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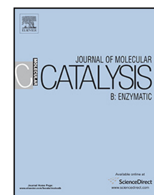

Role of Leu188 in the Fatty Acid Hydroxylase Activity of CYP102A1 from *Bacillus megaterium*

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ABSTRACT

P450 BM3 (CYP102A1) from *Bacillus megaterium* catalyzes the subterminal hydroxylation of fatty acids with 12-22 carbons at the ω-1, ω-2 and ω-3 positions. Several amino acids located at the substrate channel and active sites are known to be important for the catalytic activity of CYP102A1. The L188 residue at the C-terminus of α-helix F undergoes a large shift upon substrate binding and has frequently been found in different combinations of multiple mutations showing enhanced and altered activities. In this study, we examined the role of the L188 residue by comparing the catalytic activities of wild-type CYP102A1 and 19 mutants of L188. The mutants were made with site-directed mutagenesis and functionally expressed in *Escherichia coli*. The enzymatic properties of the mutants for a set of fatty acids (C_{10}-C_{16}) were compared to the properties of the wild-type. L188Q and L188P mutants showed especially strong increases in hydroxylase activity toward C_{10}-C_{13} fatty acids, although they did not have activity changes for C_{14}-C_{16} fatty acids. Although most mutants showed very similar overall hydroxylation rates for myristic acid, 14 mutants showed apparent changes in the regioselectivity of hydroxylation with a preference for the ω-3 position over the ω-1 position. A possible role for the L188 residue has been discussed in the context of the structure and function of CYP102A1.

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1. Introduction

Cytochrome P450 (P450 or CYP) proteins are remarkably diverse oxygenation catalysts that are found throughout all classes of life. They catalyze various oxidative metabolic reactions of endogenous and exogenous compounds [1]. Their catalytic diversity and vast substrate range with regio- and stereospecificity have high potential for many applications, including the synthesis of drug metabolites, fine chemicals, steroids, lipids, and natural products [2–5].

P450 BM3 (CYP102A1) from *Bacillus megaterium* is a self-sufficient monooxygenase because the monooxygenase domain is naturally fused to its redox partner, which is an eukaryotic-like diflavin reductase. Its natural substrates are fatty acids (C_{12} to C_{22}), which are exclusively hydroxylated at the subterminal positions (usually ω-1 to ω-3) [6]. Furthermore, this enzyme exhibits the highest catalytic activity ever detected among P450 s [6]. A number of CYP102A1 natural variants were found in a set of *B. megaterium* strains [7]. Although there were no apparent variations in hydroxylation activity toward myristic acid (C_{14}) and palmitic acid (C_{16}), the oxidation rates of lauric acid (C_{12}) for these variations was in the range of >25-fold. That study showed that diverse mutations occur naturally for CYP102A1. A set of CYP102A1 variants with high regioselectivity for the hydroxylation of the terminal (ω) position of palmitic acid was reported [8]. The variants were made through iterative cycles of random and targeted mutagenesis. The best variants identified included an alteration of additional amino acid residues outside catalytic sites in addition to substitutions at F87 and A328 in proximity to the bound substrate. This result suggests that residues distant from the bound substrate can show strong contributions to the regioselectivity for palmitic acid hydroxylation.

A large set of CYP102A1 mutants were made through directed evolution and rational design to create engineered enzymes possessing the desired catalytic activities for diverse non-natural substrates, including pharmaceuticals, short-chain hydrocarbons, and environmental chemicals [2,4,9,10]. The potential of engineered CYP102A1 for biotechnological applications such as synthetic work has been recognized [5]. Therefore, it has been

Abbreviation: δ-ALA, δ-aminolevulinic acid; BSTFA, *N,O*-bis(trimethylsilyl)trifluoroacetamide; CPR, NADPH-P450 reductase; CYP102A1, Cytochrome P450 BM3; IPTG, isopropyl-β-D-thiogalactopyranoside; MD, molecular dynamics; P450 or CYP, cytochrome P450; PCR, polymerase chain reaction; WT, wild-type.

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suggested that CYP102A1 may be used as a prototype P450 for industrial applications to produce large quantities of drug metabolites and fine chemicals [2,4,14].

CYP102A1 has several advantages as a biocatalyst because the rich information on the structures and functions of the protein is available. Crystal structures of the heme domain of WT and mutants of CYP102A1 can provide a basis for the rational design of desired activities. CYP102A1 does not need an extra reductase because the monooxygenase domain is naturally fused to its redox partner [12]. However, a complete crystal structure of the whole protein containing both the monooxygenase and reductase domains is not yet available. The capacity of CYP102A1 as a catalyst was demonstrated for the production of important chemicals, including pharmaceuticals, drug metabolites, steroids, and antibiotics [11,13]. However, practical applications of CYP102A1 in several biotechnological fields are still limited due to several technical bottlenecks such as low activity, uncoupling, NADPH limitations, and low substrate solubility [11].

During studies of site-directed mutagenesis based on the 3D structure of CYP102A1, several mutations at the substrate channel and active sites were observed to affect the catalytic activities of CYP102A1 toward fatty acids and non-natural substrates. Several combinational mutants with increased activities were found to include the amino acid residue of Leucine 188 (L188) [2,14], which is located at the C-terminus of α-helix F and around the entrance of the hydrophobic substrate-channel [15]. The F helix and adjacent G helix undergo a large shift upon substrate binding [16]. However, the role of the L188 residue itself for the catalytic activity of CYP102A1 toward fatty acids has yet to be reported.

