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Expression, purification and preliminary X-ray crystallographic analysis of nitroalkane oxidase (NAO) from *Pseudomonas aeruginosa*

Nitroalkane oxidase (NAO) is a flavin-dependent enzyme which catalyses the oxidation of nitroalkanes to the corresponding aldehydes or ketones, nitrite and hydrogen peroxide. In order to better understand the structure and function of this enzyme, NAO from *Pseudomonas aeruginosa* was purified and crystallized as a native and a selenomethionine-substituted (SeMet) enzyme. Both crystals diffracted to a resolution of 1.9 Å and belonged to the primitive orthorhombic space group $P2_1$, with unit-cell parameters $a = 70.06$, $b = 55.43$, $c = 87.74$ Å, $\beta = 96.56^\circ$ for native NAO and $a = 69.89$, $b = 54.83$, $c = 88.20$ Å, $\beta = 95.79^\circ$ for SeMet NAO. Assuming the presence of two molecules in the asymmetric unit in both crystals, the Matthews coefficients (V_M) for native and SeMet NAO were calculated to be 2.30 and 2.48 Å³ Da⁻¹, with estimated solvent contents of 46.50 and 50.37%, respectively.

1. Introduction

Nitro compounds have been broadly applied in chemical industry owing to the usefulness of nitro groups, which can be converted to other functional groups for use in intermediates, solvents and fuels or fuel additives (Fiala *et al.*, 1989; Hite & Skeggs, 1979; Gorlatova *et al.*, 1998). Some of these compounds, such as 1-nitropropane and 2-nitropropane, are expected to be toxic and some have already been shown to be carcinogens (Haas-Jobelius *et al.*, 1992). Therefore, these toxic nitrochemicals need to be converted into less harmful compounds using biocatalytic reactions (Kido *et al.*, 1978; Zhang & Tan, 2002). Among the oxidative denitrification enzymes, the flavoprotein nitroalkane oxidase (NAO) catalyses the removal of a hydride ion from a substrate, transferring electrons initially to the flavin cofactor and then to molecular oxygen as the terminal electron acceptor, leading to the formation of hydrogen peroxide and oxidized product (Gadda & Fitzpatrick, 1998). During the catalytic reaction, NAO requires the participation of flavin adenine dinucleotide (FAD) or flavin mononucleotide (FMN) as a prosthetic group, which is tightly bound or covalently attached to the protein moiety and takes part in a wide array of biological pathways involving redox reactions (Gadda, 2008; Gorlatova *et al.*, 1998; Fitzpatrick, 2004).

To date, only crystal structures of NAO from three bacteria, *Podospora anserina* (Tormos *et al.*, 2010; PDB entry 3mkh), *Fusarium oxysporum* (Nagpal *et al.*, 2006; PDB entry 2c0u) and *Streptomyces ansochromogenes* (Li *et al.*, 2011; PDB entry 3bw2), have been reported. Among them, the NAO enzymes from *P. anserina* and *F. oxysporum* contain FAD, whereas the NAO from *S. ansochromogenes* contains FMN as a cofactor and shares 43% sequence identity with NAO from *Pseudomonas aeruginosa*. Thus, further structural investigations are required for comparison with existing NAO structures and to improve understanding of the structure–function relationship and the conformational changes that accompany ligand binding. As the first step towards elucidating its structure, we report the crystallization and preliminary X-ray crystallographic analysis of NAO from *P. aeruginosa* in native and selenomethionine-substituted forms. Complete data sets were collected from both crystals at 1.9 Å resolution using a synchrotron-radiation source.

