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The role of Ile87 of CYP158A2 in oxidative coupling reaction

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ABSTRACT

Both CYP158A1 and CYP158A2 are able to catalyze an oxidative C–C coupling reaction producing biflaviolin or triflaviolin in *Streptomyces coelicolor* A3(2). The substrate-bound crystal structures of CYP158A2 and CYP158A1 reveal that the side chain of Ile87 in CYP158A2 points to the active site contacting the distal flaviolin molecule, however, the bulkier side chain of Lys90 in CYP158A1 (corresponding to Ile87 in CYP158A2) is toward the distal surface of the protein. These results suggest that these residues could be important in determining product regiospecificity. In order to explore the role of the two residues in catalysis, the reciprocal mutants, Ile87Lys and Lys90Ile, of CYP158A2 and CYP158A1, respectively, were generated and characterized. The mutant Ile87Lys enzyme forms two isomers of biflaviolin instead of three isomers of biflaviolin in wild-type CYP158A2. CYP158A1 containing the substitution of lysine with isoleucine has the same catalytic activity compared with the wild-type CYP158A1. The crystal structure of Ile87Lys showed that the BC loop in the mutant is in a very different orientation compared with the BC loop in both CYP158A1/A2 structures. These results shed light on the mechanism of the oxidative coupling reaction catalyzed by cytochrome P450.

Introduction

The cytochrome P450 (P450 or CYP)[1] enzymes belong to a superfamily of heme-containing monooxygenases responsible for biosynthesis of physiologically important compounds and metabolism of drugs and other toxic compounds [1–3]. P450s are distributed in all biological kingdoms from viruses, bacteria, fungi, and plants to mammals, including humans. More than 15,000 P450 genes have been identified in this superfamily (http://drnelson.uthsc.edu/cytochromeP450.html). However, for the majority of these P450s the biological function and endogenous substrates are unknown. There are 18 *CYP* genes in the model actinomycete *Streptomyces coelicolor* A3(2) [4,5]. CYP158A2 is a member of a highly conserved operon and catalyze phenolic oxidative C–C coupling reactions of flaviolin to biflaviolin and triflaviolin [6,7]. The flaviolin polymers are thought to provide physical protection for this soil bacterium against the deleterious effects of UV irradiation on genetic integrity [8,9]. CYP158A1 can only produce 3,3′-biflaviolin and 3,8′-biflaviolin with quite different molar ratios compared with the products from CYP158A2, which produces three isomers of biflaviolin and one triflaviolin [6]. The substrate flaviolin-bound crystal structures of CYP158A1 and CYP158A2 reveal that two molecules of flaviolin can bind to the enzymes at the same time. Both the proximal flaviolin molecules in CYP158A1/A2 are near the heme iron, however, the distal flaviolin molecules are bound in very different locations in the CYP158A1/A2 structures. In CYP158A1, the distal flaviolin is at the entrance of the substrate access channel and 9 Å away from the proximal flaviolin. In CYP158A2, the two flaviolin molecules are about 4 Å apart from each other, which could make positional sense for a coupling reaction [6]. It is unknown how these two enzymes catalyze the oxidation of the same substrate (flaviolin) while controlling stereo- and regiospecificity. Furthermore, crystal structures of CYP158A1/A2 suggest that several key residues in the active sites associated with flaviolins are different. The most striking difference is that the side chain of Ile87 points toward the active site and contacts the distal flaviolin molecule in CYP158A2, while the bulkier side chain of Lys90 in CYP158A1 (corresponding to Ile87 in CYP158A2) is orientated away from the BC loop, toward the distal surface of the protein. The sequence alignment among the CYP158 family confirmed the above differences. Ile87 in CYP158A2 is highly conserved in all other CYP158 family members (Fig. 1). In order to explore the structural basis of stereo- and regiospecificity of phenolic coupling reactions of CYP158A1/A2, we characterized mutants Ile87Lys and Lys90Ile of CYP158A2 and CYP158A1, respectively and determined the crystal structure of the Ile87Lys mutant of CYP158A2.

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[1] *Abbreviations used:* P450 or CYP, cytochrome P450; PCR, polymerase chain reaction; LC/MSMS, Liquid Chromatography–mass spectrometry; SER-CAT, Southeast Regional Collaborative Access Team.

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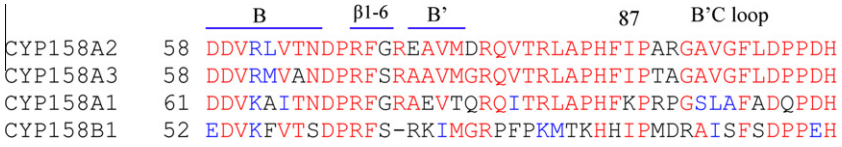

Fig. 1. B-helix and BC loop alignment of the four members of the CYP158 family with CYP158A2 from *Streptomyces coelicolor* A3(2). The alignment shows the variant amino acids in the BC loop secondary structural elements. Ile87 in CYP158A2 is conserved in CYP158A3 from *Streptomyces avermitilis* and CYP158B1 from *Saccharopolyspora erythraea*, but not in CYP158A1.

Herein, we show that the Ile87Lys mutant in CYP158A2 significantly changes the ratios of the dimerization products converting CYP158A2 into a CYP158A1-like monooxygenase. However, when the corresponding Lys90 in CYP158A1 is mutated to isoleucine as found in CYP158A2, the catalytic activity of Lys90Ile does not change at all compared with the wild type CYP158A1. The crystal structure of Ile87Lys suggests that the overall BC loop topology of the mutant is very different from the BC loop in both wild type CYP158A1/A2 structures. Our results indicate that the overall active site environments and multiple residues contribute to the regiospecificity during catalysis and together shed light on the mechanism of the unusual P450-catalyzed C–C bond formation.

