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# Crystallization and preliminary X-ray analysis of CYP153C1 from *Novosphingobium aromaticivorans* DSM12444

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Cytochrome P450 (CYP) enzymes constitute a large family of haemoproteins that catalyze the monooxygenation of a great variety of endogenous and exogenous organic compounds. In common with other members of the CYP153 family of alkane hydroxylases, CYP153C1 from the oligotrophic bacterium *Novosphingobium aromaticivorans* DSM 12444 can bind linear alkanes such as heptane, octane and nonane. Here, the production, purification and crystallization of CYP153C1 and the collection of high-resolution diffraction data to 1.77 Å resolution are reported. The crystals belonged to space group $P2_12_12_1$, with unit-cell parameters $a$ = 61.0, $b$ = 96.3, $c$ = 149.8 Å, $\alpha = \beta = \gamma = 90.0^\circ$. Preliminary X-ray diffraction data analysis revealed that the asymmetric unit is most likely to contain two protein molecules.

## 1. Introduction

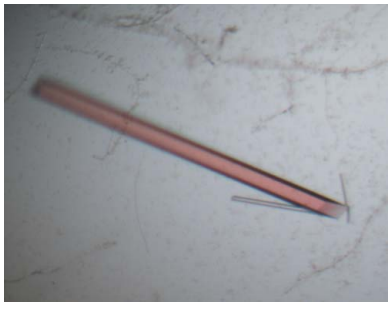
Cytochrome P450 (CYP) enzymes are a large and diverse superfamily of haemoproteins found in all domains of life. Bacterial cytochromes P450 are generally soluble enzymes which are involved in a wide variety of metabolic processes including the biosynthesis of biologically functional molecules and the metabolism of xenobiotics such as alkanes, polycyclic aromatic hydrocarbons and pesticides (Ortiz de Montellano, 2005; Sigel *et al.*, 2007). The primary function of P450 enzymes is C—H bond oxidation, which can be represented as

$$R{-}H + O_2 + 2e^- + 2H^+ \rightarrow R{-}OH + H_2O.$$

Other activities such as epoxidation, heteroatom oxygenation, dealkylation, desaturation and even dehalogenation have also been found (Isin & Guengerich, 2007). The majority of CYP enzymes utilize NAD(P)H as the source of electrons and these are delivered one at a time *via* electron-transfer proteins (Hannemann *et al.*, 2007). The selective oxidation of C—H bonds by CYP enzymes holds great promise for applications in organic synthesis, biotechnology and bioremediation (Bell *et al.*, 2003, 2007; Bernhardt, 2006).

The oligotrophic organism *Novosphingobium aromaticivorans* degrades a broad range of aromatic compounds, including polycyclic aromatic hydrocarbons (Fredrickson *et al.*, 1995). It has also been implicated in the initiation of primary biliary cirrhosis (Selmi & Gershwin, 2004). In 2006, genome sequencing of *N. aromaticivorans* DSM 12444 was completed by the US DOE Joint Genome Institute, revealing a variety of genes involved in the catabolism and transport of aromatic compounds, including many oxygenase genes (Romine *et al.*, 1999). 16 potential cytochrome P450 genes from 11 different CYP bacterial families were detected by genomic analysis. The production of 12 of these has been reported and substrates for the majority have been identified (Bell & Wong, 2007). The range of substrates is broad and includes alkanes, terpenoid compounds and substituted aromatic hydrocarbons (Bell & Wong, 2007; Yang *et al.*, 2010, 2011; Bell *et al.*, 2010; Ma *et al.*, 2011). The ability of *N. aromaticivorans* to metabolize different classes of compounds undoubtedly plays an important role in the ability of this bacterium to survive in the oligotrophic environments where it is found (Fredrickson *et al.*, 1995).

Two members of the CYP153 family are found in *N. aromaticivorans*: CYP153C1 and CYP153D1. One of these, CYP153C1 (Gene



ID 3916208, Saro_0220), has been reported to be an alkane-binding enzyme (Bell *et al.*, 2007). The CYP153 family are terminal alkane hydroxylases and are distributed in variety of bacteria, including those isolated from oil-contaminated environments (van Beilen *et al.*, 2006; Wang *et al.*, 2010; Kubota *et al.*, 2005; Maier *et al.*, 2001). CYP153C1 consists of 410 amino-acid residues and its molecular weight is 46.6 kDa. Optimal binding to CYP153C1 was observed for heptane, octane and nonane, as indicated by the 80% high-spin haem content on substrate binding. The high-spin haem content was lower for the binding of decane (40%) and hexane (10%) (Bell & Wong, 2007). The selective catalytic oxidation of simple alkanes under mild conditions is one of the most difficult chemical reactions to achieve using conventional synthetic methods (Bell *et al.*, 2003; Glieder *et al.*, 2002). Medium-chain-length terminal alcohols are intermediates in the synthesis of, for example, flavour compounds and insect pheromones. The three-dimensional structure of the native enzyme and its complexes with alkane substrates will help to elucidate the substrate-specificity and properties of this enzyme. In this paper, we report the production, purification and crystallization of CYP153C1 and preliminary analysis of the X-ray diffraction data.

