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Crystallization and preliminary crystallographic analysis of 2-aminophenol 1,6-dioxygenase complexed with substrate and with an inhibitor

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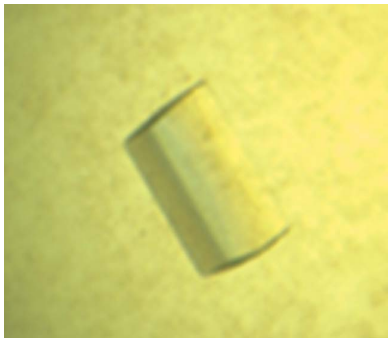
Dioxygen activation implemented by nonhaem Fe^{II} enzymes containing the 2-His-1-carboxylate facial triad has been extensively studied in recent years. Extradiol dioxygenase is the archetypal member of this superfamily and catalyzes the oxygenolytic ring opening of catechol analogues. Here, the crystallization and preliminary X-ray analysis of 2-aminophenol 1,6-dioxygenase, an enzyme representing a minor subset of extradiol dioxygenases that catalyze the fission of 2-aminophenol rather than catecholic compounds, is reported. Crystals of the holoenzyme with Fe^{II} and of complexes with the substrate 2-aminophenol and the suicide inhibitor 4-nitrocatechol were grown using the cocrystallization method under the same conditions as used for the crystallization of the apoenzyme. The crystals belonged to space group $C2$ and diffracted to 2.3–2.7 Å resolution; the crystal that diffracted to the highest resolution had unit-cell parameters a = 270.24, b = 48.39, c = 108.55 Å, β = 109.57°. All X-ray data sets collected from diffraction-quality crystals were suitable for structure determination.

1. Introduction

Nature typically employs metal centres within enzymes to activate molecular oxygen and to carry out various oxidative transformations such as drug metabolism and the biodegradation of environmental pollutants. A specific class of these enzymes contain a mononuclear nonhaem Fe^{II} and a 2-His-1-carboxylate facial triad at the active site which serves as a versatile platform for catalyzing a wide range of reactions and have attracted great attention in recent years owing to growing concern about the huge amounts of aromatic pollutants that are released worldwide (Bruijnincx *et al.*, 2008; Koehntop *et al.*, 2005; Kovaleva & Lipscomb, 2008*b*; Straganz & Nidetzky, 2006).

Extradiol dioxygenase is the archetypal member of the 2-His-1-carboxylate enzymes and catalyzes the oxygenolytic ring opening of aromatic compounds involved in microbial catabolism (Kovaleva & Lipscomb, 2008*b*; Vaillancourt *et al.*, 2006). The substrates are catechol analogues in most cases, but can also include noncatecholic compounds such as gentisate, hydroquinone, salicylate and 2-aminophenol (Vaillancourt *et al.*, 2006). Profound mechanistic insight into catecholic substrate catalysis has recently been achieved owing to the increasing availability of structural, spectroscopic and computational data (Horsman *et al.*, 2005; Kovaleva & Lipscomb, 2007, 2008*a*; Sato *et al.*, 2002; Shu *et al.*, 1995; Vaillancourt *et al.*, 2002). However, it still remains unclear whether or not the canonical mechanism applies to all enzymes, in particular those that catalyze the fission of non-catecholic substrates that lack vicinal hydroxyl substituents, as none of these enzymes has been crystallized to date.

2-Aminophenol 1,6-dioxygenase (APD) represents a minor subset of enzymes that display no sequence or structural similarity to the majority of extradiol dioxygenases such as 2,3-dihydroxybiphenyl 1,2-dioxygenase (DHBD), catechol 2,3-dioxygenase (C23O) and homoprotocatechuate 2,3-dioxygenase (HPCD) (Vaillancourt *et al.*, 2006; Wu *et al.*, 2006) but that share a remote evolutionary ancestor with protocatechuate 4,5-dioxygenase (4,5-PCD; Noda *et al.*, 1990; Sugimoto *et al.*, 1999). APD from various sources has been reported to form an $\alpha_2\beta_2$ heterotetramer (Davis *et al.*, 1999; Takenaka *et al.*,



1997; Wu *et al.*, 2005). In this study, the enzyme identified from *Comamonas testosteroni* strain CNB-1 also has a heterotetrameric functional unit, with 217 amino acids in subunit α (29 kDa) and 315 amino acids in subunit β (35 kDa). APD catalyzes the cleavage of 2-aminophenol (2AP) analogues at the position *ortho* to the hydroxyl substituent, generating a 2-aminomuconic 6-semialdehyde product, and thereby plays a vital role in the reductive pathway of microbial biodegradation of nitrobenzene (NB) and chlolorinated nitrobenzene (CNB) (Wu *et al.*, 2005, 2006). It shows amazing substrate selectivity towards 2AP, but is strongly inhibited by catechol analogues (Fig. 1). As the substrate 2AP differs from catechol in only one substituent (amino or hydroxyl), APD is hence an intriguing research target for studying the substrate specificity of enzymes that oxidize noncatecholic compounds.

