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Construction and engineering of a thermostable self-sufficient cytochrome P450

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ABSTRACT

CYP175A1 is a thermophilic cytochrome P450 and hydroxylates β-carotene. We previously identified a native electron transport system for CYP175A1. In this report, we constructed two fusion proteins consisting of CYP175A1, ferredoxin (Fdx), and ferredoxin–NADP$^+$ reductase (FNR): H_2N-CYP175A1-Fdx-FNR-COOH (175FR) and H_2N-CYP175A1-FNR-Fdx-COOH (175RF). Both 175FR and 175RF were expressed in *Escherichia coli* and purified. The V_{max} value for β-carotene hydroxylation was 25 times higher with 175RF than 175FR and 9 times higher with 175RF than CYP175A1 (non-fused protein), although the k_m values of these enzymes were similar. 175RF retained 50% residual activity even at 80 °C. Furthermore, several mutants of the CYP175A1 domain of 175RF were prepared and one mutant (Q67G/Y68I) catalyzed the hydroxylation of an unnatural substrate, testosterone. Thus, this is the first report of a thermostable self-sufficient cytochrome P450 and the engineering of a thermophilic cytochrome P450 for the oxidation of an unnatural substrate.

Introduction

Cytochrome P450s (P450s) contribute to vital processes in various organisms and require two electrons provided by electron transport systems to activate molecular oxygen, leading to the introduction of atomic oxygen into allylic positions, double bonds, or even non-activated C–H bonds [1]. Thus, P450s have great potential for the biodegradation of environmental contaminants and synthesis of fine chemicals. However, they have some disadvantages such as limited stability, low levels of activity, and requirement for redox partner(s) [1,2]. The discovery of thermophilic P450s may solve the first problem, limited stability. To date, two thermophilic P450s, CYP119 and CYP175A1, have been isolated and characterized. CYP119 from *Sulfolobus solfataricus* exhibits high thermal stability (T_m = 91 °C) [3], and hydroxylates lauric acid [4,5]. The electron transport system for CYP119 is unique. It is composed of ferredoxin (Fdx) and 2-oxoacid:ferredoxin oxidoreductase (OFOR) and utilizes pyruvate as an electron donor instead of the NAD(P)H usually used in electron transport systems for P450s [4,5]. CYP175A1 from *Thermus thermophilus* HB27 also exhibits high thermal stability (T_m = 88 °C) [6], and hydroxylates β-carotene at the 3- and 3′-positions [7]. We recently identified a native electron transport system for CYP175A1, which utilized NADPH rather than NADH and was composed of Fdx and a new type of ferredoxin–NADP$^+$ reductase (FNR) [8].

The use of self-sufficient P450s (P450s fused with their electron transport system) can solve the remaining two problems, low levels of activity and the requirement for redox partner(s). The first example of a self-sufficient P450 was P450 BM3 isolated from *Bacillus megaterium* [9]. This P450 consists of a P450 heme domain (N-terminal) and a diflavin reductase domain (C-terminal). Furthermore, recent genome-based analyses have since revealed several other classes of self-sufficient P450s [10–13]. In 1987, Murakami et al. reported an artificial fusion protein consisting of rat CYP1A1 and rat NADPH–P450 reductase [14]. Numerous fusion proteins consisting of P450s and electron transport systems have now been constructed (e.g., [15–20]), providing useful tools for enzymatic and mechanistic studies [18].

In this report, we constructed two fusion proteins consisting of CYP175A1, Fdx, and FNR and characterized them. Furthermore, we engineered the CYP175A1 domain of 175RF for the oxidation of testosterone, which is not normally a substrate of CYP175A1.

Materials and methods

Materials. Emulgen 911 was a gift from Kao Chemical (Tokyo, Japan). KOD Plus DNA polymerase was purchased from Toyobo (Osaka, Japan), NADPH from Oriental Yeast (Tokyo, Japan), testosterone from Sigma Chemical Co. (St. Louis, MO), β-carotene, potassium ferricyanide, isopropyl-β-D-thiogalactopyranoside (IPTG), and phenylmethanesulfonyl fluoride (PMSF) from Wako Pure Chemical Industries (Osaka, Japan), Tween 20 from Bio-Rad Laboratories (Hercules, CA), and 6β-hydroxytestosterone from

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Daiichi Pure Chemicals (Tokyo, Japan). Recombinant CYP175A1, Fdx, and FNR were prepared as described previously [8].

