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Crystallization and preliminary X-ray studies of azoreductases from *Bacillus* sp. B29

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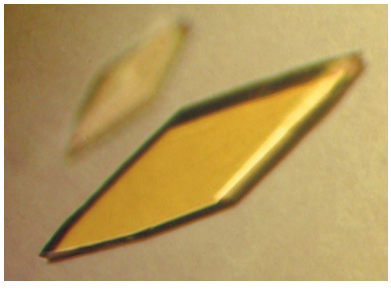
Azoreductases from *Bacillus* sp. B29 are NADH-dependent flavoenzymes which contain a flavin mononucleotide (FMN) as a prosthetic group and exist as homodimers composed of 23 kDa subunits. These enzymes catalyze the reductive degradation of various azo compounds by a ping-pong mechanism. In order to determine the structure–function relationship of the azo-dye reduction mechanism, an X-ray crystallographic study of azoreductases was performed. Selenomethionine-labelled AzrA (SeMet-AzrA) and AzrC were crystallized by the hanging-drop vapour-diffusion method. A crystal of SeMet-AzrA diffracted to 2.0 Å resolution and was determined to belong to space group $P2_12_12_1$, with unit-cell parameters a = 56.9, b = 69.0, c = 105.4 Å. The native crystals of AzrC belonged to space group $C2$, with unit-cell parameters a = 192.0, b = 56.6, c = 105.5 Å, β = 115.7°, and diffracted to 2.21 Å resolution.

1. Introduction

Azo dyes are major synthetic pigments that contain one or more azo bonds (—N═N—) and are widely used in the printing, dyeing and food-colouring industries because of their ease of synthesis, chemical stability and range of uses (Griffiths, 1984). However, their stability causes pollution when the dyes are released into the environment. Furthermore, many of these dyes have toxic and mutagenic potential (Chung & Cerniglia, 1992). These problems have given rise to a demand for azo-dye degradation technology. Wastewater degradation systems for azo dyes based on physicochemical treatment are efficient but have high running costs and consume energy (Kariminiaae-Hamedaani *et al.*, 2007). In contrast, the biological treatment of azo dyes does not have similar problems. Research has been carried out on dye-degrading enzymes and many such enzymes have been characterized to date (Chen, 2006). Azobenzene reductases, designated azoreductases (EC 1.7.1.6), catalyze the reduction of azo dyes in the presence of NAD(P)H (Mueller & Miller, 1949), transforming a number of azo dyes into colourless aromatic amines by cleaving azo groups. Such enzymes are the focus of research into the development of biodegradation systems for azo dyes.

Previously, we have reported the molecular cloning and characterization of four azoreductases from *Bacillus* sp. B29 (Ooi *et al.*, 2007, 2009) and *Geobacillus stearothermophilus* (Matsumoto *et al.*, 2009). These isozymes are NADH-dependent flavoenzymes that contain a flavin mononucleotide (FMN) as a prosthetic group and exist as homodimers composed of 23 kDa subunits. The enzyme reaction occurs by a ping-pong mechanism that uses two moles of NADH to reduce one mole of Methyl red (4′-dimethylaminoazobenzene-2-carboxylic acid), which is a model azo compound, to 2-aminobenzoic acid and *N*,*N*′-dimethyl-*p*-phenylenediamine. The substrate specificity differs between the four azoreductases: AzrA has a wide range of substrate specificity in comparison with AzrB, AzrC and AzrG. AzrA was not only able to decolourize Methyl red but was also able to degrade sulfone-modified azo dyes such as 1-(2-pyridylazo)-2-naphthol, Orange I [*p*-(4-hydroxy-1-naphthylazo)benzenesulfonic acid], Orange II [4-(2-hydroxy-1-naphthylazo)-benzenesulfonic acid] *etc*. The source of the substrate specificity of azoreductases is unclear, although the structures of AzoA from *Enterococcus faecalis* (Liu *et al.*, 2007) and AzoR from *Escherichia*



coli (Ito *et al.*, 2006), which are homologous to the four isozymes AzrA, AzrB, AzrC and AzrG, have been solved.

To understand the substrate specificity and the detailed enzymatic reaction mechanisms of azoreductases at atomic resolution, detailed knowledge of the structures of individual azoreductases complexed with substrates are indispensable. Here, we report the crystallization and preliminary X-ray crystallographic analysis of the azoreductases AzrA and AzrC from *Bacillus* sp. B29.

