crystallized by the hanging-drop vapor-diffusion method, and the functional and structural features of MsrB have been elucidated. The crystals belong to the trigonal space group P3, with unit-cell parameters a=b=136.096, c=61.918 Å, and diffracted to 2.5 Å resolution using a synchrotron-radiation source at Pohang Light Source. The asymmetric unit contains six subunits of MsrB with a crystal volume per protein mass (V_M) of 3.37 Å^3 Da^{-1} and a solvent content of 63.5%.

Keywords: *Bacillus subtilis*, methionine sulfoxide reductase B, crystallization

Methionine sulfoxide reductases (Msrs) protect cells from oxidative damage by reducing methionine sulfoxide [Met-(O)] back to methionine [9]. According to the sulfoxide enantiomer of the substrate that they reduce (Fig. 1), two different classes of Msrs, named MsrA and MsrB, catalyze the reduction back to methionine. MsrAs were first discovered in 1981 [2], and they have shown to reduce only *S*-enantiomers of the sulfoxide [10, 13]. MsrBs were then identified in 2001, and these enzymes recognize and reduce the *R*-form of methionine sulfoxide [3]. There is, however, no sequence homology between MsrAs and MsrBs, suggesting that these proteins belong to two distinct structural families of proteins [3]. A number of studies on the catalytic mechanisms of Msrs showed that two (or three) cysteine residues are involved in the reaction and these mechanisms are found to be identical in both [1, 5, 8, 11]. Specifically, MsrBs contain a distinct conserved cysteine, defined to be the catalytic residue CysA (corresponding to Cys115 in *B. subtilis*), that generates a sulfenic acid by attacking methionine sulfoxide, and most of the enzymes possess additional conserved cysteine, CysB (Cys62 in *B. subtilis*). It has been suggested that two cysteines are involved in an entire mechanism, since CysB forms a disulfide bridge with CysA [5, 11]. By the existence of two additional CxxC motifs, MsrBs are separated in two groups. MsrB proteins containing these four cysteines are Form II MsrB ($MsrB_{II}$) proteins, and MsrB sequences lacking the two CxxC motifs are Form MsrB (MsrB) proteins [5]. For the enzyme activity of SelR, which has the methionine-*R*-sulfoxide reductase activity, a zinc atom is required to bind to these four conserved residues [4, 5]. The MsrBs from *Escherichia coli* and the human protein SelX belong to the $MsrB_I$ group [3, 6], whereas those of the *Neisseria* strains (*N. gonorrhoeae* and *N. meningitidis*) are $MsrB_{II}$ group [8, 11]. MsrB from *B. subtilis* is $MsrB_{II}$ group, depending on the sequence alignment. Until now, only one crystal structure of the MsrB domain of PilB from *N. gonorrhoeae* is available in the PDB [7]. The primary amino acid sequence of MsrB from *B. subtilis* has 57% identity with the MsrB domain of PilB from *N. gonorrhoeae* [7, 14]. The PilB protein from *N. gonorrhoeae* has three domains, MsrA and MsrB reductase domains and N-terminal thioredoxin-like domain, which may function to reduce the active sites of the downstream MsrA/B domains [7]. On the other hand, each MsrA and MsrB activity in most organisms, including *B. subtilis*, has a discrete and monofunctional protein, and therefore, there may be structural differences between *N. gonorrhoeae* and *B. subtilis*. The crystal structure of the MsrB domain from PilB was solved at 1.85 Å resolution and the result indicated that it is mainly composed of antiparallel β-strands, and that these β-strands are arranged in two β-sheets to make a barrel-like

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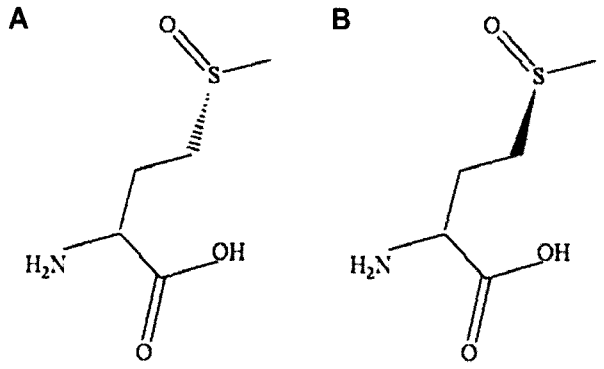

Fig. 1. Two enantiomers of methionine sulfoxide.
A. L-Methionine-*R*-sulfoxide. **B.** L-Methionine-*S*-sulfoxide.

core in opposite directions. This crystal structure suggests that Thr 403, Cys 440, His 477, His 480, Cys 495, and Asn 497 are required to bind methionine-*R*-sulfoxide and show stereospecificity with this substrate [7]. For a detailed understanding of the mechanism at the molecular level, and as a first step toward its structure elucidation, we have overexpressed MsrB from *B. subtilis*.

