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Crystallization and preliminary analysis of xenobiotic reductase B from *Pseudomonas fluorescens* I-C

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Single crystals have been obtained of xenobiotic reductase B (XenB), a flavoenzyme isolated and cloned from *Pseudomonas fluorescens* I-C. The enzyme catalyzes the NADPH-dependent elimination of nitrite from nitroglycerin with an approximately fivefold kinetic preference for the middle nitro group, primarily yielding 1,3-dinitroglycerol. X-ray diffraction data sets have been collected from native crystals to 2.3 Å resolution. The space group is $P4_12_12$, with unit-cell parameters $a = b = 140$, $c = 95.6$ Å. The asymmetric unit is likely to contain at least two XenB molecules (V_M = 3.1 Å^3 Da^{-1}, 60% solvent) and a molecular-replacement solution has been determined in order to solve the structure.

1. Introduction

Organic nitrochemicals have been distributed throughout the environment from man-made sources in vast excess over naturally occurring sources. Many of these compounds do not have any known naturally occurring counterpart and present metabolic challenges to many organisms. Moreover, because nitro-group metabolism can create reactive nitrogen intermediates (Nathan & Shiloh, 2000), the US Environmental Protection Agency often regulates sites contaminated with nitrochemicals.

Elimination of nitrite from an organic nitrochemical is an effective method to initiate catabolism that avoids some of the most reactive nitrogen intermediates. Recent studies have shown that flavoenzymes can utilize at least two mechanistic strategies to catalyze nitrate elimination and permit the organism to obtain all the nitrogen required for growth from the nitrochemical (Spain, 1995). One strategy, utilized by some lower eukaryotes, exploits enzymes that catalyze the oxidative elimination of nitrite from naturally occurring organic nitrochemicals. The best characterized examples include nitroalkane oxidase (NAO) and 2-nitropropane dioxygenase (NPD) (Tchorzewski *et al.*, 1994; Gadda & Fitzpatrick, 1998; Gorlatova *et al.*, 1998; Zhang & Tan, 2002). Another strategy, utilized by several prokaryotes, employs enzymes that catalyze reductive nitrite elimination. Indeed, several microbes that utilize nitroglycerin or other organic nitrochemicals as a sole nitrogen source have recently been isolated from the contaminated soil of World War II-era munitions-manufacturing sites (Spain, 1995; Binks *et al.*, 1996; Blehert *et al.*, 1997; French *et al.*, 1998). In particular, two nutritionally distinct *Pseudomonas* strains isolated from the same munitions-manufacturing site expressed similar flavoenzymes termed xenobiotic reductases A and B (XenA and XenB). While both catalyze NADPH-dependent reductive elimination of nitrite from nitroglycerin, the main product of XenA was 1,2-dinitroglycerol, whereas that of XenB was 1,3-dinitroglycerol (Blehert *et al.*, 1997). These two enzymes also exhibited differences in their reactions with 2,4,6-trinitrotoluene, with XenB giving ~50% yield of aromatic ring reduction products at pH 7 (Pak *et al.*, 2000). XenA and XenB are members of a class of FMN-dependent old yellow enzyme (OYE) oxidoreductases (Åkeson *et al.*, 1963; Fox & Karplus, 1994), several of which transform xenobiotic explosive compounds (Spain, 1995; Binks *et al.*, 1996; Blehert *et al.*, 1997, 1999; French *et al.*, 1998; Nivinskas *et al.*, 2000; Riefler & Smets, 2000; Williams & Bruce, 2000; Barna *et al.*, 2001; Ebert *et al.*, 2001; Meah *et al.*, 2001; Haynes *et al.*, 2002; Johnson *et al.*, 2002; Khan *et al.*, 2002; Koder *et al.*, 2002; Williams & Bruce, 2002; Orville *et al.*, 2004). The XenA and XenB enzymes have between 27 and 50% sequence identity with these homologs (Blehert *et al.*, 1999).

Clearly, the correct interactions between the enzyme active-site residues, the nitro substrate and flavin cofactor are all essential to achieve catalytic nitrite elimination. However, the structural and mechanistic elements that control these interactions, the partition between oxidative and reductive nitrite-elimination reactions and the regiospecificity of catalysis have not been well established. To that end, we have recently reported the crystallization and preliminary characterization of XenA cloned from *Pseudomonas putida* II-B (Orville *et al.*, 2004). The focus of this report is the crystallization of XenB cloned from

P. fluorescens I-C (Blehert *et al.*, 1997, 1999; Pak *et al.*, 2000).

2. Material and methods

2.1. Protein expression and purification

P. fluorescens I-C was isolated as previously reported (Blehert *et al.*, 1997) from nitro-contaminated soil at the Badger Army Ammunition Plant, Wisconsin, USA. This bacterium was enriched by its ability to obtain all nitrogen required for growth from concentrations of nitroglycerin that are toxic to nearly all other species. XenB (37 441 Da monomer) was cloned, expressed and purified as previously reported (Blehert *et al.*, 1997, 1999). The enzyme was indistinguishable when isolated from either *P. fluorescens* I-C or the *Escherichia coli* expression host, including the presence of one oxidized FMN per protein monomer.

