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Regioselective Oxidation of Lauric Acid by CYP119, an Orphan Cytochrome P450 from *Sulfolobus acidocaldarius*

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Archaebacteria *Sulfolobus acidocaldarius* contains the highly thermophilic cytochrome P450 enzyme (CYP119). CYP119 possesses stable enzymatic activity at up to 85°C. However, this enzyme is still considered as an orphan P450 without known physiological function with endogenous or xenobiotic substrates. We characterized the regioselectivity of lauric acid by CYP119 using the auxiliary redox partner proteins putidaredoxin (Pd) and putidaredoxin reductase (PdR). Purified CYP119 protein showed a tight binding affinity to lauric acid (K_d=1.1±0.1 μM) and dominantly hydroxylated (ω-1) position of lauric acid. We determined the steady-state kinetic parameters; k_{cat} was 10.8 min^{-1} and K_m was 12 μM. The increased ratio to ω-hydroxylated production of lauric acid catalyzed by CYP119 was observed with increase in the reaction temperature. These studies suggested that the regioselectivity of CYP119 provide the critical clue for the physiological enzyme function in this thermophilic archaebacteria. In addition, regioselectivity control of CYP119 without altering its thermostability can lead to the development of novel CYP119-based catalysts through protein engineering.

Keywords: P450, CYP119, lauric acid, oxidation, regioselectivity

Cytochrome P450 (P450) enzymes are found in nearly all life-forms, from bacteria to humans [3]. They are an enzyme superfamily of hemoproteins that catalyze the mixed-function oxidations involved in the metabolism of xenobiotic or endogenous chemicals [1, 9]. Interestingly, a few thermophilic P450 enzymes have been found and are of potential interest from the structural, mechanistic, and biotechnological perspectives [16]. CYP119 is the first and most extensively studied thermophilic P450 enzyme, and the early literature reported its DNA was amplified from the *Sulfolobus solfataricus* genome, but recent report has corrected that this thermophilic P450 enzyme actually had been isolated from the *Sulfolobus acidocaldarius* genome, which is closely related to *Sulfolobus solfataricus* [14, 15, 18]. The 1.5-Å resolution CYP119 structure was determined by multiple wavelength anomalous dispersion [12]. CYP119 contains the typical P450 folds with a relatively compact structure (Fig. 1). Structural analysis indicated that CYP119 undergoes a significant conformational change in the F/G region upon binding of medium to large ligands [12]. The increased salt link networks, strong hydrogen bonds, shortened loops, and optimal aromatic stacking in the structure of CYP119 contribute additively to the overall protein thermostability [12]. The natural substrate of CYP119 and its biological functions are still unknown and therefore it is considered as the so-called orphan P450. Efforts to identify substrates led first to styrene oxidation as a poor substrate, and later the hydroxylation of fatty acids of various chain lengths by the enzyme was conventionally used as a surrogate P450 reaction [7].

Various fatty acids are hydroxylated by mammalian CYP4 family enzymes. Specifically, hydroxylation regioselectivity of CYP4A is of critical physiological importance [6]. These CYP4A enzymes are characterized by their unique ability to hydroxylate the thermodynamically disfavored terminal methyl group of fatty acids in contrast to the other P450 enzymes that hydroxylate fatty acids at internal positions along the alkyl chain [9]. Therefore, these enzymes reflect the evolution of a strategy that predominantly directs the reaction to the terminal carbon. Previous studies postulated a constricted access channel to control ω-hydroxylation and identified the covalently heme-linked residues to be involved in the architecture for this narrow channel [2, 4, 5]. However, the covalent heme binding is always a

