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Purification, crystallization and preliminary X-ray analysis of cytochrome P450 219A1 from *Novosphingobium aromaticivorans* DSM 12444

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Cytochrome P450 enzymes catalyze a variety of reactions and are widely distributed in living organisms. In recent studies, the first members of five new families of cytochrome P450 enzymes have been identified, including cytochrome P450 219A1 (CYP219A1) from *Novosphingobium aromaticivorans* DSM 12444. It has also been reported that isolongifolen-9-one ($C_{15}H_{22}O$), a sesquiterpenoid ketone derivative, is a potential substrate for CYP219A1, inducing a $\geq$95% shift of the haem spin state to high spin upon binding. The CYP219A1 protein has been crystallized and single crystals have been studied by X-ray crystallography. Diffraction data were collected to 2.4 Å resolution. The crystals belonged to space group *P*6, with unit-cell parameters a = 93.1, b = 93.1, c = 98.0 Å. Preliminary X-ray diffraction data analysis revealed that the asymmetric unit contained one protein molecule.

1. Introduction

Cytochrome P450 (CYP) enzymes are versatile biocatalysts that are widely distributed in living organisms. They contain a haem group and display a characteristic absorption peak at 450 nm for the Fe^{2+}(CO) form of the enzyme (Omura & Sato, 1964). P450 enzymes are involved in the biosynthesis of endogenous compounds and they also metabolize a wide variety of xenobiotics, including polycyclic aromatic hydrocarbons (PAHs) and pesticides (Ortiz de Montellano, 2005). The primary function of P450 enzymes is C—H bond oxidation, in which the hydrocarbon substrate interacts with oxygen to form a hydroxylated metabolite and water (reviewed in Lewis & Pratt, 1998). However, other activities such as epoxidation, heteroatom oxygenation, dealkylation, desaturation and even dehalogenation have been found (Isin & Guengerich, 2007). P450 enzymes are external monooxygenases that require an external reductant, in the majority of cases NAD(P)H, to initiate their diverse functions (Hannemann *et al.*, 2007).

The oligotrophic organism *Novosphingobium aromaticivorans* degrades a broad range of aromatic compounds, including polycyclic aromatic hydrocarbons (Fredrickson *et al.*, 1995). *N. aromaticivorans* has also been implicated in initiating primary biliary cirrhosis (Selmi & Gershwin, 2004). In 2006, the 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 and many oxygenase genes (Romine *et al.*, 1999; http://genome.jgi-psf.org/finished_microbes/novar/novar.home.html). 15 potential P450 (*CYP*) genes from ten different P450 bacterial families were detected by the genomic analysis. Of these, ten belonged to existing CYP families, but the first members of five new families were identified: *CYP204A1*, *CYP219A1*, *CYP223A1*, *CYP224A1* and *CYP225A1*. The cloning and production of 12 of the P450 enzymes in the CYPome of *N. aromaticivorans* DSM 12444 have been reported and potential substrates for some have been identified (Bell & Wong, 2007). CYP219A1 was reported to bind and oxidize testosterone (Agematu *et al.*, 2006), even though the compound did not induce a haem spin-state shift when assayed by the classical type I spectrum. On the other hand, isolongifolen-9-one ($C_{15}H_{22}O$, a sesquiterpenoid ketone derivative) was found to induce a $\geq$95% spin-

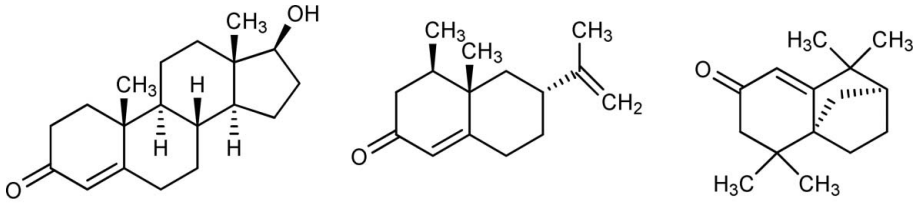

Figure 1
Substrates tested with CYP219A1.

state shift (Bell & Wong, 2007), strongly indicating that it is a potential substrate of CYP219A1 (Figs. 1 and 2; unpublished data). Interestingly, other closely related sesquiterpenoid ketones such as nootkatone were not found to induce a spin-state shift in CYP219A1.

The sequence of the *CYP219A1* gene (Gene ID 3916770, Saro_2451) reveals that the mature protein has 414 residues and that its molecular weight is 46 kDa. In this paper, we report the crystallization of the CYP219A1 protein. As structural information on cytochrome P450s has increased greatly in recent years (see review by Denisov *et al.*, 2005), the three-dimensional structure of the native enzyme, as well as that in complexes with substrates such as isolongifolene-9-one and testosterone, will help to elucidate the substrate specificity and biological function of the enzyme.

