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Crystallization and preliminary X-ray diffraction analysis of recombinant chlorocatechol 1,2-dioxygenase from *Pseudomonas putida*

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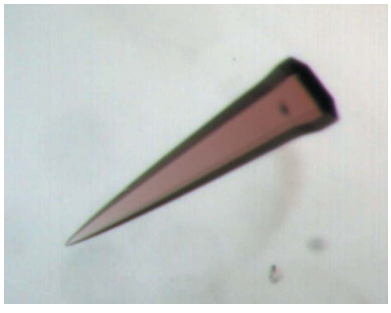
Chlorocatechol 1,2-dioxygenase from the Gram-negative bacterium *Pseudomonas putida* (Pp 1,2-CCD) is considered to be an important biotechnological tool owing to its ability to process a broad spectrum of organic pollutants. In the current work, the crystallization, crystallographic characterization and phasing of the recombinant Pp 1,2-CCD enzyme are described. Reddish-brown crystals were obtained in the presence of polyethylene glycol and magnesium acetate by utilizing the vapour-diffusion technique in sitting drops. Crystal dehydration was the key step in obtaining data sets, which were collected on the D03B-MX2 beamline at the CNPEM/MCT – LNLS using a MAR CCD detector. Pp 1,2-CCD crystals belonged to space group $P6_122$ and the crystallographic structure of Pp 1,2-CCD has been solved by the MR-SAD technique using Fe atoms as scattering centres and the coordinates of 3-chlorocatechol 1,2-dioxygenase from *Rhodococcus opacus* (PDB entry 2boy) as the search model. The initial model, which contains three molecules in the asymmetric unit, has been refined to 3.4 Å resolution.

1. Introduction

The accumulation of persistent organic pollutants (POPs) is a serious environmental problem worldwide. Industrial activities and technological advances contribute to the spread of pollutants which are highly toxic, carcinogenic, bioaccumulative and resistant to physical, chemical, photolytic and biological degradation (Ghosal *et al.*, 1985; Colborn & Smolen, 1996; Elci & Akpinar-Elci, 2009). In particular, the accumulation of POPs can be a consequence of the inappropriate use of pesticides, the production of toxic synthetic compounds such as polychlorinated biphenyls and a large list of activities that promote the incomplete combustion of organic matter and release a large amount of polycyclic aromatic hydrocarbons (PAHs) into the environment (Bamforth & Singleton, 2005).

Conventional methods such as physical removal, isolation and incineration, among others, can be used to mitigate contamination of the environment (Haritash & Kaushik, 2009). However, the application of these methodologies only works as a palliative measure. An alternative strategy, named bioremediation, is a new biotechnological approach that has shed light on revitalization techniques for contaminated sites and is based on the application of microorganisms, or their enzymes, to eliminate or reduce environmental contaminants to inert substances (Gibson & Sayler, 1992; Miller & Poindexter, 1994). The serious environmental problems caused by persistent organic pollutants have stimulated basic research in order to explore the capacity of specific microorganisms to degrade complex aromatic molecules.

Microorganisms such as algae, fungi and bacteria (Cerniglia, 1992) that are capable of reducing the concentrations of POPs and PAHs have been identified and their metabolic pathways have been elucidated. Bacteria of the genus *Pseudomonas*, including *P. putida*, have been widely studied owing to their ability to grow in different sites contaminated by PAHs (Cerniglia, 1992), nitrated and chlorinated PAHs (Wang *et al.*, 2009; Xiao *et al.*, 2006; Broderick & O'Halloran, 1991) and dioxins (Field & Sierra-Alvarez, 2008). The chlorocatechol 1,2-dioxygenase of *P. putida* (Pp 1,2-CCD) is among several enzymes



that are responsible for breaking down the aromatic ring in PAHs. This enzyme displays high affinity for several different halogenated and dihalogenated derivatives of catechol, which is the central intermediate in the metabolic pathway of aromatic compounds (Broderick & O'Halloran, 1991).

Owing to the great potential of Pp 1,2-CCD as a bioremediator, we are interested in understanding the molecular basis of its enzymatic mechanism and the structural features that are responsible for its wide spectrum of substrate specificity. It is our aim to combine biophysical and biochemical studies (Citadini *et al.*, 2005; Melo *et al.*, 2010) with structural characterization of the Pp 1,2-CCD enzyme in order to establish a correlation between structure and function. In the present work, we describe the protocol adopted in the purification process, crystallization, crystallographic characterization and phasing of the Pp 1,2-CCD enzyme as an important step towards this goal.

2. Expression and purification

The *clcA* gene (GenBank ID CAE92861.1) that encodes chlorocatechol 1,2-dioxygenase from *P. putida* was cloned into the pTYB2 expression vector (New England Biolabs Inc.), resulting in the pTYCLCA construct (Araújo *et al.*, 2000). This plasmid provides recombinant protein expression using a modified protein-splicing element (intein) in conjunction with a chitin-binding domain (CBD) as an affinity tag (Chong *et al.*, 1996, 1997).

