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The crystal structure of P450-TT heme-domain provides the first structural insights into the versatile class VII P450s

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ABSTRACT

The first crystal structure of a class VII P450, CYP116B46 from *Tepidiphilus thermophilus,* has been solved at 1.9 Å resolution. The structure reveals overall conservation of the P450-fold and a water conduit around the I-helix. Active site residues have been identified and sequence comparisons have been made with other class VII enzymes. A structure similarity search demonstrated that the P450-TT structure is similar to enzymes capable of oxy-functionalization of fatty acids, terpenes, macrolides, steroids and statins. The insight gained from solving this structure will provide a guideline for future engineering and modelling studies on this catalytically promiscuous class of enzymes.

1. Introduction

Cytochrome P450 monoxygenases (P450s or CYPs) are a widespread class of heme-containing enzymes capable of catalyzing a diverse array of oxidation reactions with important roles in biosynthetic pathways and xenobiotic metabolism [1]. Since these reactions often occur with high regio- and stereoselectivity, P450s have recently been targeted for synthetic applications [2]. The most common and synthetically attractive reaction catalyzed by these enzymes is the monooxygenation of unactivated C-H bonds under mild conditions using molecular oxygen as the sole oxidant. In order to carry out this reaction, two NAD(P)H-derived electrons are shuttled by redox partners for reductive activation of dioxygen. Two protonation steps are necessary to generate the ferryl-oxo compound I which represents the catalyst for oxygen insertion [3].

Cytochrome P450s can be classified according to the electron transport system exploited to complete their catalytic cycle [4]. Most of these P450 systems require separate redox proteins to function. However, P450 classes have been discovered in which the redox partners are fused to the heme domain of the enzyme, creating multicomponent, self-sufficient systems. These are attractive biocatalysts, as the need for identification and expression of separate redox partners is obviated.

Structural data available for P450 enzymes led to the conclusion that an overall-P450 fold is conserved and large conformational rearrangements are sometimes observed upon ligand binding [5]. Even though structural-based standard numbering systems have certainly improved the quality of P450 sequence alignments [6], the process of accurately converting sequence information into a 3D structure is complicated by the low sequence identity among P450s belonging to different families (10–30%) and by the diversity within the active site [7].

Class VII P450s represent a unique class of fused enzymes in which electrons are transferred by a phthalate family oxygenase (PFOR)-like reductase domain containing a FMN and a 2Fe–2S cluster [8]. These self-sufficient enzymes have recently received attention for their remarkable substrate promiscuity, with further efforts made to engineer them through directed evolution for higher dealkylation activity, to improve the catalytic performance in the hydroxylation of tetralin derivatives or to carry out anti-Markovnikov oxidation of styrenes [9–11]. Moreover, benefitting from the rapid expansion of available CYP sequences, we have recently reported the discovery of a panel of new class VII P450s

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with desirable biocatalytic properties such as good expression levels, thermal stability and diverse substrate scope [12,13]. In particular, CYP116B46 from *T. thermophilus* displayed not only better expression levels but also enhanced thermal stability when compared to the previously characterized CYP116B2 (P450-RhF).

Since the discovery of the first class VII P450-RhF in 2002, several more have been reported and thoroughly characterized biochemically [14–19]. However, as yet no physiological role has been determined for these enzymes with the exception of only CYP116B5 [18]. Moreover, X-ray crystal structures for the heme domain of these proteins are not available to date. In order to provide a link between the sequence and the 3D structure of this valuable class of enzymes, we have solved the first X-ray structure of the heme domain of a class VII P450. As thermally stable enzymes tend to generate protein crystals better suited for X-ray diffraction [20], we focused on CYP116B46 (UniProt A0A0K6ITW2) to get a first insight into the structure of this class of self-sufficient enzymes.

2. Material and methods

For full experimental details please refer to the Supporting Information.

2.1. Protein expression and purification

Protein expression was carried out as reported previously [12]. For protein purification, cells were resuspended in 50 mM HEPES buffer, 300 mM NaCl, 20 mM imidazole, pH 8.0 (buffer A) to a wet cell concentration of 200 mg/mL. After ultrasonication and ultracentrifugation, immobilized metal ion affinity chromatography (IMAC) was employed as a first purification step using Ni-agarose resin (Qiagen) in a gravity flow column following the manufacturer instructions. Anion-exchange chromatography was carried out using a 6-ml RESOURCE Q column (GE Healthcare) on a ÄKTA Pure system (GE Healthcare). Size-exclusion chromatography was performed in 50 mM HEPES, 300 mM NaCl, pH 8 on a ÄKTA Pure system equipped with a HiLoad 16/600 Superdex 200 pg column. Enzyme elution was monitored at 280 nm and red fractions displaying at least 50% maximal absorbance of the main peak at 280 nm were analyzed by SDS-PAGE (Fig. S1), collected, concentrated and desalted in 50 mM HEPES pH 8 using a PD-10 column. Prior to crystallization trials, protein solutions were concentrated to 20 mg/mL, as determined by NanoDrop (Thermo Scientific), using $\varepsilon_{280} = 67755\ M^{-1}\ cm^{-1}$ and stored overnight at 4 °C.