2. Materials and methods

2.1. Expression and purification

Selenomethionine-labelled AzrA (SeMet-AzrA) was expressed in *E. coli* BL21 (DE3) cells using methionine-free minimal essential medium (MEM) containing selenomethionine (Kawamura *et al.*, 2008) at low temperature (298 K) as described previously (Ooi *et al.*, 2007) but with slight modifications. Purification of SeMet-AzrA was performed using DEAE-cellulose, Phenyl-Toyopearl and Sephacryl S-200HR based on the previously reported method (Ooi *et al.*, 2007). The fractions containing SeMet-AzrA were applied onto a Resource Q anion-exchange column (GE Healthcare) and were washed with 25 m*M* Tris–HCl buffer pH 7.4. The bound proteins were eluted with a linear gradient of 0–0.6 *M* NaCl. Finally, the collected fractions were further purified on a PD-10 desalting column (GE Healthcare) equilibrated with 25 m*M* Tris–HCl buffer pH 7.4. AzrC was expressed and purified using the same procedure as described previously (Ooi *et al.*, 2009). SeMet-AzrA and AzrC were finally concentrated to 10 mg ml^{-1} by ultrafiltration using an Amicon Ultra-4 (10 000 nominal molecular-weight limit; Millipore).

2.2. Crystallization

Initial crystallization trials were performed by the sitting-drop vapour-diffusion method in 96-well plates at 293 K using a series of crystallization kits (from Hampton Research and Emerald BioStructures). Each drop was prepared by mixing 1 µl protein solution prepared as described above with the same volume of reservoir solution. The initial crystals of SeMet-AzrA were obtained using a reservoir solution composed of 0.1 *M* phosphate–citrate buffer pH 4.2 and 40%(*w*/*v*) PEG 600. Optimization of the crystallization conditions was carried out using the hanging-drop vapour-diffusion method, in which 2 µl protein solution was mixed with 2 µl reservoir solution and equilibrated against 0.5 ml reservoir solution. After varying the pH, protein concentration, precipitants and additives, the best crystals were obtained within 3 d using a protein concentration of 3.5 mg ml^{-1}, 0.1 *M* phosphate–citrate buffer pH 4.4 and 40%(*w*/*v*) PEG 600. For AzrC, multiple crystals were obtained under optimized conditions using 0.1–0.2 *M* cacodylate buffer pH 6.5, 0.2–0.26 *M* calcium acetate, 35%–40%(*w*/*v*) PEG 600 and a protein concentration of 5 mg ml^{-1}. The conditions were further optimized to improve the crystals by adding azo compounds. Single crystals were finally grown from 0.1 *M* cacodylate buffer pH 6.5, 0.24 *M* calcium acetate, 37%(*w*/*v*) PEG 600 and 1 m*M* Orange I (Tokyo Chemical Industry) within one week.

2.3. Data collection

As the crystals of SeMet-AzrA and AzrC had been grown in 35–40%(*w*/*v*) PEG 600, the crystals were directly flash-cooled at 100 K for data collection without any additional cryoprotectant. All diffraction data sets were collected using an ADSC Quantum 315 CCD detector on beamline BL41XU of SPring-8 (Hyogo, Japan). An Se-SAD data set was collected for SeMet-AzrA at a wavelength of 0.97918 Å, which was selected for data collection based on the fluorescence spectrum of the Se atom and corresponded to the maximum f''(peak). The crystal-to-detector distance was 250 mm and the oscillation angle was 1°, with an exposure time of 2 s per image. The data set for AzrC was collected at a wavelength of 1.00000 Å with a crystal-to-detector distance of 220 mm, an oscillation angle of 0.6° and an exposure time of 1 s per image. The reflections from each crystal were indexed, integrated, scaled and merged using the *HKL*-2000 package (Otwinowski & Minor, 1997).

3. Results and discussion

SeMet-AzrA was overexpressed in *E. coli* cells using MEM containing selenomethionine. The protein was highly purified by five steps of column chromatography, with a yield of 20 mg per litre of culture. The enzymatic activity of the purified protein was equivalent to that of native AzrA (data not shown). In addition, AzrC was obtained at a level similar to that reported previously (Ooi *et al.*, 2009).