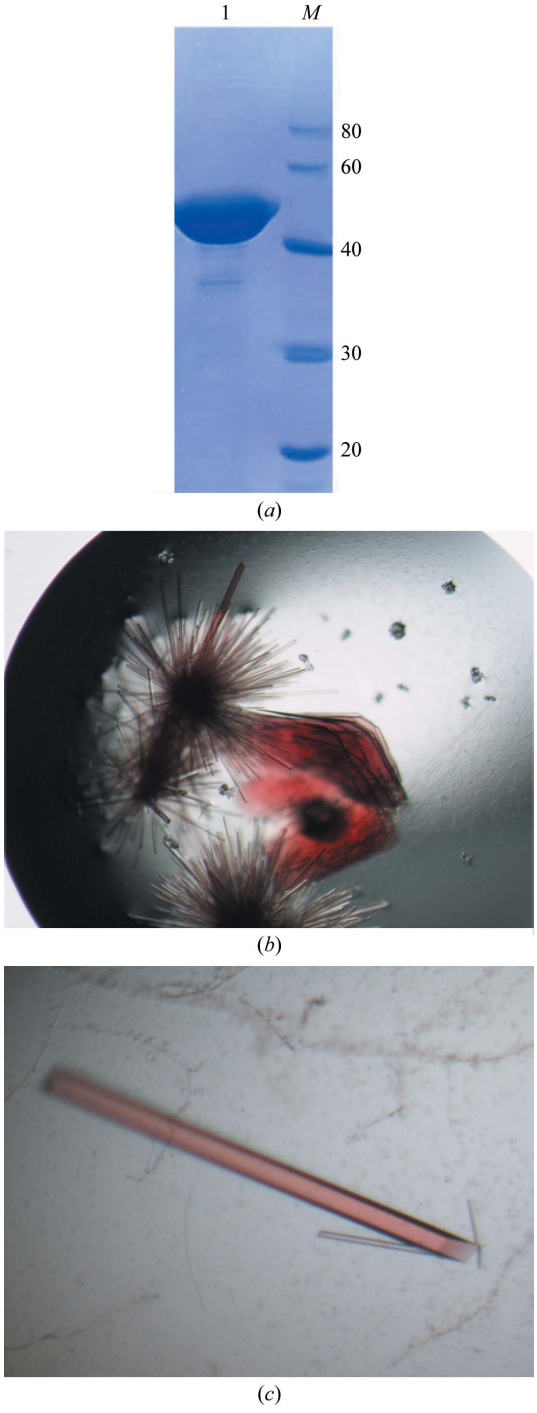



**Figure 1**
(*a*) SDS–PAGE gel of CYP153C1. Lane *M*, molecular-weight marker (labelled in kDa); lane 1, CYP153C1. (*b*) Crystals of CYP153C1 after initial screening. (*c*) Crystals of CYP153C1 from optimal conditions with approximate dimensions 0.01 × 0.03 × 0.5 mm.

## 2. Materials and methods

### 2.1. Cloning, expression and purification

The *CYP153C1* gene was excised from the plasmid pET26-CYP153C1 (Bell & Wong, 2007) and subcloned into the vector pET28a(+) (Novagen Inc.) using the restriction sites *Nde*I and *Hin*dIII, which added an N-terminal 6×His tag to the protein to assist in purification. The entire sequence of all non-native residues was MGSSHHHHHHSSGLVRGSH. The recombinant plasmid was transformed into *Escherichia coli* strain BL21 (DE3) and the transformed bacterial cells were cultured in LB medium at 310 K with 25 µg ml$^{-1}$ kanamycin. When the $OD_{600}$ of the culture reached 0.6–0.8, 0.5 m*M* IPTG was added in order to induce production of the recombinant protein. After further growth for 18 h at 301 K, the cells were harvested by centrifugation, resuspended in buffer *P* (40 m*M* potassium phosphate, 10 m*M* *β*-mercaptoethanol pH 7.4) and lysed by sonication at 277 K. The crude extracts were then centrifuged at 27 000*g* for 30 min at 277 K to remove the cell debris.