2. Materials and methods

2.1. Cloning, expression and purification

The complete *CYP219A1* gene was excised from the plasmid pET26-CYP219A1 (Bell & Wong, 2007) and subcloned into the vector pET28a(+) (Novagen Inc.) using the *Nde*I and *Hin*dIII restriction sites. The new construct adds an N-terminal His tag with sequence MGSSHHHHHHSSGLVPRGSH to the target protein for Ni^{2+}-chelating affinity chromatography. 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 (isopropyl *β*-D-1-thiogalactopyranoside) was added in order to induce the production of recombinant protein. After further growth for 8 h at 301 K, 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 to remove the cell debris.

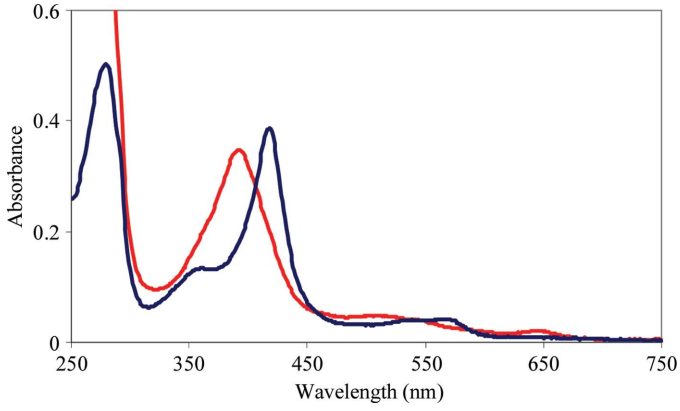

Figure 2
CYP219A1 spin-state shift with (−)-isolongifolen-9-one (blue, no substrate; red, with excess substrate).

Table 1
Data-collection and processing statistics for CYP219A1.

Values in parentheses are for the highest resolution shell.

Space group	*P*6
Unit-cell parameters (Å)	a = 93.1, b = 93.1, c = 98.07
Wavelength (Å)	1.5418
Resolution range (Å)	50.0–2.40 (2.49–2.40)
Total reflections	145011
Unique reflections	19075
Redundancy	7.6 (7.6)
Average $I/\sigma(I)$	39.5 (4.6)
R_{merge}† (%)	6.7 (49.1)
Data completeness (%)	99.6 (100)
Molecules per ASU	1
V_M (Å^3 Da^{-1})	2.72
Solvent content (%)	54.88

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

The protein supernatant solution was loaded onto an Ni^{2+}-chelating affinity column (1.0 ml Ni^{2+}–NTA agarose), which was then washed in two steps with buffer *P* containing 10 m*M* and then 20 m*M* imidazole. The desired protein was eluted with buffer *P* containing 200 m*M* imidazole and then buffer-exchanged to buffer *A* (20 m*M* HEPES, 10 m*M* *β*-mercaptoethanol) by ultrafiltration. The protein solution in buffer *A* was concentrated to 500 µl and injected onto a Resource Q column (Amersham-Pharmacia Biotech), which was then eluted with a gradient of 0–1 *M* NaCl in buffer *A*. The CYP219A1 protein was eluted at ∼190 m*M* NaCl.

2.2. Crystallization

The purified CYP219A1 protein was concentrated to 50 mg ml^{-1} in crystallization buffer (20 m*M* HEPES, 200 m*M* KCl, 10 m*M* *β*-mercaptoethanol pH 7.4). The hanging-drop vapour-diffusion method was used with Crystal Screens I and II (Hampton Research) to screen

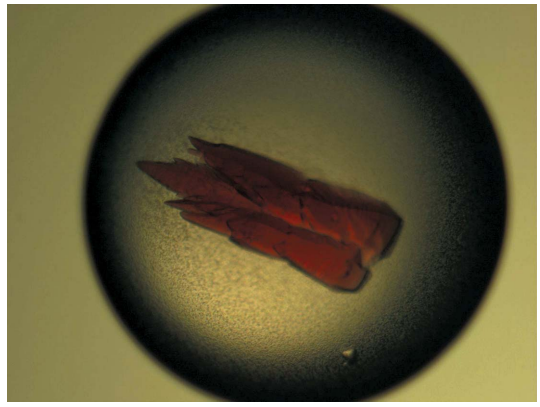

Figure 3
Crystals of cytochrome P450 219A1.