Construction of 175RF. The scheme for the construction of 175RF is shown in Fig. S1. The primers used for the construction are shown in Table S1. To remove the stop codon of pET-FNR [8] and introduce a linker sequence (Fig. S1A), FNR was amplified by PCR with pET-FNR as a template, primer 1 as a forward primer, and primer 2 as a reverse primer deleting the stop codon and incorporating both a SpeI site encoding Thr-Ser and a BamHI site. The PCR product was digested with NdeI and BamHI, the fragment was ligated into the expression vector pET-21a (Novagen, Madison, WI) using NdeI and BamHI sites, and the construct was designated pET-FNR-TS. To generate the FNR-Fdx fusion protein (Fig. S1B), Fdx was amplified by PCR with pET-Fdx [8] as a template, primer 3 as a forward primer incorporating both a SpeI site and a linker sequence encoding Gly-Asp-Ala, and primer 4 as a reverse primer. The PCR product was digested with SpeI and BamHI, the fragment was ligated into pET-FNR-TS using SpeI and BamHI sites, and the construct was designated pET-FNR-Fdx. To introduce a linker sequence into pET-FNR-Fdx (Fig. S1C), FNR-Fdx was amplified by PCR with pET-FNR-Fdx as a template, primer 5 as a forward primer incorporating a KpnI site encoding Gly-Thr, a linker sequence encoding Ser, and a BamHI site, and primer 4 as a reverse primer. The PCR product was digested with BamHI, the fragment was ligated into the expression vector pET-21a using a BamHI site, and the construct was designated pET-GTS-FNR-Fdx. To generate the CYP175A1-FNR-Fdx fusion protein (175RF) (Fig. S1D), CYP175A1 was amplified by PCR with pET-CYP175A1 [8] as a template, primer 6 as a forward primer, and primer 7 as a reverse primer incorporating both a KpnI site and a linker sequence encoding Ala-Asp. The PCR product was digested with NdeI and KpnI, the fragment was ligated into pET-GTS-FNR-Fdx using NdeI and KpnI sites, and the construct was designated pET-175RF. All PCRs were carried out at 94 °C for 5 min and then 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 68 °C for 2 min using KOD Plus DNA polymerase. The construction of 175FR is described in Supplemental materials and methods.

Expression and purification of fusion proteins. *Escherichia coli* BL21 (DE3) Codon Plus cells were transformed with either pET-175FR or pET-175RF. An overnight culture (50 ml) of the transformant was inoculated into 1 liter of 2× YT medium containing chloramphenicol and ampicillin, and then the culture was incubated at 37 °C until the OD_{600} was 1.0. Expression of the fusion protein was induced with 1.0 mM IPTG for 20 h at 25 °C. Cells expressing 175FR or 175RF were harvested by centrifugation, and the fusion protein was purified using ammonium sulfate fractionation, a butyl Sepharose 4 Fast Flow (Amersham Biosciences), and a Mono S HR5/5 column (Pharmacia) (see details in Supplemental materials and methods). The purified protein was dialyzed against 50 mM potassium phosphate buffer, pH 7.4, containing 10% glycerol and 300 mM KCl and stored at −80 °C. The concentration of the protein was determined from reduced CO-difference spectrum using an extinction coefficient of 91 mM^{-1} cm^{-1} at 450 nm [21].

Construction of 175RF mutants. 175RF mutants were constructed with the megaprimer method [22]. The mutagenic primer (Table S2), primer 7 (Table S1), and 175RF or 175RFm1 as a template (Table S2) were used for the first round of amplification. The PCR products were then used in the second round of amplification as a primer in conjunction with primer 6 (Table S1) and CYP175A1 (WT) or CYP175A1 (m1) as a template (Table S1). The products were ligated into pET-GTS-FNR-Fdx as described for the construction of 175RF. Furthermore, these mutants were expressed and purified in the same method as 175RF.

Enzyme assays. β-Carotene hydroxylation activity and ferricyanide reduction activity were measured as described previously (see details in Supplemental materials and methods) [8]. Testosterone hydroxylation activity was measured in buffer (50 mM potassium phosphate buffer, pH 7.4, 10% glycerol; total volume, 400 μl) containing testosterone (500 μM) and 175RF or mutants (1 μM). The reaction mixture was incubated at 60 °C for 3 min, and then the reaction was initiated by the addition of NADPH (final concentration, 1.0 mM). After 15 min at 60 °C, ice-cold ethyl acetate (2 ml) was added to stop the reaction. The metabolites were extracted and analyzed by HPLC as described [23] with minor modifications. The analysis was performed using a HPLC system (Prominence, Shimadzu, Kyoto, Japan) equipped with an ODS-100Z column (150 × 4.6 mm, Tosoh, Tokyo, Japan) and a gradient of acetonitrile, methanol, and water at a flow rate of 1 ml/min. The metabolites were monitored at 254 nm.