The expression and purification protocols were adapted from previously reported work (Araújo *et al.*, 2000). The pTYCLCA construct was used to transform *Escherichia coli* BL21 (DE3) host cells. A single colony was added to 20 ml Luria broth (LB) medium containing 100 µg ml^{-1} ampicillin and cultured overnight at 310 K (with shaking at 250 rev min^{-1}). The culture was diluted (1:100) in LB medium containing 100 µg ml^{-1} ampicillin supplemented with 0.31 m*M* $FeSO_4$ and incubated at 310 K (with shaking at 250 rev min^{-1}) until the A_{600} reached 0.5–0.6. The cells were induced with 0.5 m*M* isopropyl β-D-1-thiogalactopyranoside (IPTG) and growth was continued for 5 h at 295 K. After induction, the cells were harvested by centrifugation at 277 K and 6800*g*, suspended in lysis buffer (20 m*M* Tris, 500 m*M* NaCl, 0.5 m*M* PMSF pH 8.0) and disrupted by sonication for 15 × 30 s (with 60 s intervals) using a Misonix XL 2000 sonicator with 9 W power. Following centrifugation at 16 000*g* for 30 min (277 K) the supernatant was loaded onto a chromatographic column (C10/10, GE Healthcare) packed with 5 ml chitin resin (Biolabs) pre-equilibrated with 14 column volumes (cv) of lysis buffer at 1 ml min^{-1} and washed with 25 cv at 0.5 ml min^{-1}. The elution process began by incubation with 30 m*M* of the reducing agent dithiothreitol (DTT) diluted in Tris–HCl buffer consisting of 20 m*M* Tris, 50 m*M* NaCl pH 8.0 and fractions were eluted after 16 h incubation at 277 K. The purified protein migrated as one clear band on 12% SDS–PAGE.

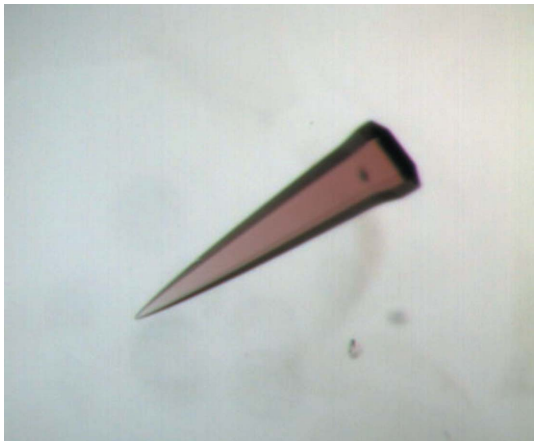


Figure 1
Crystal of chlorocatechol 1,2-dioxygenase from *P. putida*.

3. Crystallization

Prior to crystallization, the recombinant Pp 1,2-CCD was dialyzed against Tris–HCl buffer consisting of 20 m*M* Tris, 50 m*M* NaCl pH 8.0 and was concentrated to 22 mg ml^{-1} based on the molar extinction coefficients of tryptophan, tyrosine and cysteine residues (Pace *et al.*, 1995). This method gave a calculated extinction coefficient for the recombinant protein of 34 505 M^{-1} cm^{-1}. The sparse-matrix method (Jancarik & Kim, 1991) as implemented in commercially available screening kits (Crystal Screen, Crystal Screen 2 and PEG/Ion; Hampton Research) was used in initial crystallization experiments using the vapour-diffusion method in sitting drops and hanging drops. Equal volumes (2 µl) of protein and reservoir solution were mixed, equilibrated against 500 µl reservoir solution and kept at 295 K. Microcrystals appeared within 10 d in 0.2 *M* magnesium acetate tetrahydrate and 20% polyethylene glycol 3350 (PEG/Ion Screen formulation No. 25; Hampton Research). Several efforts were made to optimize the crystallization experiment by screening a wide range of crystallization variables (pH, precipitant concentration, temperature and additives). Reddish-brown crystals were obtained at 295 K using sitting drops in the presence of 0.2 *M* magnesium acetate tetrahydrate and 14% PEG 8000. The hexagonal pyramid-shaped crystals usually appeared within 2 d and reached maximum dimensions of 0.2 × 0.2 × 0.5 mm in 10 d (Fig. 1).