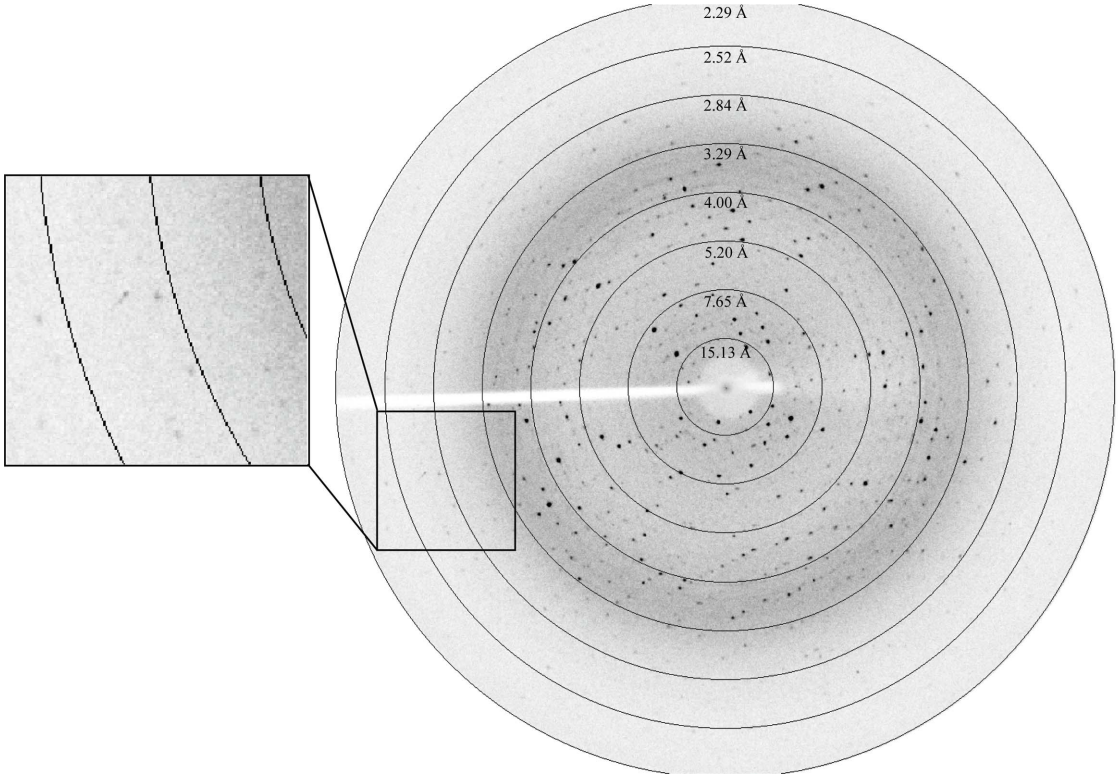

Figure 4
A typical diffraction pattern of the cytochrome P450 219A1 crystal. An enlarged image is shown on the left.

for CYP219A1 crystals at 291 K in 16-well plates. 1 µl protein solution (50 mg ml^{-1}) was mixed with 1 µl reservoir solution and equilibrated against 200 µl reservoir solution. Several crystals were obtained from Crystal Screen I condition No. 15 (0.2 *M* ammonium acetate, 0.1 *M* sodium cacodylate pH 6.5, 30% PEG 8000), but none of them were suitable for X-ray diffraction analysis. Conditions were then optimized by varying the precipitant concentration and buffer pH of Crystal Screen I condition No. 15. After 8 d, single crystals that gave good diffraction were grown under the optimized conditions of 0.2 *M* ammonium acetate, 0.1 *M* sodium cacodylate pH 6.3, 28% PEG 8000 (Fig. 3).

2.3. Crystallographic data collection and processing

Before data collection, crystals were soaked in a cryoprotectant solution consisting of 0.2 *M* ammonium acetate, 0.1 *M* sodium cacodylate pH 6.3, 28% PEG 8000, 20%(*v*/*v*) glycerol. X-ray diffraction data were collected from flash-cooled CYP219A1 crystals (at 100 K in a stream of nitrogen gas) on a MAR 345 image plate (MAR Research, Hamburg) using an in-house Rigaku MicroMax-007 rotating-anode X-ray generator (Fig. 4) operating at 40 kV and 20 mA. The intensity set was indexed, integrated and scaled with the *HKL*-2000 package (Otwinowski & Minor, 1997). Data-collection results are summarized in Table 1.

3. Results and discussion

The complete *CYP219A1* gene was subcloned into the pET28a(+) vector and the protein was produced in *E. coli* strain BL21 (DE3). The His tag in the new vector enabled purification by metal-affinity chromatography and anion-exchange chromatography. Crystal Screens I and II were used for preliminary screening and several crystals were obtained. After optimization, single crystals were obtained that showed good diffraction using the in-house X-ray source. The diffraction data collected extended to 2.4 Å resolution.

The CYP219A1 crystal belonged to space group *P*6, with unit-cell parameters a = 93.1, b = 93.1, c = 98.0 Å. It was estimated that the asymmetric unit contained one protein molecule with 55% solvent content by Matthews coefficient analysis (Matthews, 1968). We attempted to use cytochrome P450 pikC (PDB code 2bvj; 34% identity; Sherman *et al.*, 2006) as a model to solve the phase problem for CYP219A1 by molecular replacement using the program *Phaser* (Read, 2001), but were unsuccessful. We then tried P450 eryf (PDB code 1z8q, chain *A*; Nagano *et al.*, 2005) and CYP199A2 (PDB code 2fr7, chain *A*; Bell *et al.*, 2008) models as well as truncated versions of the three models. Unfortunately, the structure of CYP219A1 could not be solved and further work on solution of the native structure is in progress. Preparation of a selenomethionyl derivative of CYP219A1 is also under way in order to determine the structure by single-wavelength or multiple-wavelength anomalous dispersion using synchrotron radiation. We also intend to investigate the soaking of the potential substrates isolongifolene-9-one and testosterone into the CYP219A1 enzyme in order to examine substrate binding at the structural level. The CYP219A1–substrate complex structures should help to elucidate the detailed molecular mechanism of this enzyme.

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