The full length of the MsrB gene from the genomic DNA of *B. subtilis* was amplified by polymerase chain reaction, using two primers (forward primer: 5'-AATTAAGGAACATATGATGGCGTACAATAAAGAAGAAAAAA-3'; reverse primer: 5'-AATTCTCGAGTTTATTAAAGAGATGCAGATAGCTTTC-3'). The PCR product was digested with NdeI and XhoI, and ligated into the pET22b vector (Novagen), which contains a hexahistidine tag at the C-terminus. The resulting plasmid was transformed into *E. coli* BL21 (DE3) strain (Novagen), and the cells were grown at 37°C in Luria-Bertani medium supplemented with ampicillin (50 μg/ml). The expression of the recombinant MsrB protein was induced by 0.5 mM isopropyl-D-thiogalactopyranoside at an optical density of about 0.45 at 600 nm, and the cells were grown for an additional 12 h at 18°C. It should be noted that we failed to solve the structure by molecular replacement method using the *N. gonorrhoeae* MsrB crystal structure as a search model. Therefore, selenomethionine (SeMet)-substituted protein was also prepared for multiple-wavelength anomalous dispersion (MAD) experiments. The SeMet-substituted protein was overexpressed in methionine auxotroph *E. coli* B834 (DE3) cells, using the M9 minimal culture medium containing extra amino acids and selenomethionine. Cells were then harvested by centrifugation at 6,000 ×*g* for 30 min at 4°C. The pelleted cells were suspended in buffer A (10 mM KH_2PO_4/K_2HPO_4 buffer, pH 6.8) and homogenized by sonication. The crude lysate was centrifuged at 13,000 ×*g* for 30 min at 4°C. The supernatant was loaded onto a Mini-Trap affinity column (Amersham Biosciences) equilibrated with buffer A. The protein was eluted with a linear gradient of 0–0.5 M imidazole in buffer A. The fractions containing

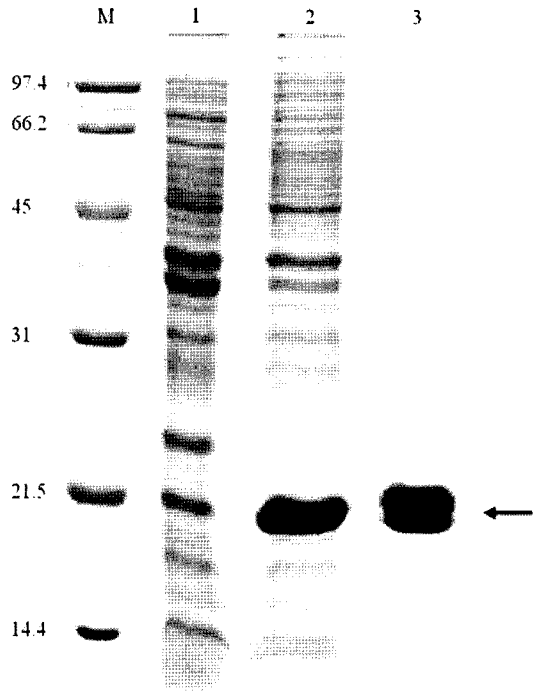

Fig. 2. Expression and purification of MsrB protein as judged by SDS-PAGE.
Lane M, molecular mass protein marker (kDa); lane 1, uninduced cell lysate; lane 2, induced cell lysate; lane 3, purified MsrB protein.

MsrB were pooled, concentrated, and washed with buffer B (10 mM KH_2PO_4/K_2HPO_4 buffer, 5 mM DTT, pH 6.8). The high concentration of dithiothreitol (DTT) protects sulfur atoms of cysteine residue and selenomethionine in the SeMet-substituted protein from oxidation. Final purification of MsrB protein was achieved by gel filtration on a SephacylTM S-100 (Amersham Biosciences), which was previously equilibrated with buffer B. The purified protein was concentrated to 50 mg/ml for crystallization experiment. The homogeneity of the protein purified was confirmed by gel electrophoresis (Fig. 2). For molecular-mass estimation, SDS-PAGE under denaturing conditions was carried out using Precision Protein Standard (Bio-rad) as reference proteins. Initial crystallization screening was performed using the Crystal Screen I and II kits, JBScreen kit, MembFac Screen kit, Natrix Screen kit (Hampton Research), and Wizard I and II kits (Emerald Biostructures Inc.) by the hanging-drop vapor-diffusion method at 22°C. Each hanging drop was prepared by mixing 1 μl of protein solution and 1 μl of reservoir solution over 0.5 ml of reservoir solution. Initial crystals were obtained after 3–5 days in the conditions containing ammonium sulfate, and lithium sulfate and various shapes of microcrystals were formed under these conditions. First optimization attempt was carried out in a solution containing 0.1 M Tris-Cl (pH 8.0), 0.1 M NaCl, and 1.1 M ammonium sulfate. Under this condition, however, piled needle-shaped or amorphous crystals were grown in 3–5 days at 4°C, and these crystals

