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Expression, purification, crystallization and preliminary X-ray analysis of *para*-nitrophenol 4-monooxygenase from *Pseudomonas putida* DLL-E4

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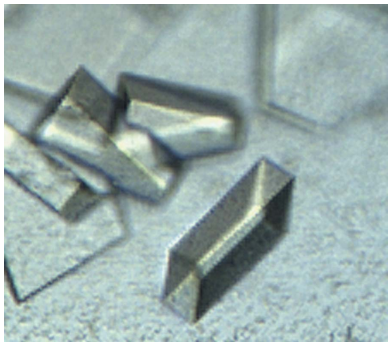

Para-nitrophenol 4-monooxygenase (PnpA) plays an important role in bacterial degradation of *para*-nitrophenol by oxidative release of the nitro group from the aromatic ring to form *p*-benzoquinone. In order to understand the structural basis of the function of this enzyme, PnpA was cloned, expressed in *Escherichia coli* and purified. PnpA was crystallized by the hanging-drop vapour-diffusion technique with PEG 4000 as precipitant. The PnpA crystals belonged to space group $P2_12_12_1$, with unit-cell parameters a = 54.47, b = 77.56, c = 209.17 Å, and diffracted to 2.24 Å resolution.

1. Introduction

Para-nitrophenol (PNP; also known as 4-nitrophenol or 4-NP) is the most common and important pollutant in terms of quantities manufactured and the extent of environmental contamination. It is used in the synthesis of medicines, dyes, explosives, leather colouring, wood preservatives and rubber chemicals (Uberio & Bhattacharya, 1997). Microbial degradation of PNP has been studied for more than 20 years, but the genes involved in the degradation pathways have only recently been cloned (Wataru *et al.*, 2004; Takeo *et al.*, 2003, Perry & Zylstra, 2007). Degradation of PNP by microorganisms commonly takes place through two different pathways in pure cultures: *via para*-benzoquinone (HQ) or 4-nitrocatechol (4-NC) under aerobic conditions (Wataru *et al.*, 2004). In Gram-negative bacteria, PNP is transformed to benzoquinone by monooxygenase and then reduced to hydroquinol by benzoquinone reductase, as in a *Moraxella* sp. (Spain *et al.*, 1991); in Gram-positive strains, PNP is first converted to 1,2,4-benzenetriol by monooxygenases as in *Rhodococcus* sp. PN1 (Takeo *et al.*, 2003), *R. opacus* SAO101 (Wataru *et al.*, 2004) and *Arthrobacter* sp. JS443 (Perry & Zylstra, 2007). Three distinctive PNP degradation-related gene clusters have been reported in the above-mentioned Gram-positive bacteria; they each encode a specific two-component flavin-diffusible monooxygenase (TC-FDM). A single-component PNP 4-monooxygenase which converts PNP to *para*-benzoquinone has recently been cloned and sequenced from *Pseudomonas* sp. WBC-3 (Zhang *et al.*, 2009). *P. putida* DLL-E4 (Liu *et al.*, 2003) was isolated from soil polluted with methyl parathion (MP) in our laboratory; it can utilize PNP as a sole carbon and nitrogen source. We cloned a 9.2 kbp gene cluster from this strain which is involved in the catabolism of PNP and exhibited PNP 4-monooxygenase activity. We successfully identified the *pnpA* gene (GenBank accession No. FJ376608) from the fragment by SEFA PCR (Wang *et al.*, 2007) and successfully expressed it in *Escherichia coli* (paper in preparation). The gene shares 87% identity in nucleotide sequence with the PNP 4-monooxygenase gene from *Pseudomonas* sp. strain WBC-3. PnpA can release nitrite from both PNP and 4-NC. It contains 418 amino acids, with a calculated molecular mass of 46.6 kDa. Both PnpA and TC-FDM can use FAD and NADH as cofactors to convert 4-NC to 1,2,4-trihydroxybenzene, but their reaction mechanisms are different. Analysis of their crystal structures will shed light on the catalytic mechanisms of these enzymes. In this report, we describe the expression, purification, crystallization and initial X-ray analysis of PnpA.