Crystals of SeMet-AzrA grew to approximate dimensions of 0.3 × 0.3 × 0.15 mm under the conditions described above (Fig. 1). A

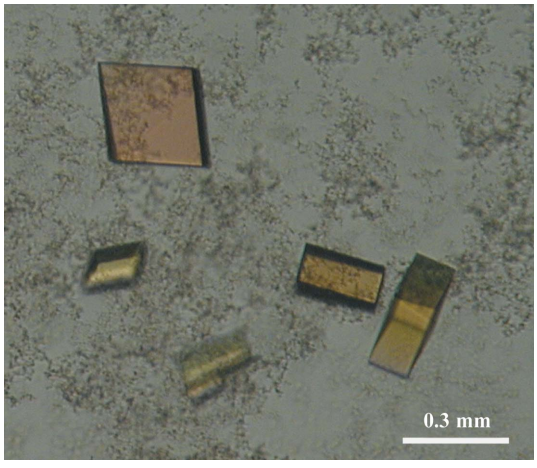


Figure 1
Crystals of SeMet-AzrA. The approximate dimensions of the crystals were 0.3 × 0.3 × 0.15 mm.

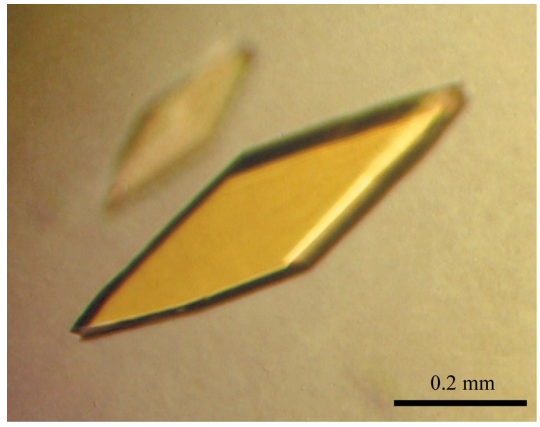


Figure 2
Crystals of AzrC. The approximate dimensions of the crystals were 0.6 × 0.2 × 0.15 mm.

Table 1
Summary of crystallographic data.

Values in parentheses are for the highest resolution shell.

	SeMet-AzrA (SAD)	AzrC
Wavelength (Å)	0.97918	1.0000
Space group	$P2_12_12_1$	$C2$
Unit-cell parameters (Å, °)	a = 56.9, b = 69.0, c = 105.4	a = 192.0, b = 56.6, c = 105.5, β = 115.7
Resolution range (Å)	50.0–2.0 (2.07–2.0)	50.0–2.21 (2.25–2.21)
No. of unique reflections	28543	50874 (2391)
Redundancy	9.9 (9.4)	4.5 (3.3)
Completeness (%)	99.7 (99.0)	99.7 (95.5)
$\langle I/\sigma(I)\rangle$	10.25 (2.32)	8.985 (2.706)
$R_{\rm merge}$† (%)	10.7 (39.9)	11.6 (41.9)
Wilson B factor (Å^2)	19.9	24.7

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

diffraction data set was obtained with a resolution range of 50–2.00 Å. The crystal belonged to space group $P2_12_12_1$, with unit-cell parameters a = 56.9, b = 69.0, c = 105.4 Å. The asymmetric unit contained a dimer of SeMet-AzrA, giving a crystal volume per protein mass ($V_{\rm M}$; Matthews, 1968) of 2.3 Å^3 Da^{-1} and a solvent content of 45.9%. Single crystals of AzrC grown under the condition described above were found to be small, measuring less than 0.2 × 0.08 × 0.05 mm (Fig. 2). A diffraction data set was obtained from an AzrC crystal with a resolution range of 50–2.21 Å. The crystal belonged to space group $C2$, with unit-cell parameters a = 192.0, b = 56.6, c = 105.5 Å, β = 115.7°. The asymmetric unit contained two dimers of AzrC, giving a $V_{\rm M}$ of 2.8 Å^3 Da^{-1} and a solvent content of 56.3%. The diffraction data statistics are summarized in Table 1.

Previous attempts to determine the structure of AzrA by the molecular-replacement method have been unsuccessful. Studies are currently under way to determine the structure of SeMet-AzrA using the single-wavelength anomalous diffraction (SAD) method. Once we have obtained the structure of SeMet-AzrA, we will attempt to resolve the structure of AzrC by the molecular-replacement method using the structure of SeMet-AzrA as a search model, as the two proteins show 41% amino-acid sequence identity.

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