2.2. Crystallization

Crystallization conditions for XenB were determined from sparse-matrix screens and optimized using additive and detergent screens from Hampton Research (Laguna Nigel, CA, USA). Typically, 2 µl protein (5–10 mg ml^{-1} in 20 m*M* HEPES buffer pH 7.0) was mixed with an equal volume of reservoir solution on a silanized cover slip and equilibrated at 277 K or room temperature by vapor diffusion in 24-well Linbro plates. XenB crystals typically required more than three weeks to grow when the drops were suspended over well solutions containing 100 m*M* PIPES buffer pH 5.5–6.5 with approximately 24% polyethylene glycol (PEG 4000, 6000 or 8000) and 200 m*M* $MgSO_4$. The crystals first appeared as long needles at the border of a phase-separated region of the drops. The addition of 1 µl detergent (*e.g.* 0.7 m*M* HEGA-10, 0.25 m*M* *n*-decanoylsucrose or 0.2 m*M* Zwittergent 3-12; Anatrace Inc., Maumee, OH, USA) to the drop reduced phase separation and increased the reproducibility of crystal formation.

2.3. X-ray diffraction data collection and analysis

Crystals were harvested with a nylon loop and transferred to cryoconditions, which included either briefly passing the crystals through mother liquor augmented with at least 17% glycerol or transferring the crystals to Paratone-N (Hampton Research). The crystals were flash-frozen by either rapidly plunging them into liquid N_2 or by exposing them to the cold-stream of the X-ray source. All diffraction data were collected at beamline 9-2 of the Stanford Synchrotron Radiation Laboratory from crystals held at approximately 100 K. The detector was an ADSC Quantum 4R CCD detector (Poway, CA, USA). Various exposure times, crystal-to-detector distances and oscillation ranges per frame were used (Table 1). The data were integrated with *MOSFLM* (Powell, 1999) and internally scaled with *SCALA* from the *CCP*4 suite of programs (Collaborative Computational Project, Number 4, 1994). Molecular replacement was carried out with the *MOLREP* routine in *CCP*4 by searching for two or three molecules in the asymmetric unit with an input search molecule derived from morphinone reductase (PDB code 1gwj; Barna *et al.*, 2002) and the resolution range 28–3.0 Å. Rigid-body refinement of the potential solutions took place using *REFMAC*5 from *CCP*4.

Table 1
Data-collection statistics for XenB.

Data were collected at SSRL beamline 9-1 with 0.9778 Å X-rays and a Quantum 4R detector. Values for the highest resolution shell of data are given in parentheses.

Data set	Native 1	Native 2
Distance (mm)	225	250
Exposure time (s)	20	10
Oscillation per image (°)	0.75	1.0
No. images	132	90
Resolution range (Å)	28–2.3	30–2.3
Space group	$P4_12_12$	$P4_12_12$
Unit-cell parameters		
$a = b$ (Å)	140.4	139.9
c (Å)	95.6	94.8
Total reflections	305018	225745
Unique reflections	40812	35056
Multiplicity	7.4 (4.9)	6.2 (3.7)
Completeness (%)	92.1 (97.1)	87.2 (87.2)
R_{sym}†	0.126 (0.41)	0.095 (0.34)
$I/\sigma(I)$‡	4.7 (1.8)	6.2 (2.2)
Mosaic spread (°)	∼0.8	∼0.6

† $R_{sym}(I)$ gives the average agreement between the independently measured intensities such as $\sum_h \sum_i |I_i - I| / \sum_h \sum_i I$, where I is the mean intensity of the i observations of reflection h. ‡ $I/\sigma(I)$ is the root-mean-square value of the intensity measurements divided by their estimated standard deviation.

3. Results and discussion

Crystals of XenB were birefringent elongated needles (Fig. 1) that were intensely yellow when viewed with unpolarized light. They took much longer to appear than the equilibration of the hanging drops suspended over various well solutions. The enzyme did not precipitate in the drops during the incubation period; the drops often remained clear for several months, especially when incubated at 277 K. If detergents were omitted from the drops, they often separated into clear and yellow phases. The addition of various detergents reduced the tendency for phase separation and increased the reproducibility of obtaining crystals, but did not significantly reduce the time required for crystallization. Thus, it appears that crystal nucleation may be the rate-limiting step in the crystallization of XenB.