The supernatant containing the protein was loaded onto an $Ni^{2+}$-chelating affinity column (1.0 ml $Ni^{2+}$–NTA agarose) which had been equilibrated with buffer *P* and washed twice with buffer *P* containing 10 and 20 m*M* imidazole, respectively. CYP153C1 was eluted with buffer *P* containing 300 m*M* imidazole and the eluent was buffer-exchanged into buffer *A* (20 m*M* HEPES, 10 m*M* *β*-mercaptoethanol pH 7.4) by ultrafiltration. The protein solution in buffer *A* was concentrated to 1 ml, injected onto a Resource Q column (GE Healthcare, USA) and eluted with a gradient of 0–1 *M* NaCl in buffer *A*. CYP153C1 eluted at ∼100 m*M* NaCl. The purity of the protein was checked by SDS–PAGE (Fig. 1*a*). The protein concentration was calculated by UV absorbance measurements at 280 nm wavelength with an extinction coefficient of 65 430 $M^{-1}$ cm$^{-1}$ for full-length CYP153C1 predicted using the *ProtParam* software from the ExPASy proteomics server.

### 2.2. Crystallization

Purified CYP153C1 was concentrated to 50 mg ml$^{-1}$ in buffer consisting of 20 m*M* $KH_2PO_4$, 200 m*M* KCl, 10 m*M* *β*-mercaptoethanol pH 7.4. The hanging-drop vapour-diffusion method was used with Crystal Screen, Crystal Screen 2 and Index (Hampton Research, USA) to screen for CYP153C1 crystals at 291 K in 16-well plates manufactured by Tianjin Xiangyushun Macromolecule Technology Ltd, People's Republic of China. Two drops (1 µl each) of protein solution at 30 or 50 mg ml$^{-1}$ protein were mixed with 1 µl reservoir solution separately and equilibrated against 200 µl reservoir solution. After 4 d, several dark red crystals were obtained from both drops using Crystal Screen condition No. 17 and Index condition Nos. 74 and 75. However, none of the crystals were suitable for X-ray diffraction analysis because of their non-single thin plate morphology and poor

**Table 1**
Data-collection and processing statistics for CYP153C1.

Values in parentheses are for the highest resolution shell.

| | |
|---|---|
| Space group | $P2_12_12_1$ |
| Unit-cell parameters (Å) | $a$ = 61.0, $b$ = 96.3, $c$ = 149.8 |
| Wavelength (Å) | 1.5418 |
| Resolution range (Å) | 50.0–1.77 (1.83–1.77) |
| Total reflections | 156860 |
| Unique reflections | 86960 |
| Multiplicity | 7.1 (6.6) |
| Average $I/\sigma(I)$ | 24.9 (5.2) |
| $R_{\rm merge}$† (%) | 7.6 (38.3) |
| Data completeness (%) | 94.6 (87.5) |
| Molecules per asymmetric unit | 2 |
| $V_{\rm M}$ (Å$^3$ Da$^{-1}$) | 2.44 |
| Solvent content (%) | 49.7 |

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

X-ray diffraction. Further optimization was undertaken using Crystal Screen condition No. 17 owing to the higher crystallinity of the crystals obtained (Fig. 1*b*). The precipitant concentration, buffer, pH and protein concentration were all varied. Long single rod-shaped and good diffraction-quality crystals were eventually obtained when the protein concentration was lowered to 20 mg ml$^{-1}$. After two weeks, single crystals that gave high-resolution data were grown under optimized conditions consisting of 0.2 *M* lithium sulfate, 0.1 *M* 2-(*N*-morpholino)ethanesulfonic acid (MES) pH 6.5 and 20%(*w*/*v*) polyethylene glycol 4000 (Fig. 1*c*).

### 2.3. Crystallographic data collection and processing

X-ray diffraction data were collected to 1.77 Å resolution from CYP153C1 crystals flash-cooled at 100 K in a nitrogen-gas stream. Data were collected on a Rigaku R-AXIS HTC image plate using Cu $K\alpha$ radiation from an in-house Rigaku MicroMax-007 rotating-anode X-ray generator operating at 40 kV and 30 mA (Fig. 2). No cryoprotectant was used. A total of 360 frames of images were collected with an exposure of 3 min and 0.3° oscillation per image. The intensity set was indexed, integrated and scaled with the *HKL*-2000 package (Otwinowski & Minor, 1997). Molecular replacement was carried out with the program *Phaser* (Read, 2001) from the *CCP*4 suite. Data-collection statistics are listed in Table 1.