2. Materials and methods

2.1. Cloning and expression

The NAO gene was amplified from the genomic DNA of *P. aeruginosa* by polymerase chain reaction (PCR) using specific primers. The forward primer contained an *Nde*I restriction site (shown in bold) and had the sequence 5'-GGG AAT TCC **ATA TGA** CTG ACC GCT TCA CGC -3', while the reverse primer contained an *Xho*I recognition site (shown in bold) and had the sequence 3'-CCG **CTC GAG** TCA GCC GCG CAG TTC C-5'. The PCR-amplified DNA fragment was purified, digested with *Nde*I and *Xho*I, and ligated into the pET-28a vector (Novagen, USA), which adds six consecutive histidines at the N-terminus. The recombinant plasmids were transformed into chemically competent *Escherichia coli* strains BL21 (DE3) for native NAO and B834 (DE3) for selenomethionine-substituted (SeMet) NAO using a heat-shock technique. The *E. coli* BL21 and B834 transformants were grown in Luria–Bertani medium and in M9 minimal medium containing extra amino acids and selenomethionine, respectively, along with 50 $\mu\text{g ml}^{-1}$ kanamycin. Protein expression was induced by adding 0.2 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) when the cells reached an optical density at 600 nm of about 0.6; the cells were then grown for an additional 12 h at 291 K followed by centrifugation at 5000g for 30 min at 277 K.

2.2. Purification

The harvested cell pellets were suspended in buffer A (20 mM Tris–HCl pH 7.9, 500 mM NaCl, 5 mM imidazole) and lysed by sonication on ice, after which the lysate was centrifuged at 20 000g for 40 min at 277 K. The supernatant was loaded onto an Ni^{2+} -chelated HiTrap Chelating HP column (GE Healthcare, USA) which was equilibrated in buffer A. The bound protein was eluted with a linear gradient of buffer B (20 mM Tris–HCl pH 7.9, 500 mM NaCl, 1 M imidazole). Fractions containing NAO were identified by SDS–PAGE and were purified to the final state by gel-filtration chromatography using a HiLoad 16/60 Superdex 200 column (GE Healthcare, USA) which was equilibrated with buffer C (20 mM Tris–HCl pH 7.9, 200 mM NaCl, 2 mM DTT). The soluble fractions containing native or SeMet NAO were pooled together and concentrated to 14 and 10 mg ml^{-1} , respectively, using an Amicon Ultra-15 centrifugal filter device (Millipore, USA). Protein purity was monitored by 15% PAGE and staining was carried out with the Bradford assay.

2.3. Crystallization and preliminary X-ray analysis

Preliminary crystallization screens for the native and SeMet NAO enzymes were performed by the sitting-drop vapour-diffusion method (0.2 μl protein solution and 0.2 μl reservoir solution equilibrated against 100 μl reservoir solution) using several commercial screens: Wizard I, Wizard II (Emerald BioStructures Inc.), Crystal Screen, Crystal Screen 2 and PEG/Ion (Hampton Research, USA). After screening, large single crystals were grown from the PEG/Ion screen: 0.2 M ammonium fluoride, 20% polyethylene glycol 3350 for the NAO crystal and 0.2 M ammonium fluoride, 10% polyethylene glycol 3350 for the SeMet-substituted crystal. Crystal growth was scaled up to the hanging-drop vapour-diffusion method in 24-well VDX plates (Hampton Research). Each hanging drop was prepared by mixing 1 μl protein solution and 1 μl reservoir solution and was equilibrated over 500 μl reservoir solution. Crystals appeared within 3 d and grew to full size within one week. The crystal dimensions of the native and SeMet NAO crystals were $0.1 \times 0.05 \times 1.2$ and $0.07 \times 0.05 \times 1.0$ mm, respectively (Fig. 1). Both crystals were transferred to a cryoprotection solution consisting of 25% polyethylene glycol 400

in reservoir solution. Data sets were collected to resolutions of 1.9 Å from the native crystal on beamline 5C and 1.9 Å from the SeMet-substituted crystal on beamline 7A at the Pohang Light Source (Pohang, Republic of Korea) using an ADSC Quantum 210 CCD detector (Fig. 2). A total range of 360° was covered with 1.0° oscillation and 10 s exposure per frame. The wavelength of the synchrotron X-rays was 1.0000 Å at beamline 5C. The subsequent single-wavelength anomalous diffraction (SAD) data were collected at the peak ($\lambda = 0.97951$ Å) wavelength of the Se *K*-edge absorption profile. The crystal-to-detector distance was set to 150 mm. All data were indexed, integrated and scaled using the *HKL*-2000 software package (Otwinowski & Minor, 1997).