Results

Construction, expression, and purification of fusion proteins

We constructed two fusion proteins consisting of CYP175A1, Fdx, and FNR: H_2N-CYP175A1-Fdx-FNR-COOH (175FR) and H_2N-CYP175A1-FNR-Fdx-COOH (175RF). Five small hydrophilic amino acids were inserted at the junction between each component to act as a flexible hinge allowing mobility of each protein and increasing the interaction between each component [16]. The peptide linker between CYP175A1 and Fdx or FNR was composed of Ala-Asp-Gly-Thr-Ser, while that between Fdx and FNR was composed of Thr-Ser-Gly-Asp-Ala. The fusion proteins were expressed in *E. coli* and purified. The purified fusion protein was >90% pure, as judged by SDS–PAGE (Fig. S3). The reduced CO-difference spectra of 175FR and 175RF both showed an absorption peak at 450 nm but did not show a peak at 420 nm which is characteristic of an inactive P450 (Fig. S3).

pH-Dependency and kinetic parameters of fusion proteins

We determined the optimal pH of the two fusion proteins (Fig. 1A). In both cases, the optimal pH was pH 7.0–7.4, and the β-carotene hydroxylation activity was markedly reduced at pH 5.0. This dependency on pH was significantly different from that of a reconstitution system composed of CYP175A1, Fdx, and FNR, the optimum pH of which was pH 5.0 [8]. The difference is consistent with the pH-dependency of the ferricyanide reduction activity of the FNR domain in the fusion protein (Table S3). The kinetic parameters of both the reconstitution system and fusion proteins were determined to characterize the hydroxylation of β-carotene (Fig. 1B). The k_m values of 175FR and 175RF for β-carotene were comparable with CYP175A1 (non-fused protein) in the reconstitution system. The V_{max} value for the hydroxylation, however, was 25 times higher with 175RF than 175FR and 9 times higher with 175RF than CYP175A1 (non-fused protein).

Electron transfer pathway of fusion proteins

There are two pathways catalyzing the hydroxylation of β-carotene by a fusion protein, intermolecular electron transfer and intramolecular electron transfer. As described previously [15,18,19], the activity should increase exponentially as the concentration of enzyme increases in the case of the intermolecular electron transfer pathway, whereas it should increase linearly in the case of the intramolecular electron transfer pathway. As a control, the activity of the reconstitution system increased exponentially (Fig. 2A), indicating that electrons are transferred from NADPH via FNR and Fdx to CYP175A1 by the intermolecular pathway. On the other hand, the activities of both 175FR and 175RF increased linearly with their concentrations (Fig. 2A), indicating that

