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Crystal structure of *p*-nitrophenol 4-monooxygenase PnpA from *Pseudomonas putida* DLL-E4: The key enzyme involved in *p*-nitrophenol degradation

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ABSTRACT

p-Nitrophenol 4-monooxygenase PnpA, the key enzyme in the hydroquinone pathway of *p*-nitrophenol (PNP) degradation, catalyzes the monooxygenase reaction of PNP to *p*-benzoquinone in the presence of FAD and NADH. Here, we determined the first crystal structure of PnpA from *Pseudomonas putida* DLL-E4 in its apo and FAD-complex forms to a resolution of 2.04 Å and 2.48 Å, respectively. The PnpA structure shares a common fold with hydroxybenzoate hydroxylases, despite a low amino sequence identity of 14–18%, confirming it to be a member of the Class A flavoprotein monooxygenases. However, substrate docking studies of PnpA indicated that the residues stabilizing the substrate in an orientation suitable for catalysis are not observed in other homologous hydroxybenzoate hydroxylases, suggesting PnpA employs a unique catalytic mechanism. This work expands our understanding on the reaction mode for this enzyme class.

1. Introduction

p-Nitrophenol (PNP) is an important class of nitrophenolic pollutant and widely used in the manufacture of pesticides, dyes, explosives, and drug intermediates [1]. Due to its potential toxicity and persistence in the environment, the US ATSDR and US EPA had put PNP on its “Priority Pollutants List”. Many PNP-degrading bacteria have been isolated, and their degradation characteristics have been extensively studied, which makes them be useful and irreplaceable in the environmental bioremediation [2–6]. At present, two pathways for PNP degradation have been elucidated. The hydroxyquinol (BT) pathway is initiated by a two-component monooxygenase, usually found in Gram-positive bacteria [7]. The hydroquinone (HQ) pathway is mostly discovered in Gram-negative bacteria, initiated by a single-component monooxygenase [8].

Pseudomonas putida DLL-E4 has the ability to grow on PNP as the sole source of carbon, nitrogen, and energy, and HQ was detected as the key intermediate in PNP degradation. A 9.2-kb gene cluster involved in the catabolic pathway has been cloned [9]. The enzymes involve the stepwise conversion of PNP to β-ketoadipate correspond to PnpA-F in other PNP degrading bacteria, such as *Pseudomonas* sp. WBC-3 [10], *Pseudomonas* sp. 1–7 [8], *Pseudomonas* sp. NyZ402 [11] and *Burkholderia* sp. SJ98 [12]. Although biochemical and genetic characterization of the PNP degradation have been clearly illustrated, many challenges remain in understanding the structural mechanism of each key enzyme in the pathway. Recently, crystal structure of *p*-benzoquinone reductase PnpB [13], hydroquinone 1,2-dioxygenase PnpCD [14] and γ-hydroxymuconic semialdehyde dehydrogenase PnpE [15] from *Pseudomonas* sp. WBC-3 were reported. However, the structure of *p*-nitrophenol 4-monooxygenase PnpA are not available, except for our primary X-ray analysis of PnpA from *P. putida* DLL-E4 [16].

PnpA is a FAD-dependent monooxygenase, key enzyme in the hydroquinone pathway of PNP degradation, catalyzes the

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hydroxylation reaction of PNP to *p*-benzoquinone with the release of nitrite. Moreover, PnpA may selectively catalyze *p*-substituted other than *m*- or *o*-substituted nitrophenol with the preference of NADH [9]. According to the sequence and function, PnpA can be assigned to the Class A flavoprotein monooxygenases (FPMO), almost exclusively hydroxylating the aromatic rings. The preponderance understanding of Class A FPMO comes from the investigations of *p*-hydroxybenzoate hydroxylase (PHBH) and phenol hydroxylase (PHHY) [17]. Two of these enzymes introduce one hydroxyl group at the *m*-position of the phenolic substrate, resulting double hydroxyl groups present on the aromatic ring. PnpA produce a quinone with hydroxylation and denitrogenation at *p*-position, the mechanism of this substrate has not been reported. In order to provide a basis to elucidate how PnpA functions, we determined the first crystal structure of PnpA. Through enzyme-substrate docking, we identified the binding pocket of PnpA and studied its catalytic mechanism.

2. Materials and methods

2.1. Protein expression and purification

E. coli BL21 (DE3) cells carrying pET29a (+)-*pnpA* were cultured at 37 °C in LB medium to OD_{600} about 0.6, followed by induction with 0.2 mM IPTG at 16 °C for 24 h. The cells were harvested by centrifugation and resuspended in buffer A (20 mM HEPES pH 7.0, 500 mM sodium chloride, 5 mM imidazole, 10% (v/v) glycerol), and lysed by ultrasonication. Cell extracts were centrifuged at 20,000 × g for 15 min at 4 °C. The supernatant was applied to Ni-NTA agarose columns (Invitrogen Biosciences) and eluted with buffer A containing 50 mM, 100 mM, 200 mM, and 300 mM imidazole. The protein was further purified using a Superdex 200 column (GE Healthcare, Beijing, China) equilibrated with 20 mM HEPES, 50 mM sodium chloride, 5% (v/v) glycerol, pH 7.0. The purified protein was concentrated to 10 mg ml^{-1} with Amicon Ultra Centrifugal Filter Units (30 K, Merck Millipore, Billerica, USA) for crystallization.