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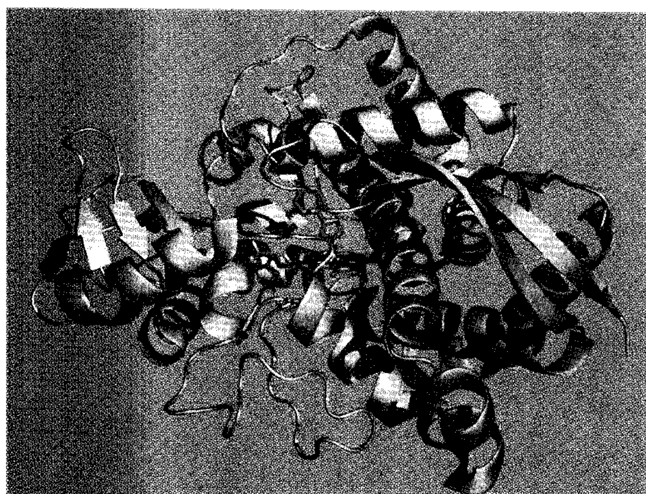

Fig. 1. High-resolution X-ray crystal structure (PDB entry: 1F4T) using the program PYMOL [12].
A ribbon diagram of the CYP119-4–phenylimidazole complex with heme and 4-phenylimidazole shown in the active site.

prerequisite for high ω-hydroxylation regioselectivity. We previously showed that CYP52A21, which does not bind its prosthetic heme covalently, catalyzes the hydroxylation of lauric acid with a 14:1 ω:(ω-1) ratio [6, 10].

In this investigation, we have examined the regioselective oxidation of fatty acid specific for CYP119 enzyme during completion of its P450 catalytic function. The regioselectivity of CYP119 could provide the critical understanding of its physiological role in the thermophilic microorganism. In addition, owing to the powerful catalysis of CYP119 in potential applications, the elucidation of CYP119 regioselectivity may have advantages in terms of industrial production of useful chemicals using metabolic oxidation.

MATERIALS AND METHODS

Chemicals

Lauric acid, sodium dithionite, and NADH were purchased from Sigma (St. Louis, MO, U.S.A.) or Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). IPTG was purchased from Anatrace (Maumee, OH, U.S.A.). [^{14}C] Lauric acid was purchased from Sigma (St. Louis, MO, U.S.A.). Other chemicals were of the highest grade commercially available.

Enzymes

Recombinant CYP119 enzyme was purified as previously described with some modifications [8]. Purified putidaredoxin (Pd), putidaredoxin reductase (PdR), and rat P450 4A1 expressed in *E. coli* were kindly provided by Professor P. R. Ortiz de Montellano (UCSF, CA, U.S.A.).

Lauric Acid Hydroxylation Assays

Lauric acid hydroxylation by CYP119 was carried out using CYP119/Pd/PdR reconstitution systems. The reaction mixtures consisted of 0.5 μM P450, 2.5 μM Pd, and 0.5 μM PdR in 100 μl of 50 mM ammonium acetate buffer (pH 4.5), along with a specified concentration of 1:5 mixture of the ^{14}C-labeled to unlabeled lauric acid (12.5–250 μM). An NADH was used to start the reactions. Incubations were generally done for 30 min at 24, 37, 60, or 80°C and terminated with addition of 150 μl of 6% acetic acid in acetonitrile followed by vortex mixing and centrifugation.

HPLC Analysis

Analyses were performed on a reverse-phase HPLC column (Alltech Econosil, C18, 3.2×100 mm) coupled with a radioisotope detector (Packard Radiomatic Flow-One Model A500). Metabolites were eluted at a flow rate of 0.6 ml/min using linear gradients of acetonitrile:0.1% acetic acid from 40:60 to 95:5 for 30 min.

Spectral Binding Titrations

Purified CYP119 was diluted to 1 μM in 100 mM potassium phosphate buffer (pH 7.4) and divided between two glass cuvettes. Spectra (350–500 nm) were recorded with subsequent additions of substrates (from a methanol stock [19]) using a CARY100 spectrophotometer (Varian, Palo Alto, CA, U.S.A.). The difference in absorbance between the difference wavelength maximum (390 nm) and minimum (420 nm) was plotted against *versus* the substrate concentration [17].