2.2. Crystallization, data collection and structure determination

Crystals of CYP116B46 (P450-TT) were obtained by mixing 200 nL of protein with 200 nL of condition A12 (0.1 M MES 6.5 22% v/v PEG Smear Broad) from the BCS screen (Molecular Dimensions Ltd., Newmarket, UK). Drops were incubated at 4 °C for 24 h prior to inspection. Single crystals suitable for data collection were cryo protected with the addition of 15% Peg 200 prior to plunge cooling in liquid nitrogen.

Data were collected from a single cryo protected crystal of P450-TT at beamline i04-1 (Diamond Light Source). All data were indexed, scaled and subsequently integrated with Xia2. Structure determination was initially performed by molecular replacement in Phaser using a search model derived from the previously solved cytochrome P450 structure (5GWE). A combination of automated and manual rebuilding and refinement in Phenix and COOT were used to produce a complete model. Validation with both Molprobity and PDB_REDO were integrated into the iterative rebuild process. Complete data collection and refinement statistics are available in Table S1. The atomic coordinates and structure factors of P450-TT structure have been deposited in the RCSB Protein Data Bank under the accession code 6GII.

2.3. Sequence analysis

Sequence alignments were performed with ClustalW [21]. The output files were then used to run ESPrint [22] (for the characterized class VII P450s). Conservation analyses were carried out using the 3DM™ system (subfamily K9GPR5, https://www.bio-prodict.nl/) [23]. The 3DM™ system provides multiple sequence alignments of protein superfamilies based on multiple structure alignments, thereby generating a 3D numbering scheme (3DM standard numbering). When a 3DM standard numbering could not be assigned, class VII sequences were aligned using the CYPED database [24]. Amino acid composition of characterized class VII P450 heme domains were computed starting from ClustalW alignments using MEGA7 [25].

2.4. Structural analysis

Structures were visualized and figures generated with PyMOL (the PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC). The CAVER plug-in in PyMOL [26] was employed using standard parameters and a probe radius of 1.4 Å to identify paths leading to the buried active site. Structural superposition were performed in COOT [27] and the output files used to generate structure-based sequence alignments in Chimera (Match- > Align, residue-residue cutoff distance of 5 Å).

3. Results and discussion

3.1. Overall structure

The substrate-free crystal structure of CYP116B46 (P450-TT) was determined at 1.9 Å resolution from residue 28 to 443 by molecular replacement using the recently solved structure of CYP288A2 (PDB 5GWE) as search model [28].

Overall, P450-TT adopts the triangular P450 fold composed of 19 α-helices and 10 β-strands divided into 3 sheets embedding the heme (Fig. 1A, the structural nomenclature of Hasemann et al. was adopted where possible and secondary structural elements annotated with ESPrint) [29]. Close inspection of the protein surface does not reveal an open tunnel leading to the cofactor. Analysis of the structure using the CAVER 3.0 tool enabled identification of a putative tunnel ~14 Å long and with a bottleneck of ~1.7 Å (Fig. 1B). This tunnel is mainly lined by hydrophobic residues (Table S2) but its opening appears too small to accommodate organic molecules (for comparison, methane radius is 1.9 Å) [30]. Therefore, opening motions might be needed to accommodate the diverse range of substrates accepted by this class VII P450 [12].

The heme-iron is hexacoordinated, with Cys385 acting as the proximal ligand and water as the sixth ligand (Fig. 1C). The proximal ligand is part of the so-called “cysteine-pocket”, which includes Phe378, Gly379, Tyr380, Gly381, Gln384, Leu386, Gly387 and Arg388. The K-helix bears the sequence ^{307}IPXAXEECLR^{316} in which we can recognize the well described EXXR motif followed by the conserved Arg369 in the so-called “meander” (the loop between K′-helix and the cysteine-pocket) [29].

The heme propionates are either coordinated to the protein chain directly through charged residues or indirectly through water, allowing the insertion of the heme cofactor in the interior of the molecule (Fig. 1D). In particular, the A-ring propionate interacts with N^{ε} of His125, and Arg129 side chain, while the D-ring propionate hydrogen bonds with the main chain amide group of Tyr380,

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