3. Results and discussion

The DNA fragment encoding the NAO gene from *P. aeruginosa* was successfully cloned into the pET-28a vector. The protein, which contains 351 amino acids with a calculated molecular weight of 36.7 kDa, was overexpressed in *E. coli* BL21 (DE3) and B834 (DE3). The encoded NAO was purified using an Ni^{2+} -chelated HiTrap Chelating HP column and gel filtration. Crystals suitable for X-ray analysis were obtained by the hanging-drop vapour-diffusion method

Figure 1
Crystals of NAO protein from *P. aeruginosa*. (a) Native crystal; (b) SeMet-substituted crystal. The crystal dimensions of the native and SeMet-substituted crystals were $0.1 \times 0.05 \times 1.2$ and $0.07 \times 0.05 \times 1.0$ mm, respectively.

Figure 2

X-ray diffraction pattern of an SeMet-substituted crystal obtained using an ADSC Quantum 210 CCD detector. The box shows an enlargement of an area containing high-resolution spots.

Table 1

Data-collection statistics.

Values in parentheses are for the outermost resolution shell.

	Native	SeMet-substituted, peak
Space group	$P2_1$	$P2_1$
Wavelength (Å)	1.0000	0.97951
Unit-cell parameters (Å, °)	$a = 70.06$, $b = 55.43$, $c = 87.74$, $\beta = 96.56$	$a = 69.89$, $b = 54.83$, $c = 88.20$, $\beta = 95.79$
Resolution range (Å)	50–1.9 (1.93–1.90)	50–1.9 (1.93–1.90)
Total/unique reflections	199848/49473	227644/50675
Multiplicity	4.0 (2.2)	4.5 (2.3)
Completeness (%)	93.5 (82.2)	97.3 (88.9)
$R_{\text{merge}}^{\dagger}$ (%)	8.5 (37.0)	8.5 (37.4)
$\langle I/\sigma(I) \rangle$	16.9 (2.1)	13.7 (1.8)

$^{\dagger} R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ represents the i th observed intensity of reflection hkl and $\langle I(hkl) \rangle$ represents the average intensity of reflection hkl .

under the following conditions: 0.2 *M* ammonium fluoride, 20% polyethylene glycol 3350 for the native crystal and 0.2 *M* ammonium fluoride, 10% polyethylene glycol 3350 for the SeMet-substituted crystal. Both crystals diffracted to a resolution of 1.9 Å and belonged to the primitive orthorhombic space group $P2_1$, with unit-cell parameters $a = 70.06$, $b = 55.43$, $c = 87.74$ Å, $\beta = 96.56^\circ$ for native NAO and $a = 69.89$, $b = 54.83$, $c = 88.20$ Å, $\beta = 95.79^\circ$ for SeMet NAO. Assuming the presence of two molecules per asymmetric unit in both crystals, the Matthews coefficients (V_M) for the native and the SeMet NAO crystals were calculated to be 2.30 and 2.48 Å³ Da^{−1}, with estimated solvent contents of 46.50 and 50.37%, respectively (Matthews, 1968). The data-collection statistics are summarized in Table 1.

Structural determination was performed by SAD phasing using the *AutoSol* routine from *PHENIX* (Adams *et al.*, 2002) and by the molecular-replacement method using *MOLREP* from the *CCP4* package (Winn *et al.*, 2011) with the crystal structure of NAO from

S. ansochromogenes (PDB entry 3bw2; 43% identity; Li *et al.*, 2011) as a search model. Further refinements of the model structure using the experimental phases are currently in progress and the structural details will be reported separately.

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