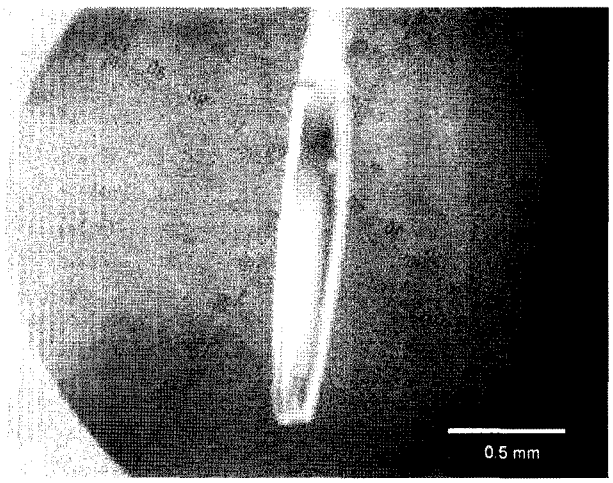

Fig. 3. A SeMet-substituted crystal of methionine sulfoxide reductase B from *Bacillus subtilis*.
Its approximate dimensions is 0.3×0.3×1.0 mm.

were not suitable for diffraction studies. Optimization of crystallization condition with a reservoir solution containing 0.1 M HEPES (pH 7.25) and 1.2 M lithium sulfate at 4°C produced the best quality of native crystals for data collection; A long and rod-shaped crystal was obtained within 6 days and used for x-ray diffraction data collection (Fig. 3). A crystal of the SeMet-substituted protein was transferred to cryoprotection solution containing 0.1 M Tris-HCl (pH 7.5), 0.05 M lithium sulfate, 30% PEG 400, and 10% glycerol. It was then scooped up in a cryoloop, frozen in liquid nitrogen, and mounted on the goniometer in a stream of cold nitrogen. X-ray diffraction data were then collected from the cooled crystals using an ADSC Quantum CCD 210 detector at the Beamline 4A at Pohang

Table 1. Data collection statistics.

	SeMet (MsrB)		
	λ1 (edge)	λ2 (peak)	λ3 (remote)
Space group	P3		
Wavelength (Å)	0.97962	0.9795	0.9718
Cell dimension (Å)	a=b=136.096, c=61.918 Å α=β=90, γ=120°		
Resolution range (Å)	20.0–2.5 (2.59–2.50)		
Observed reflections	282,173	283,793	281,169
Unique reflections	42,726	42,897	42,843
Redundancy	6.6 (2.4)	6.6 (2.5)	6.6 (2.3)
Completeness (%)	96.5 (74.4)	96.9 (77.9)	96.7 (76.3)
R_{sym} (%)	12.1 (39.4)	13.1 (35.0)	12.1 (34.7)
I/sigma (I)	10.57 (1.54)	10.79 (1.58)	10.57 (1.54)
No. molecules in AU	6		
V_M (Å^3/Da)	3.37		

Values in parentheses are for the highest resolution shell.
R_{sym}: $\Sigma_{hkl,i}|I_{hkl,i}-\langle I_{hkl}\rangle_i|/\Sigma_{hkl,i}I_{hkl,i}$, where I represents the observed intensity, $\langle I\rangle$ the average intensity, and i the counts through all symmetry-related reflections.

Accelerator Laboratory (Pohang, South Korea). The three-wavelength diffraction data from the SeMet-substituted crystal were collected for resolution of 2.5 Å at −173°C. Data were processed and scaled using the program HKL2000 [12]. Table 1 summarizes the statistics for data collection. The asymmetric unit contains six molecules of monomer, giving a crystal volume per protein mass (V_M) of 3.37 Å^3 Da^{-1} and a solvent content of 63.5% by volume. The determination of MsrB structure by the MAD method is currently in progress, and the structural details will be described in a separate paper.

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