RESULTS

Binding of Lauric Acid to CYP119

Titration of purified CYP119 with lauric acid showed an increase at 390 nm and a decrease at 420 nm (Fig. 2). This is a typical type I substrate binding spectral change, suggesting displacement of the distal iron water ligand upon binding of lauric acid to the protein. The calculated K_d value was 1.1±0.1 μM (Fig. 2).

Regioselective Oxidation of Lauric Acid by CYP119

A reconstituted system containing CYP119, Pd, and PdR catalyzed the oxidation of lauric acid (Fig. 3A). The major

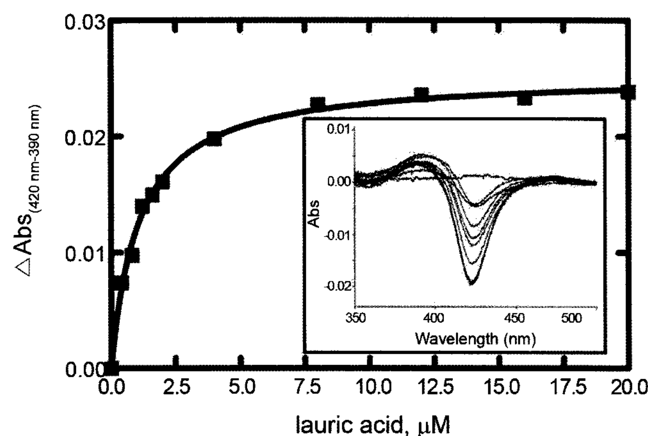

Fig. 2. Binding titration of CYP119 with lauric acid: a plot of $\Delta A_{390-420\ nm}$ versus concentration of lauric acid.
The inset shows titration spectra with increasing concentrations of lauric acid.

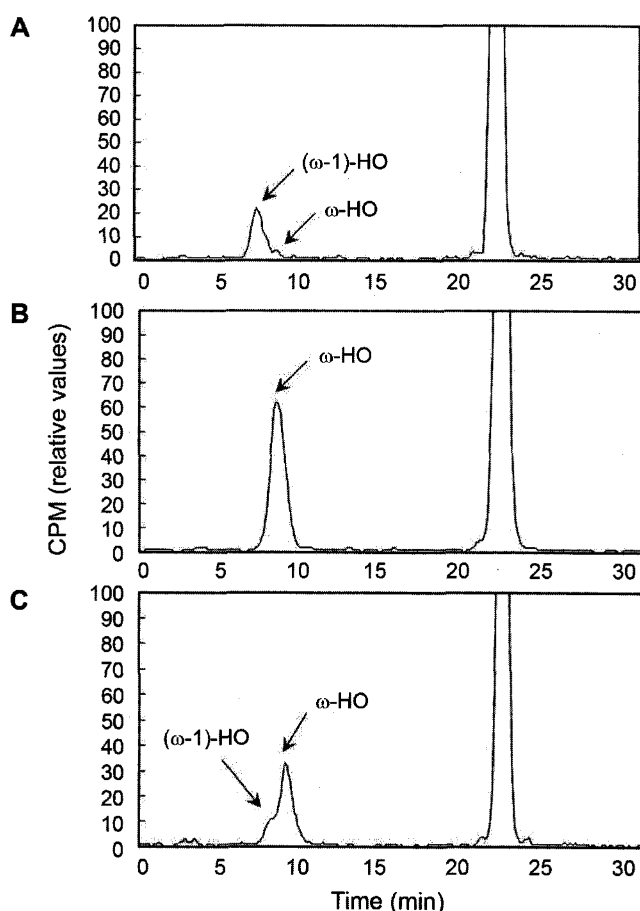

Fig. 3. HPLC profiles of lauric acid hydroxylation.
t_R of (ω-1)-hydroxylation, 8 min; t_R of ω-hydroxylation, 9 min. **A.** Lauric hydroxylation by CYP119. **B.** Lauric acid hydroxylation by P450 4A1. **C.** Co-injection of lauric acid hydroxylation products from CYP119 and P450 4A1.