2. Materials and methods

2.1. Purification

Wild-type 2-aminophenol-1,6-dioxygenase from *C. testosteroni* strain CNB-1 (APD_{CNB-1}) was purified from laboratory-cultured bacterial cells as described previously (Wu *et al.*, 2005). In brief, the protein was precipitated from the cell lysate using ammonium sulfate and was then purified using a three-step purification protocol: (i) size-exclusion chromatography on a HiLoad 16/600 Superdex 200 column (GE Healthcare), (ii) ion-exchange chromatography on a HiLoad 5/5 Mono Q column (GE Healthcare) and (iii) another round of size-exclusion chromatography using the same Superdex 200 column.

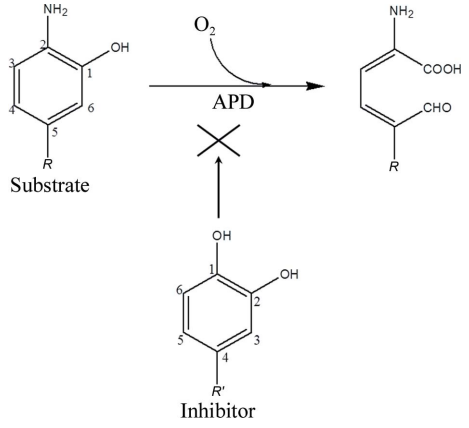


Figure 1
The ring-opening reaction of aromatic compounds catalyzed by APD. 2-Aminophenol analogues are substrates of APD, with the products being 2-amino-6-semialdehyde analogues. The catalysis is inhibited by catechol analogues. R = H or Cl; R' = H or NO_2.

2.2. Crystallization

The purified wild-type apoenzyme was concentrated to approximately 10 mg ml^{-1} and stored in 50 m*M* Tris–HCl pH 8.0, 5% glycerol, 2 m*M* DTT, 0.2 m*M* EDTA. All crystallization trials were carried out by hand using the hanging-drop vapour-diffusion method at room temperature under aerobic conditions. The drop in each well was formed by mixing 1 µl protein solution with 1 µl reservoir solution and was equilibrated against 400 µl reservoir solution. Initial conditions were screened using approximately 500 commercial crystallization conditions from the Crystal Screen, Crystal Screen 2, Index, SaltRx, PEG/Ion, PEG/Ion 2 and PEGRx kits (all from Hampton Research, California, USA). Microcrystals were grown under several conditions, all of which contained PEG as the major precipitant. Nearly 200 drops were set up for optimization, including grid screening of pH and precipitant concentration and the use of various additives. The optimum condition for the growth of apoenzyme (Fe^{2+}-free APD) crystals was 0.1 *M* sodium cacodylate pH 6.5, 25%(*w*/*v*) PEG 3350, 0.2 *M* sodium chloride.

Crystals of the holoenzyme (Fe^{2+}-containing APD) were grown under the same conditions as used for the metal-free protein by cocrystallization in the presence of 5 m*M* $FeSO_4$. Similarly, the complexes of APD with its substrate 2AP and the suicide inhibitor 4-nitrocatechol (4NC) were also grown by cocrystallization under the same conditions by the addition of 5 m*M* $FeSO_4$ and 5 m*M* 2AP or of 5 m*M* $FeSO_4$ and 5 m*M* 4NC to the reservoir solution, respectively.

2.3. Data collection and evaluation

The crystals used for data collection were dipped into cryoprotectant (reservoir solution supplemented with 10% glycerol) for approximately 10 s before being mounted in nylon cryoloops (Hampton Research) and flash-cooled in a stream of liquid nitrogen at 95 K. Native diffraction data for the apoenzyme and the APD–2AP complex were collected on an in-house Rigaku R-AXIS IV^{++} image plate using Cu $K\alpha$ radiation (λ = 1.5418 Å) from a rotating-