2. Materials and methods

2.1. Expression and purification

The *pnp*A gene was amplified from the 9.2 kbp PNP degradation-related cluster in *P. putida* DLL-E4 by PCR using primers pnpAF (5′-CATATGGGCCGTGATAGGAGACAC-3′) and pnpARhis (5′-CTCGAGAGCCCCTACCAGTTCATGCTCTG-3′). The primers contained *Nde*I and *Xho*I restriction-enzyme cloning sites in the 5′ and 3′ regions, respectively. The gene was cloned downstream of a T7 promoter and a C-terminal six-His-tag clamp in the vector pET29b(+) (Novagen) and the constructed plasmid was sequenced by Invitrogen (Shanghai, People's Republic of China). The verified construct was then transformed into *E. coli* BL21 (DE3) for expression of PnpA. The cells harbouring the plasmid were incubated overnight at 303 K in automatic induction medium (Studier, 2005) supplemented with 50 mg l^{-1} kanamycin. The cells were harvested, washed and resuspended in equilibration buffer *A* (20 m*M* Tris–HCl pH 8.5, 100 m*M* NaCl, 20 m*M* imidazole, 10% glycerol) at 277 K and lysed by ultrasonication. The cell extract was clarified by centrifugation at 20 000*g* for 15 min and the supernatant was then applied onto an Ni–NTA agarose column (Invitrogen Biosciences) pre-equilibrated with buffer *A*. The fusion protein was eluted with a linear gradient of imidazole from 20 to 200 m*M*. The fractions containing PnpA were further purified by passing them through a pre-equilibrated size-exclusion Sephadex G-100 column (Amersham Biosciences) with buffer *B* (10 m*M* Tris–HCl pH 8.5, 10% glycerol) and then concentrated to 30 mg ml^{-1} with a Millipore Centriprep (Amicon Ultra 10 000 molecular-weight cutoff, 15 ml capacity). The protein was confirmed to be homogeneous by SDS–PAGE (Laemmli, 1970) and the purified protein was then immediately used for crystallization.

2.2. Crystallization screening and optimization

The PnpA crystallization conditions were initially screened by sparse-matrix screening with commercial crystallization kits (Hampton Research) using the sitting-drop vapour-diffusion method in 96-well Intelli-Plates (Art Robbins Instruments). The concentrations of PnpA used were 30 and 10 mg ml^{-1} and the crystallization screening was carried out at 277 and 293 K, respectively. The initial screens included the sparse-matrix Crystal Screens 1 and 2, a systematic PEG–pH screen and the PEG/Ion Screen. 50 µl mother liquor was used as the reservoir solution and 1 µl PnpA was mixed with 1 µl reservoir solution in the drop support. The crystallization conditions were optimized in 24-well Linbro plates by the hanging-drop vapour-diffusion method; the droplet was prepared on a siliconized cover slip by mixing 3 µl protein solution with 3 µl precipitant solution and was equilibrated over 500 µl reservoir solution.

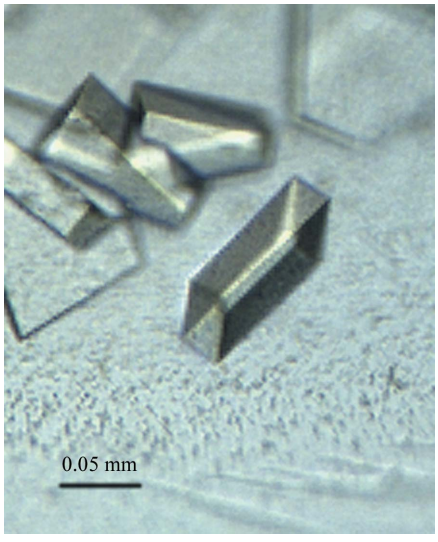

Figure 1
Crystals of *para*-nitrophenol 4-monooxygenase PnpA from *P. putida* DLL-E4. The scale bar is 0.05 mm in length.