X-ray diffraction data-collection statistics for the two data sets are presented in Table 1. Analysis of the data suggested that the crystals belong to a primitive tetragonal space group. Both data sets included reflec-

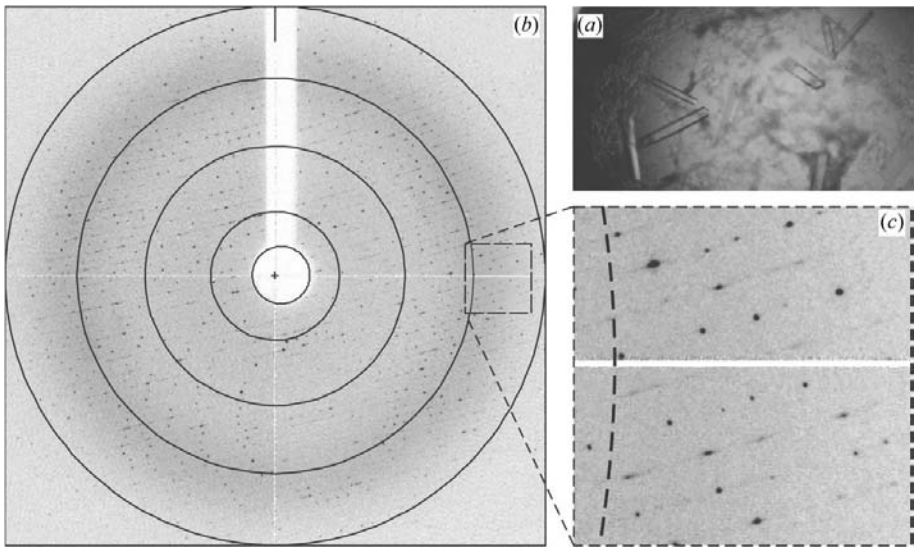

Figure 1
(*a*) Crystals of XenB measuring ∼0.3 × 0.05 mm photographed with polarization. (*b*) The X-ray diffraction pattern for native 1 with a 20 s exposure and 0.75° oscillation about the vertical axis. The arcs indicate 2.7, 3.6, 5.5, 10.9 and 28 Å resolution, respectively. (*c*) An expanded view of the high-resolution diffraction perpendicular to the oscillation axis.

tions along the (*h*00) principal axis that exhibited systematic absences. For example, the native 1 and native 2 data sets had average values for $I/\sigma(I)$ for (even, 0, 0) reflections of 26.4 and 17.2, respectively, whereas the (odd, 0, 0) reflections yielded average values of 0.09 and 0.18, respectively. However, there were no reflections observed for the principal axis (00*l*) for any of the data sets. Therefore, the space-group determination remained ambiguous based upon reflection-intensity data, but they were all consistent with space groups $P42_12$, $P4_12_12$, $P4_22_12$ or $P4_32_12$. A reasonable assumption of the weight per unit volume suggested that two XenB molecules were present in the asymmetric unit (V_M = 3.2 or 2.1 Å^3 Da^{-1}, 61 or 41% solvent content, respectively; Matthews, 1968).

Morphinone reductase (PDB code 1gwj; Barna *et al.*, 2002) has 50% sequence identity to XenB and was used to prepare a monomeric molecular-replacement search model comprised of 276 residues out of the 349 residues in full-length XenB. All four space groups and both native data sets were analyzed for molecular-replacement solutions. For each data set the best solutions were for two molecules in the asymmetric unit in space group $P4_12_12$. These yielded correlation coefficients of 0.58 and 0.57, which were significantly better than analogous trials in the other space groups (the correlation coefficients ranged between 0.45 and 0.49). Moreover, the crystal packing, solvent content and interpretable electron-density maps are all consistent with two XenB molecules in space group $P4_12_12$. Refinement of the atomic models to the high-resolution limit for each data set are in progress.

Widely divergent flavoenzymes catalyze a remarkable range of reactions in biology (Fraaije & Mattevi, 2000; Massey, 2000). The results from XenB, in comparison to our emerging results for XenA (Orville *et al.*, 2004), will help define the structural basis of regiospecific reductive nitrite elimination by flavoenzymes (Blehert *et al.*, 1999; Williams & Bruce, 2000, 2002). Moreover, by extending the comparisons to enzymes such as NAO and NPD, which catalyze oxidative nitrite-elimination reactions, broader mechanistic insights can be achieved.

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