Experimental procedures

Site-directed mutagenesis of the CYP158A1 and CYP158A2 genes

The CYP158A1/A2 proteins were genetically engineered to contain a His4-tag at the carboxy-terminus using a polymerase chain reaction (PCR)-based strategy as described previously [6,7]. The QuikChange mutagenesis kit (Stratagene) was used for construction of site-directed mutants. The primers were: CYP158A2 Ile87Lys forward: 5′-CGCCCCCCACTTCAAGCCTGCCCGCGGCG-3′; CYP158A2 Ile87Lys reverse: 5′-CGCCGCGGGCAGGCTTGAAGTGGGGGGCG-3′; CYP158A1 Lys90Ile forward: 5′-CGCCCCGCACTTCATCCCGCGGCCCGGCTCGC-3′; CYP158A1 Lys90Ile reverse: 5′-GCGAGCCGGGCCGCGGGATGAAGTGCGGGGCG-3′. The integrity of the mutant sequences was confirmed by DNA sequencing.

Expression and purification of mutants of CYP158A1/CYP158A2

The expression and purification of the mutants were performed as described previously [7]. Recombinant proteins were produced in *Escherichia coli* BL21 (DE3) pLysS competent cells. Briefly, the cells were cultured in LB broth containing 50 μg/ml ampicillin overnight. The transformed *E. coli* inoculated (1:100) in 1 l of Terrific Broth containing 100 μg/ml ampicillin were grown at 37 °C and 240 rpm. After induction with 1 mM isopropyl-β-D-thiogalactopyranoside and the addition of 1 mM δ-aminolevulinic acid, growth was continued for an additional 20 h at 27 °C and 190 rpm. The cells were harvested by centrifugation and resuspended in 100 ml of lysis buffer. Cells were broken by freeze-thawing and the cytosol isolated following centrifugation at 100,000*g*. The soluble mutants were purified by metal (Ni^{2+}) affinity chromatography (Qiagene) followed by S-Sepharose/Q-Sepharose (Amersham Bioscience) chromatography [7].

Spectral substrate binding and activity assays

Spectral and catalytic activity assays were carried out as reported previously for CYP158A2 using flaviolin as a substrate [7]. Absorbance difference spectra were recorded using a double beam Shimadzu UV-2401PC spectrophotometer. The interaction of flaviolin with mutants was examined by perturbation of the heme Soret spectrum. Ile87Lys and/or Lys90Ile were divided between two tandem cuvettes. Variant concentrations of flaviolin in 5% methanol were added to the sample cuvette to give a final ligand concentration in the range of 1–150 μM. An equal volume of 5% methanol was added to the reference cuvette, and the difference spectrum recorded after each titration. The Hill coefficient was obtained from a plot of $\log_{10}(\Delta A_{(382-418)}/[\Delta A_{max} - \Delta A_{(382-418)}])$ vs. $\log_{10}$[flaviolin]. K_d values were estimated by fitting plots of $\Delta A_{382-418}$ vs. [flaviolin]. For activity assays, Ile87Lys and/or Lys90Ile were reconstituted in 400 μl of 20 mM Tris–HCl buffer (pH 8.2) and flaviolin (0.26 μmol). Following incubation of the reaction mixture for 5 min on ice, the reconstituted enzyme solution was placed in a shaking water bath at 37 °C. The reaction was initiated by the addition of NADPH to a final concentration of 1 mM and was carried out for 2 h in a 1.5-ml test tube, at which time the reaction was terminated by addition of 4 μl of concentrated HCl. Subsequently the mixtures were extracted with ethyl acetate. The product formation was analyzed by LC/MSMS (Liquid Chromatography–mass spectrometry) [7]. UV detection was measured at 254 nm. The kinetic parameters were calculated by a nonlinear regression fit to the Michaelis–Menten equation using the GraphPad Prism software (GraphPad Software Inc., San Diego, CA).

Crystallization and data collection

Crystals of Ile87Lys of CYP158A2 were obtained using hanging-drop vapor diffusion, in which 2 μl of a 20 mg/ml protein solution was mixed with an equal volume of 0.1 M bis–Tris (pH 6.5), 1.2 M ammonium dihydrogen phosphate. At 20 °C, the ligand-free crystals appeared within a few days; to attempt to generate the substrate flaviolin-bound crystals, flaviolin (1 mM, containing 2% (v/v) methanol) was mixed with Ile87Lys protein solution before crystallization. The crystals belong to the monoclinic space group *P*2(1) (Table 1). Full diffraction data were collected at 100 K at the Southeast Regional Collaborative Access Team (SER-CAT) 22-BM beamline at the Advanced Photon Source, Argonne National Laboratory, Argonne, IL. The X-ray data were processed and scaled with the HKL package programs HKL2000 [10].

Structure determination

The structure of the CYP158A2 Ile87Lys mutant was determined by molecular replacement using the program PHASER [11] and the substrate-free CYP158A2 structure (Protein Data Bank code: 1SE6) as a search model. The initial model was built in COOT [12] and refinement was performed using CNS1.3 [13]. There were two molecules of Ile87Lys in the asymmetric unit. Final refinement statistics are given in Table 1. The coordinates and associated structure factors have been deposited with the Protein Data Bank (accession codes: 3TZO). Figures were generated by PyMOL [14]. Attempts to generate complexes with flaviolin in the active site of Ile87Lys failed because no electron densities corresponding to flaviolin were over the heme in the structure.