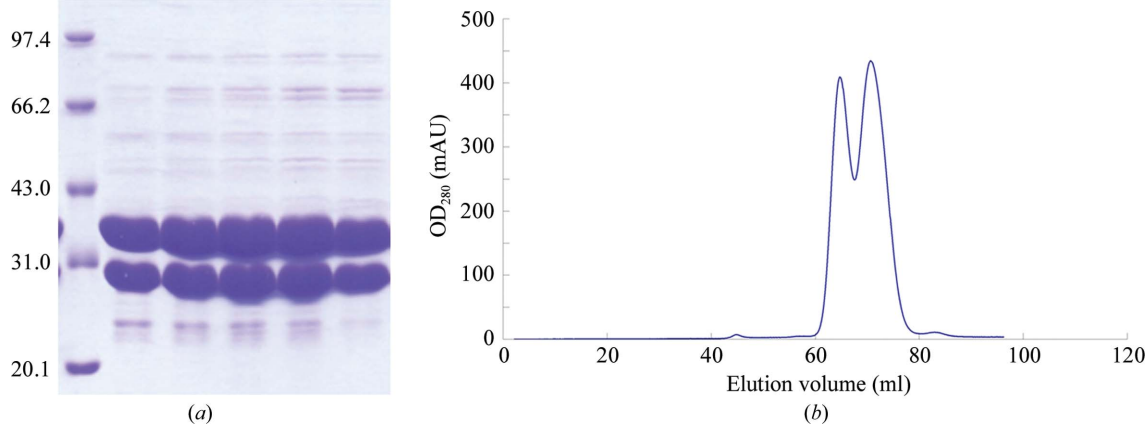


Figure 2
Purification profiles of the apoenzyme (Fe^{2+}-free APD). (*a*) The eluted fractions after the first round of size-exclusion chromatography and ion-exchange chromatography analyzed by SDS–PAGE. The two major bands showing apparent molecular masses of 30 and 35 kDa correspond to the α subunit and the β subunit, respectively. The left lane contains molecular-mass markers (labelled in kDa). (*b*) Chromatogram of the second round of gel filtration on a HiLoad 16/600 Superdex 200 column. The two absorption peaks contained the (discarded) octamer and the tetramer of APD.

Table 1
Summary of X-ray data collection.

Values in parentheses are for the highest resolution shell.

	Fe^{2+}-free APD	Active APD	APD–2AP	APD–4NC
Wavelength (Å)	1.5418	1.0000	1.5418	1.0000
Space group	*C*2	*C*2	*C*2	*C*2
Unit-cell parameters				
a (Å)	269.73	265.39	270.24	272.73
b (Å)	48.26	47.47	48.3	48.26
c (Å)	108.44	107.38	108.55	108.12
β (°)	109.44	109.47	109.57	109.61
Resolution (Å)	39.78–2.40 (2.53–2.40)	47.95–2.70 (2.85–2.70)	39.87–2.30 (2.42–2.30)	69.34–2.50 (2.64–2.50)
Completeness (%)	91.8 (82.5)	98.1 (93.7)	97.8 (86.5)	91.0 (81.6)
No. of unique reflections	48049 (6247)	34673 (4756)	58265 (7456)	42370 (5494)
Mutliplicity	4.5 (4.4)	3.2 (2.6)	5.2 (3.9)	3.2 (2.9)
R_{merge}† (%)	7.3 (32.3)	10.3 (33.3)	10.9 (35.6)	4.7 (33.6)
Average $I/\sigma(I)$	20.6 (4.4)	11.5 (2.7)	15.7 (2.7)	18.2 (3.2)

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$.

anode generator operating at 40 kV and 20 mA with a 0.1 mm confocal incident-beam diameter. X-ray data collections for the other crystals were performed on beamline NW12A at the Photon Factory, KEK, Japan using an ADSC Quantum 210 CCD detector. Diffraction data for the holoenzyme and the complex with 4NC were collected at a wavelength of 1.0 Å.

All X-ray diffraction data were indexed, integrated and scaled using *iMOSFLM* (Battye *et al.*, 2011) and *SCALA* from the *CCP*4 program suite (Winn *et al.*, 2011).

3. Results and discussion

Consistent with previous reports of APD from other sources, two major bands with apparent molecular masses of 30 and 35 kDa were observed on an SDS–PAGE gel loaded with the enzyme after purification by ammonium sulfate precipitation, size-exclusion chromatography and anion-exchange chromatography (Fig. 2*a*). These bands had the same masses as the calculated values for the α subunit and the β subunit, respectively. On the elution profile of the second round of size-exclusion chromatography using a Superdex 200 column pre-equilibrated with 20 m*M* Tris–HCl, 10% ethanol, 1 m*M* DTT, 0.5 m*M* ascorbate, two absorption peaks were eluted at 65 and 75 ml with estimated molecular masses of 280 and 140 kDa (Fig. 2*b*), corresponding to APD octamers and tetramers, respectively. The formation of the octamer was probably a consequence of the low ionic strength of the elution buffer. The tetramer fraction was pooled and subsequently concentrated for crystallization, while the octamer fraction was discarded. The purified enzyme was assumed to be in the apo form as it did not show any catalytic activity in the absence of Fe(II).