4. Data collection and data processing

Although the Pp 1,2-CCD crystals displayed an excellent external morphology with well shaped faces and good size, the initial X-ray diffraction experiments showed a severe limitation in terms of diffraction quality. Most of the crystals did not diffract at all or in the best-case scenario data were limited to 8–10 Å resolution. Moreover, the high anisotropy and the overlap of the diffraction spots suggested the presence of crystalline disorder and a large crystallographic axis. Pre- and post-crystallization treatments (Chayen, 1997; Heras & Martin, 2005), the use of additives and the use of a microfocus X25 beamline (at NSLS, Brookhaven National Laboratory) were tested in attempts to improve data quality. From hundreds of crystals tested, only those submitted to dehydration showed a consistent improvement in data quality. Several experimental setups for crystal dehydration were tested and the best results were obtained by immersing the crystals for a short time (30–90 s) in cryoprotectant solution containing an excess of $(x + 2)$% or $(x + 4)$% of the precipitant agent PEG 8000 (x = 14) and 0.2 *M* magnesium acetate tetrahydrate supplemented with 25%(*v*/*v*) glycerol and flash-frozen directly in a nitrogen stream at 100 K. Dehydration was then employed routinely before any data collection.

The two best data sets were collected on beamline D03B-MX2 at the Brazilian Synchrotron Light Laboratory (CNPEM/MCT – LNLS) using a MAR CCD (MAR Research) detector. The data sets were processed using the *MOSFLM* (Leslie, 1992) and *SCALA* (Evans, 1997) programs. Crystals of recombinant Pp 1,2-CCD belonged to the hexagonal space group $P6_122$ or $P6_522$, as determined on the basis of systematic absences for the 00*l*, $l = 6n$ reflections and analysis of the

Table 1
Data-collection and processing statistics.

Values in parentheses are for the outer resolution shell.

	Crystal 1	Crystal 2
Space group	$P6_122/P6_522$	$P6_122/P6_522$
Unit-cell parameters (Å)	$a = b = 98.84$, $c = 425.33$	$a = b = 97.57$, $c = 423.60$
Solvent content (%)	64.35	63.27
V_M (Å^3 Da^{-1})	3.45	3.35
Molecules per asymmetric unit	3	3
Data collection		
Temperature (K)	100	100
Wavelength (Å)	1.741	1.459
Resolution range (Å)	60.76–3.95 (4.16–3.95)	59.83–3.40 (3.58–3.40)
Unique reflections	11741	17523
Data completeness (%)	100.0 (100.0)	99.9 (100.0)
Mean $I/\sigma(I)$	22.0 (7.4)	9.6 (3.1)
Multiplicity	25.4 (26.2)	8.6 (8.9)
Anomalous multiplicity	14.7 (14.5)	
R_{merge}† (%)	14.4 (54.3)	21.3 (69.2)

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

probability based on the Laue group using *POINTLESS* (Evans, 2006) and *phenix.xtriage* (Adams *et al.*, 2010). The data-collection and processing statistics are summarized in Table 1. The calculated Matthews coefficient (V_M) for three molecules of protein in the asymmetric unit is 3.35 Å^3 Da^{-1}, with 63.27% of the unit cell occupied by solvent (Matthews, 1968).

5. Structure determination

The best attempt at phasing the data by molecular-replacement techniques was obtained with *Phaser* (McCoy *et al.*, 2007). Using the coordinates of 3-chlorocatechol 1,2-dioxygenase (PDB entry 2boy; Ferraroni *et al.*, 2004), which shares approximately 40% sequence identity with Pp 1,2-CCD, as a search model we found a partial solution with two protein chains in the asymmetric unit. This two-chain model presented an unusually high solvent content (∼77%). Moreover, the two molecules found in the asymmetric unit did not pack in the functional form observed for this class of proteins (Vetting & Ohlendorf, 2000; Ferraroni *et al.*, 2004, 2006; Earhart *et al.*, 2005; Matera *et al.*, 2010). As an alternative strategy, we attempted to explore the presence of the iron cofactor as an anomalous scatterer by collecting a data set at the absorption edge (Table 1). We applied the MR-SAD protocol as implemented in *PHENIX* (Adams *et al.*, 2010), which placed three Fe atoms in the asymmetric unit. The locations of two Fe atoms were consistent with the MR monomer structure. The third iron was positioned on the anomalous peak and the third protein molecule was rotated to fit both the iron coordination and the electron density. Moreover, in this arrangement the two monomers within the functional dimeric Pp 1,2-CCD structure are related by twofold crystallographic symmetry. The resulting model containing three protein chains was used as a search model for a new cycle of molecular replacement in *Phaser* against our best-resolution data set (3.4 Å).

Initial refinement was performed using the *phenix.refine* program (Adams *et al.*, 2010). Owing to the low-resolution data, it was necessary to establish strong geometric and noncrystallographic symmetry restraints. Moreover, application of bulk-solvent correction and simulated-annealing routines was indispensable to conduct model refinement. Partial refinement reached an R factor of 21.6% and an R_{free} of 27.8%. Iterative manual building and refinement of the model are currently under way.

Comparison of the structure of the chlorocatechol 1,2-dioxygenase from *P. putida* with those of other dioxygenases will be explored in order to provide new structural insights into the molecular mechanism of this enzyme.

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