product of the reaction was (ω-1)-hydroxylated lauric acid (t_R=8 min), although the ω-hydroxylated lauric acid (t_R=9 min) was also obtained as a minor product. In comparison, the hydroxylation reaction of lauric acid by rat P450 4A1 produced mainly ω-hydroxylated product (t_R=9 min, Fig. 3B), and co-injection of the reaction products from CYP119 and P450 4A1 reactions showed two separate peaks (Fig. 3C). Previous study reported that rat P450 4A1 regioselectively catalyzes the ω-hydroxylation of lauric acid [4]. These results suggested that CYP119 and P450 4A1 produced regiochemically different major metabolites; (ω-1)-hydroxylated lauric acid versus ω-hydroxylated lauric acid.

Steady-State Kinetic Analysis of Lauric Acid Hydroxylation
In order to more precisely evaluate the enzymatic activity, the steady-state kinetic parameters were obtained by measuring the final reaction products [(ω-1)- and ω-hydroxylated lauric acid]. The reactions were carried out as in the same condition above, for 10 min. Kinetic parameters were estimated using nonlinear regression analysis with

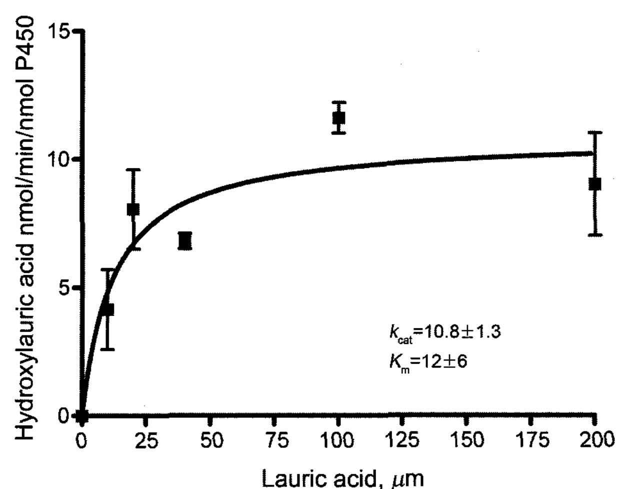

Fig. 4. Steady-state kinetics of (ω-1)-hydroxylation of lauric acid by CYP119.
Each point represents the mean ± SD of a duplicate assay. k_{cat} 10.8±1.3 nmol of hydroxylated lauric acid formed/min/nmol of P450; K_m 12±6 μM.

Graph-Pad Prism software (San Diego, CA, U.S.A.). A turnover number (k_{cat}) of lauric acid hydroxylation of 10.8 min^{-1} and a Michaelis–Menten constant (K_m) of 12 μM were calculated (Fig. 4).

Temperature Effect of Hydroxylation Regioselectivity
The conversion ratios to ω- or (ω-1)-hydroxylated products of lauric acid by CYP119 were compared with varing temperatures (24, 37, 60, and 80°C). CYP119 catalyzed the increased ratio of ω-hydroxylated product with increase in the reaction temperature (Fig. 5). Reaction at 24°C produced only 2.5% of ω-hydroxylated lauric acid, whereas reaction at 80°C catalyzed the less regioselective hydroxylated product.

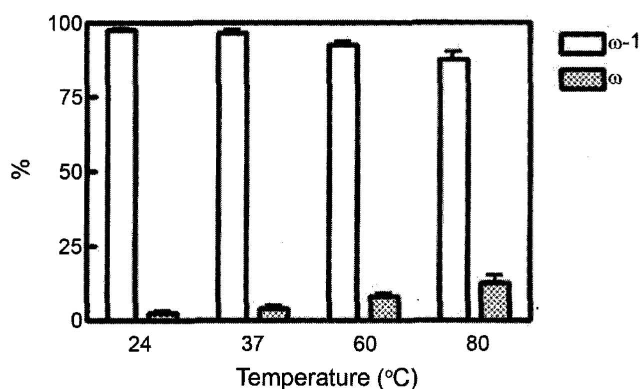

Fig. 5. Temperature effect of regioselectivity for lauric acid hydroxylation by CYP119.
The relative amount of (ω-1)-hydroxylated versus ω-hydroxylated product ratio is represented as a percentage.