The crystal structure of the CYP102A1 heme domain has been solved for the substrate-free form [17]. The structure shows that L188 is an amino acid of the hydrophobic pocket of the substrate access channel [17,18]. Because soaking the crystals in solutions containing arachidonate causes a conformational change, the access channel may undergo a large opening/closing motion [18]. The F/G loop and β-sheet regions, which have a close connection with the substrate access channel, move closer together when a substrate binds, which results in a closure of the substrate access channel [18]. Because L188 is located at the C-terminus of α-helix F, which forms a lid covering the active site together with helix G [17], the F helix and the adjacent G helices undergo a large shift upon substrate binding [17]. These results suggest that L188 is one of the important residues in the substrate access channel for catalytic activity. Recently, MD simulation suggested that the L188 is involved in hydrogen-bonding interactions, with the substrate at the entrance of the active sites [19]. The interactions may induce a huge conformational rearrangement and involve the determination of regio- and stereoselectivity.

In this study, the role of the L188 residue was examined by a comparative study of 20 different amino acids. Nineteen mutants of L188 were made by site-directed mutagenesis and functionally expressed in *Escherichia coli* to compare fatty acid hydroxylase activity to wild-type (WT) CYP102A1. All of the mutants showed apparent catalytic activity toward typical CYP102A1 substrates, which are fatty acids. The role of L188 in the catalytic activity and conformation of CYP102A1 will be discussed.

2. Materials and Methods

2.1. Materials

Isopropyl-β-D-thiogalactopyranoside (IPTG), glucose-6-phosphate, glucose-6-phosphate dehydrogenase, δ-aminolevulinic acid (δ-ALA), reduced β-nicotinamide adenine dinucleotide phosphate (NADPH), fatty acids, and *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) were obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Methods

2.2.1. Construction, protein expression and purification

Nineteen mutants of L188 residue were created with the polymerase chain reaction (PCR) using Stratagene's QuikChange XL site-directed mutagenesis kit with a template *pCW*BM3 WT plasmid [7]. The forward mutagenic oligonucleotides for each mutant are shown (Supplemental Table S1). All of the CYP102A1 L188 mutants generated in this study were verified by DNA sequencing at Genotech (Daejeon, Republic of Korea) to confirm the presence of the intended mutations and were transformed into *E. coli* strain DH5αF'-IQ using standard procedures. WT and L188 mutants were expressed in *E. coli* and purified as described previously [20]. The P450 concentration was determined by Fe^{2+}-CO versus Fe^{2+} difference spectra [21].

2.2.2. Fatty acid hydroxylation analysis

Typical steady-state reactions for the oxidation of fatty acid were carried out in 0.5 ml of 100 mM potassium phosphate buffer (pH 7.4) for 20 min at 37 °C [22]. The reaction mixtures contained WT or mutant enzymes (0.1 μM), substrate (0.5-2.0 mM), and an NADPH regeneration system (10 mM glucose-6-phosphate, 0.5 mM $NADP^{+}$, and 1 IU/ml yeast glucose-6-phosphate dehydrogenase). The reaction was stopped with 50 μl of 20% trichloroacetic acid and extracted with 1 ml of ice-cold dichloromethane (containing 0.1 mM internal standard). After centrifugation of the reaction mixture, the organic solvent was removed under nitrogen gas and the residue was dissolved in *N,O*-bis(trimethylsilyl) trifluoroacetamide (70 μl) containing trimethylchlorosilane (1%, v/v). The solution was transferred to a glass vial and incubated at 75 °C for 20 min to yield trimethylsilylated products. A gas chromatograph analysis was carried out on a Shimadzu QP2010 using a Rtx®-5 column (column length, 30 m; internal diameter, 0.25 mm; film thickness, 0.1 μm) (Shimadzu Corporation, Tokyo, Japan), with electron-impact ionization. The derivatized samples were separated with a GC oven temperature program of 70 °C for 1 min, followed by an increase to 170 °C (25 °C/min), 200 °C (5 °C/min), and then to 280 °C (20 °C/min). The oven was finally held at 280 °C for 5 min. The injection volume was 3 μl. The MS source and interface were maintained at 250 and 280 °C, respectively, and a solvent delay for 4 min was used. The mass spectra were collected using electron ionization at 70 eV.

The products were identified by their characteristic mass fragmentation patterns as described previously [14,22]. The turnover numbers of the hydroxylation of fatty acids by L188 mutants were determined by a GC-FID detector (Shimadzu GC2010 with FID detector). The same approximate procedure was used for the regioselectivity of the hydroxylated products of fatty acid oxidation. The distribution of products was based on the relative peak area of the chromatogram of GC and mass fragmentation patterns using hydroxylated products at the ω position as standards.

2.2.3. NADPH oxidation

NADPH consumption (ε_{340} = 6,220 M^{-1} cm^{-1}) was monitored by measuring absorbance at 340 nm during the initial 2 min of each reaction. The reaction mixtures contained 1.0 mM fatty acid and P450 enzyme (0.1 μM) in a 1 ml volume of 100 mM potassium phosphate (pH 7.4). The reactions were initiated with the addition of NADPH to a final concentration of 200 μM [23]. The rates of absorbance changes at 340 nm were converted into activity units (moles of NADPH oxidized per minute per mole of enzyme) [24].

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