## 3. Results and discussion

CYP153C1 was purified using metal-affinity and anion-exchange chromatography. SDS–PAGE analysis showed that the purity of the protein was higher than 95%. Therefore, size-exclusion chromatography was not used for further purification. Hampton Research Crystal Screen kits were used for preliminary screening and protein crystals were obtained from several conditions, all of which contained lithium sulfate, PEG 3350 or PEG 4000. During optimization, two different types of crystal shape were obtained. One was in the form of thin plates and the other in the form of long rods. The diffraction data from the rod-like crystals were of much higher quality than those from the plate form. Therefore, we further optimized the conditions by varying the buffer pH, precipitant concentration, protein concentration and temperature. Red-coloured fine rod crystals were obtained after several weeks. These crystals showed very good diffraction using the in-house X-ray source and diffraction data were collected to 1.77 Å resolution.

The data were initially indexed in space group *P*222. After scaling using *SCALEPACK*, it was confirmed that the correct space group was $P2_12_12_1$ based on analysis of systematic absences. The CYP153C1 crystals belonged to space group $P2_12_12_1$, with unit-cell parameters $a$ = 61.0, $b$ = 96.3, $c$ = 149.8 Å, $\alpha = \beta = \gamma = 90.0^\circ$.

Based on crystal-packing analysis the asymmetric unit is most likely to contain two molecules, with 49.7% solvent content and a Matthews coefficient of 2.44 Å$^3$ Da$^{-1}$ (Matthews, 1968). However, no noncrystallographic symmetry peaks were found in a self-rotation map or a native Patterson map (data not shown). Accordingly, we cannot exclude the possibility that there is only one molecule per asymmetric unit with 74.9% solvent content and a Matthews coefficient of 4.89 Å$^3$ Da$^{-1}$. Resolution of the crystal packing will have to await solution of the structure.

We attempted to use several P450 structures as models to solve the phases of CYP153C1 by molecular replacement with the program *Phaser* (Read, 2001) from the *CCP*4 suite (Winn *et al.*, 2011). For

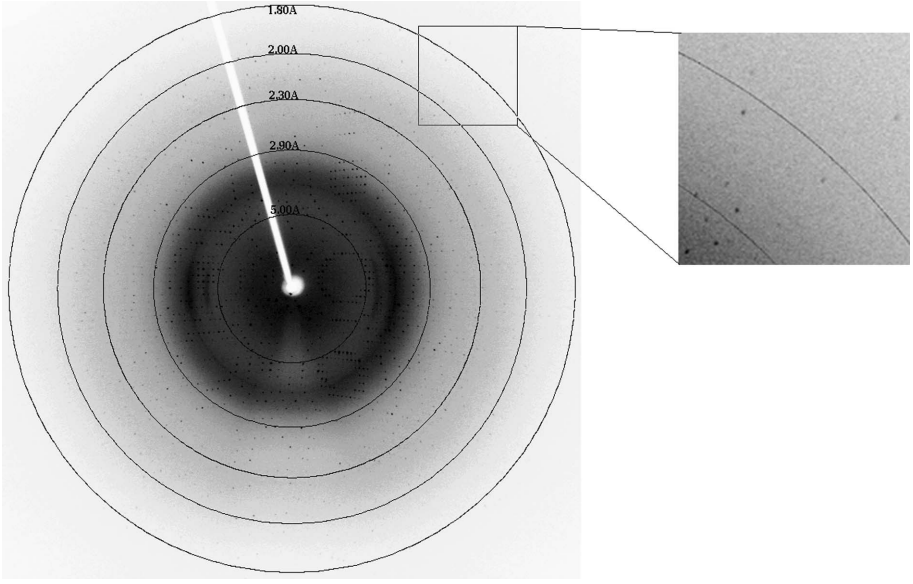



**Figure 2**
A typical diffraction pattern of a CYP153C1 crystal.

example, the structures of CYP124A1 with phytanic acid (PDB entry 2wm4; 33% sequence identity; Johnston *et al.*, 2009), CYP125A1 with cholest-4-en-3-one (PDB entry 2x5w; 29% sequence identity; Ouellet *et al.*, 2010) and CYP108A1 (PDB entry 1cpt; 28% sequence identity; P450terp; Hasemann *et al.*, 1994) were tried on the basis of sequence homology and their substrate range. However, none of the models allowed us to solve the phases of CYP153C1. Trials with *AMoRe* (Navaza, 2001) and *MOLREP* (Vagin & Teplyakov, 2010) were similarly unsuccessful. Presumably, the structure of CYP153C1 differs compared with the CYP enzymes used as models.

Further work on a selenomethionine derivative of CYP153C1 is under way in order to solve the protein structure. Since there are 17 Met residues in one molecule, site-directed mutagenesis may have to be used to obtain soluble expression protein. Substrate-soaking experiments to obtain the substrate-bound complex will also be undertaken. The role of the CYP153 family in catalyzing the terminal oxidation of alkyl side chains and alkanes and the mechanisms of substrate binding and selective hydroxylation will be of particular interest (Johnston *et al.*, 2009, 2011; van Beilen *et al.*, 2006).

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