DISCUSSION

CYP119 was identified from the *Sulfolobus acidocaldarius* genome and known as the first purified cytochrome P450 from thermophilic organisms. So far, three thermophilic P450 enzymes have been crystallized and their structures determined; $P450_{st}$ from *Sulfolobus tokadaii*, CYP175A1 from *Thermus thermophilus*, and CYP119 [16]. The additional thermophilic P450 enzymes will be discovered with increased numbers of available genomes of thermophilic organisms [16].

The endogenous substrates of CYP119 in *S. acidocaldarius* are not known. Lauric acid is considered to be a relatively good ligand and used as a surrogate substrate for CYP119. Lauric acid gives rise to a conventional Type I binding titration spectrum upon binding to the enzyme (Fig. 2). The calculated K_d value of 1.1 μM in this study indicates a tight binding affinity and a previous study reported a similar K_d value [7]. Styrene, another surrogate substrate, binds somewhat less tightly (K_d=28 μM) [16]. The tight binding affinity of fatty acids suggested that the CYP119 enzyme may play an important role in lipid oxidative metabolism of *S. acidocaldarius*.

Fatty acids are commonly hydroxylated by the mammalian CYP4 enzymes, which possess their unique ability to hydroxylate the thermodynamically disfavored ω-position of fatty acids [10]. A proposed reasonable mechanism was that covalent heme binding to CYP4 enzymes helps the projection of the fatty acid for the ω-hydroxylation [10]. However, a recently characterized fungal P450, CYP52A21, does not bind its prosthetic heme covalently but catalyzes the lauric acid with a 14:1 ω:(ω-1) ratio [6]. Our previous study showed that an access channel in CYP52A21 contains such a small size as that of CYP4A1 [6]. Therefore, the basic strategy used to enforce ω-hydroxylation is that P450 enzymes should possess a sterically restricted architecture for the narrow access channel. CYP102 ($P450_{BM3}$) from *Bacillus megaterium* is the best known bacterial fatty acid hydroxylase [11]. Unlike mammalian CYP4 enzymes or CYP52A21, CYP102 oxidizes fatty acids predominantly at the (ω-1) position and the X-ray crystal structure implies the more spacious active site in the protein [11]. The preferential (ω-1)-hydroxylation of lauric acid by CYP119 in this study and the X-ray crystallographic analysis indicated that CYP119 has a relatively larger active site like CYP102 without ligand and the large conformational shift of active site complements specific substrate (Fig. 1) [16]. The physiological role of selective (ω-1)-hydroxylation of fatty acid by CYP119 or CYP102 is not clear. The selective (ω-1)-hydroxylation may possibly play an important role in lipid metabolism of microorganisms. Thermal stability was a notable feature of thermophilic CYP119 enzyme. Puchkaev *et al.* [13] showed that the unusual aromatic clusters contributed the thermal stability of CYP119. Finding the increasing ratio of ω:(ω-1) hydroxylation at high temperature was unexpected, but the total catalytic activity was not changed in varing temperatures (Fig. 5). A possible interpretation is that 80°C may be a tolerable temperature to CYP119 but not to its electron transfer partners (Pd, and PdR); therefore, the unstable function of Pd and PdR results in the change of regioselectivity.

Thermophilic CYP119 enzyme is of special interest from the structural, mechanistic, and biotechnological points of view. The development of a bioengineering system using CYP119 can present a prospective application in industrial bioreactions for the production of useful chemicals. The investigation of CYP119 regioselectivity in this study may provide the foundation for future work on its application as catalysts in the bioengineering industry.

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