The optimal crystallization condition was obtained by screening a number of conditions using PEG 3350 and salt as the precipitant within the pH range 5.0–8.0. After optimization, single crystals of Fe^{2+}-free protein with approximate dimensions of 0.1 mm were obtained at pH 6.5. Visible crystals usually appeared 2–3 d after drop setup and grew to maximal size within one week. A crystal was flash-cooled and a complete diffraction data set was collected to 2.4 Å resolution using an in-house Rigaku rotating-anode X-ray source (λ = 1.5418 Å). The crystal was monoclinic, space group *C*2, with unit-cell parameters a = 269.73, b = 48.26, c = 108.44 Å, $\alpha = \gamma = 90$, β = 109.44°, giving a value of 2.57 $Å^3\,Da^{-1}$ for the Matthews

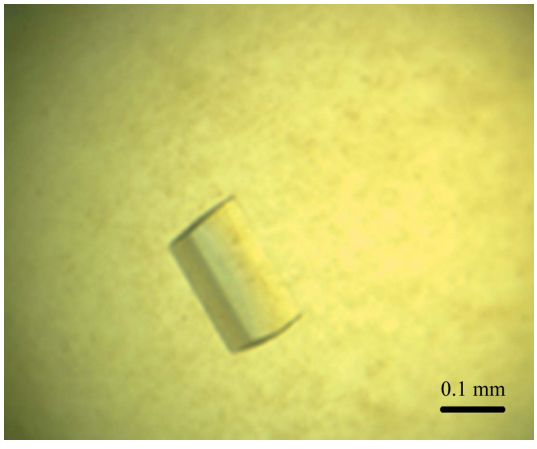


Figure 3
An optimized crystal of the APD–2AP complex obtained using 0.1 *M* sodium cacodylate buffer pH 6.5, 25% PEG 3350, 0.2 *M* sodium chloride with the addition of 5 m*M* $FeSO_4$ and 5 m*M* 2AP.

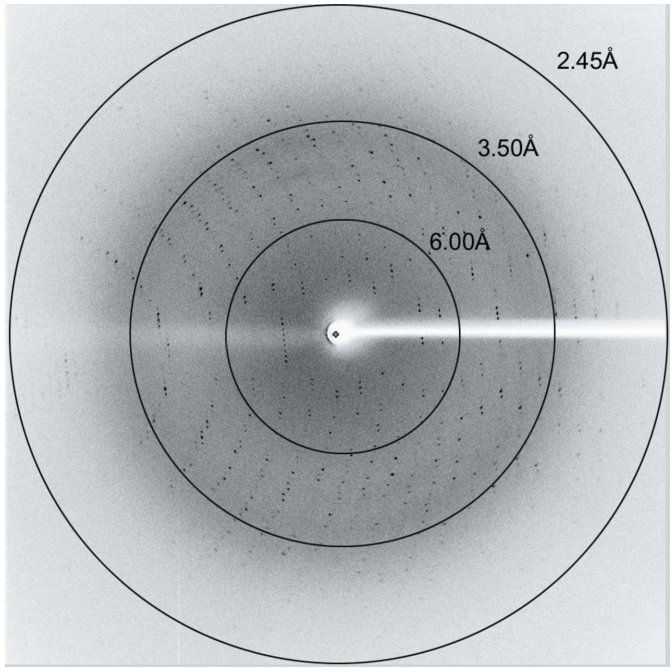


Figure 4
A diffraction image of a crystal of the APD–2AP complex obtained on an in-house Rigaku R-AXIS IV^{++} image plate.

coefficient (Matthews, 1968) and a solvent content of 52.1% assuming the presence of one tetramer in the asymmetric unit. Other statistics are given in Table 1.

The holoenzyme and the complexes with 2AP and with 4NC were crystallized in the same space group by cocrystallization. The largest single crystal from APD–2AP cocrystallization reached dimensions of 0.2 × 0.1 × 0.1 mm (Fig. 3) and diffracted to 2.3 Å resolution using an in-house Rigaku rotating-anode X-ray source (Fig. 4). The unit-cell parameters for this crystal were $a = 270.24$, $b = 48.39$, $c = 108.55$ Å, $\alpha = \gamma = 90$, $\beta = 109.57^{\circ}$. The diffraction data sets for the active enzyme containing Fe^{2+} and for the APD–4NC complex were collected on beamline NW12A at the Photon Factory, KEK, Japan ($\lambda = 1.0000$ Å). All data-collection results are summarized in Table 1 and a representative diffraction image is shown in Fig. 4.

No enzyme from the extradiol dioxygenase superfamily that acts on noncatecholic substrates has been crystallized to date. Mechanistic understanding of the minor subsets of this large protein class would definitely benefit from the crystal structure determination of APD, which will give us an opportunity to determine whether enzymes that are effective towards noncatecholic compounds utilize a similar catalytic mechanism to the canonical mechanism revealed by the published structures or use a unique mechanism of their own.

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