2.2. Crystallization and data collection

Apo-PnpA crystals were obtained from the condition containing 10% isopropanol, 0.1 M Tris-HCl, pH 8.5,13.5% (*w/v*) PEG 4000 and 5% glycerol and grow at 293K by the sitting-drop vapor-diffusion method by mixing 4 μl protein solution (5.2 mg mL^{-1}) with 4 μl reservoir solution. In order to obtain the complex structures, 0.01 mM FAD and 0.5 mM PNP were co-crystallized with PnpA in the above mentioned conditions. Before fiash-cooling in liquid nitrogen, all crystals were immersed briefly in a cryo-buffer consisting of reservoir solution plus 10% glycerol. The X-ray diffraction data were collected on BL17U beamline at the Shanghai Synchrotron Radiation Facility (Shanghai, China) using a MAR 225 CCD detector. The diffraction images were processed using HKL2000 [18].

2.3. Structure determination and refinement

The crystal structure of PnpA was solved by molecular replacement (MR) method with Phaser MR program [19] using the structure of aklavinone-11-hydroxylase RdmE from *Streptomyces purpurascens* (PDB: 3IHG, 30% sequence identity with PnpA) as the search model. Further refinement was carried out by using the programs Refmac5 [20] and Coot [21]. The figures of the protein and ligand structures were generated with PyMol [22]. The structures for apo-PnpA and PnpA-FAD complex have been deposited in the Protein Data Bank with access codes of 6AIO and 6AIN, respectively.

2.4. Substrate docking

The pdb file of PNP was generated using PRODRG2 Server [23]. Substrate docking was carried out by using AutoDock [24]. All water molecules were removed from the original Protein Data Bank file. Polar hydrogen atoms were added and Gasteiger charge, atomic solvation parameters, and fragmental volumes were assigned to the protein using AutoDock Tools (ADT). The program AutoGrid was used to generate the grid maps. The potential substrate binding site was implicated by the structure of PHBH (PDB: 1K0J). The grid size was set to be 30 × 30 × 30 with 0.375 Å. All the complex candidates were evaluated and ranked in terms of the binding energy by using the standard energy score function implemented in the docking program, but also according to criteria established by previous studies on mechanism and substrate selectivity in this enzyme class.

3. Results and discussion

3.1. Overall structure of PnpA

The structure of the apo-PnpA was obtained first, after which the PnpA-FAD complex structure was obtained from the co-crystallization with 0.01 mM FAD and 0.5 mM PNP. The FAD complex was more complete in each subunit, all the protein regions as well as the FAD moiety were clearly defined, except the N-terminal 8 residues and C-terminal 15 residues and the His_6 tag. Besides, Val47 to Pro51 and Gly173 to Arg175 was absent in the apo-PnpA. The overall rmsd between the two structures is 0.35 Å over 740 backbone Ca atoms (Fig. S1). Final data collection and refinement statistics for PnpA are summarized in Table 1. The apo-PnpA and PnpA-FAD complex were determined at a resolution of 2.04 Å and 2.48 Å, respectively. PnpA was crystallized in the space group

Table 1
Data collection and refinement statistics for PnpA.

	apo-PnpA	PnpA-FAD complex
Data Collection		
Space group	$P2_12_12_1$	$P2_12_12_1$
Resolution [Å]	30–2.04 (2.11–2.04)	30–2.48 (2.57–2.48)
Cell dimensions		
a, b, c [Å]	53.84, 76.32, 208.25	54.43, 77.18, 210.24
α, β, γ (°)	90.00, 90.00, 90.00	90.00, 90.00, 90.00
Unique reflections	55036 (5337)	32252 (3154)
Molecules per asymmetric unit	2	2
Redundancy	5.9 (5.4)	7.3 (7.4)
Completeness [%]	99.1 (97.7)	99.4 (93.7)
Mean $I/\sigma(I)$	25.09 (13.4)	19.37 (4.04)
R_{merge}[a] [%]	5.2 (9.5)	8.9 (49.3)
Refinement		
Unique reflections	52274 (3702)	30628 (2066)
R_{work}[a] (95% of data)	0.173 (0.181)	0.193 (0.240)
R_{free}[a] (5% of data)	0.217 (0.233)	0.245 (0.260)
R.m.s.d. bonds [Å]	0.0183	0.0137
R.m.s.d. angles [°]	1.840	1.638
Ramachandran plot		
Most favored [%]	97.8	97.2
Allowed [%]	2.2	2.8
Disallowed [%]	0	0
Number of non-H atoms		
Protein	6110	6149
Water	619	74
Ligand		53
Average B-factors [$Å^2$]		
Protein	22.37	48.70
Water	30.10	45.09
Ligand		69.58

Values in parentheses are for the highest resolution shell.
[a] $R_{merge} = \sum_{hkl}\sum_i|I_i(hkl)-\langle I(hkl)\rangle|/\sum_{hkl}\sum_i I_i(hkl)$.

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