Table 1
Data-collection statistics for PnpA crystal.

Values in parentheses are for the highest resolution shell.

Wavelength (Å)	0.94722
Resolution (Å)	50–2.24 (2.28–2.24)
Unit-cell parameters	a = 54.47, b = 77.56, c = 209.17
Crystal system	Orthorhombic
Space group	$P2_12_12_1$
Total reflections	250942
Unique reflections	42724
Average redundancy	5.9 (5.2)
Molecules per ASU	2
V_M (Å^3 Da^{-1})	2.33
Completeness (%)	97.9 (90.9)
R_{merge}† (%)	7.8 (24.2)
Mean $I/\sigma(I)$	12.0 (8.14)

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the ith observation of reflection hkl and $\langle I(hkl)\rangle$ is the mean intensity for all observations of hkl.

2.3. Data collection

Diffraction data were collected from a single crystal on beamline BL17U of Shanghai Synchrotron Radiation Facility (Shanghai, People's Republic of China) using a MAR 225 CCD area detector (MAR Research). The crystal was harvested using a nylon loop (Hampton Research) and flash-cooled in liquid nitrogen. Cryoprotection was achieved by soaking the crystal in 20 m*M* HEPES pH 7.0, 15% PEG 4000, 15% isopropanol, 25% glycerol. 166 consecutive images were collected using an oscillation angle of 1° with 5 s exposure per frame and a crystal-to-detector distance of 250 mm. The diffraction data for the crystal were integrated and scaled using *HKL*-2000 (Otwinowski & Minor, 1997). The X-ray data statistics are summarized in Table 1.

2.4. Analytical methods

The PNP 4-monooxygenase (PnpA) activity was determined by measuring the nitrite released from the substrate (PNP or 4-NC). The reaction mixture was prepared from 1 ml 50 m*M* PBS buffer pH 8.0 by adding 0.4 m*M* NADH, 0.04 m*M* FAD, 0.15 m*M* substrate (final concentrations) and suitable amounts of protein. The reaction was initiated by addition of the protein and continued for 30 min at 293 K. The released nitrite in the reaction mixture was determined by the method adopted by White *et al.* (1996) immediately after the reaction time had been reached. One unit of enzyme activity was defined as the amount of activity required to catalyze the oxidization of 1 µmol substrate (PNP or 4-NC) per minute at 293 K. The protein concentration was determined by the Bradford method (Bradford, 1976) using bovine serum albumin as the standard.

3. Results and discussion

Diffraction-quality oblique parallelepiped-shaped crystals were obtained using the hanging-drop vapour-diffusion method at 293 K by mixing 3 µl 10 mg ml^{-1} protein with 3 µl reservoir solution and

equilibrating against 500 µl reservoir solution (20 m*M* HEPES pH 7.0, 15% PEG 4000 and 15% 2-propanol). The crystals reached dimensions of up to 0.025 × 0.1 × 0.1 mm (Fig. 1) after 2 d. The collected data had 97.9% completeness, with an overall $R_{\rm merge}$ of 7.8% on intensities; the crystal belonged to the orthorhombic space group $P2_12_12_1$. Based on a subunit molecular weight of 47.3 kDa (including the six histidine residues added to the C-terminus of PnpA during cloning), there were two molecules in the asymmetric unit and the Matthews coefficient $V_{\rm M}$ was 2.33 Å^3 Da^{-1}, corresponding to a solvent content of 47.35% (Matthews, 1968). The estimated mosaicity of the data was 0.117°. An attempt at phasing by molecular replacement using *Phaser* (McCoy *et al.*, 2007) from the *CCP*4 package (Collaborative Computational Project, Number 4, 1994) with the structure of 3-hydroxybenzoate hydroxylase from *Comamonas testosteroni* (PDB code 2dkh; Hiromoto *et al.*, 2006), which shares 21.9% sequence identity, as the search model yielded no promising results. Further attempts to solve the structure by the multi-wavelength anomalous diffraction (MAD) method (Hendrickson *et al.*, 1990) using selenomethionine-substituted protein are currently under way.

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