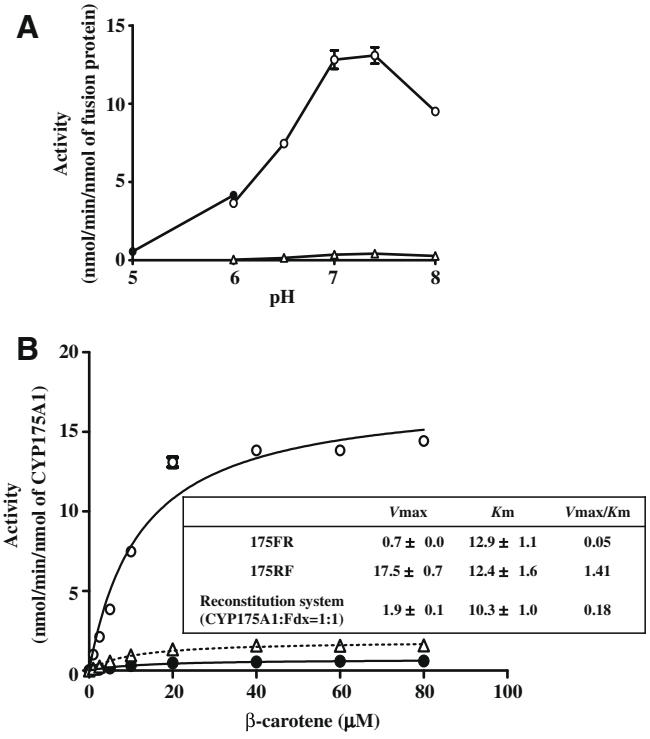

Fig. 1. pH-dependency and kinetic analysis of fusion proteins. (A) Effect of pH on β-carotene hydroxylation activity. The reactions were performed at the indicated pH value in the presence of 20 μM β-carotene, 0.8% Tween 20, 1 mM NADPH, and 175FR (triangles, 60 nM) or 175RF (circles, 30 nM) at 65 °C for 2 min. The buffers used in this experiment were 50 mM potassium acetate buffer containing 10% glycerol of the pH range 5.0–6.0 (closed circles) and 50 mM potassium phosphate buffer containing 10% glycerol of the pH range 6.0–8.0 (open circles and triangles). (B) Kinetic analysis for β-carotene hydroxylation by 175FR, 175RF, and CYP175A1 (non-fused protein). In the kinetic analysis of the fusion proteins, the reaction mixture contained 0.8% Tween 20, β-carotene (1–80 μM), 1 mM NADPH, and 175FR (60 nM) or 175RF (30 nM) in 50 mM potassium phosphate buffer, pH 7.4, containing 10% glycerol. For CYP175A1 (non-fused protein), the reconstitution system contained CYP175A1 (30 nM), Fdx (30 nM), FNR (30 nM), 0.8% Tween 20, β-carotene (1–80 μM), and 1 mM NADPH in 50 mM potassium acetate buffer (pH 5.0) containing 10% glycerol. The reactions were performed at 65 °C for 2 min. Closed circles and solid line, 175FR; open circles and solid line, 175RF; open triangles and dotted line, CYP175A1. Inset shows k_m and V_{max} values for β-carotene hydroxylation. The values represent means ± SD of triplicate experiments.

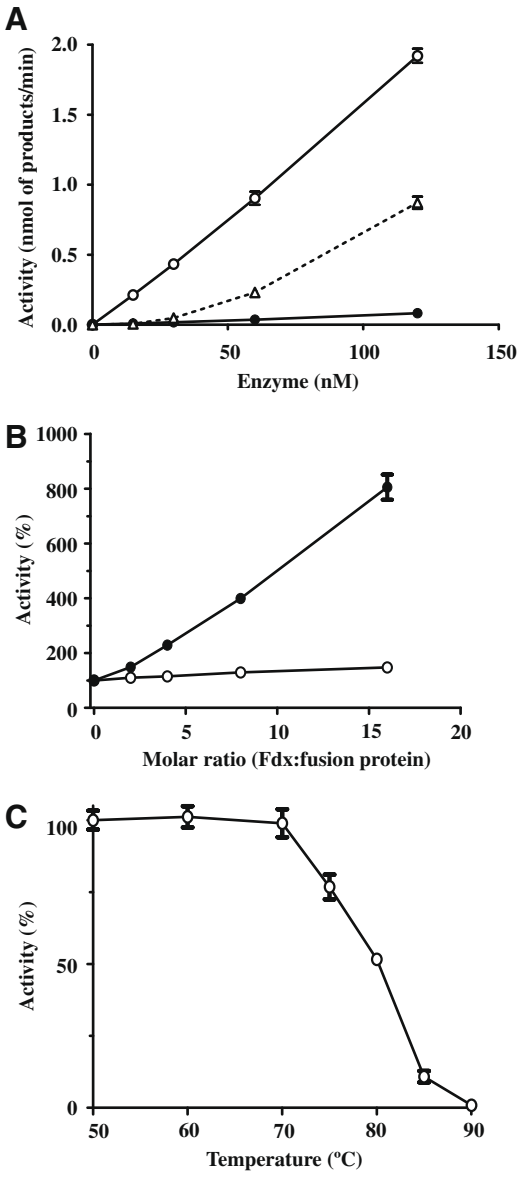

Fig. 2. Characterization of hydroxylation of β-carotene by fusion proteins. (A) Effect of the concentrations of proteins on β-carotene hydroxylation activity. As CYP175A1 (non-fused protein), equimolar concentrations (15–120 nM) of CYP175A1, Fdx, and FNR were incubated in 50 mM potassium acetate buffer (pH 5.0) containing 10% glycerol, β-carotene (80 μM), and NADPH (1 mM) at 65 °C for 2 min. As the fusion protein, the reaction mixture contained β-carotene (80 μM), NADPH (1 mM), and 175FR (15–120 nM) or 175RF (15–120 nM) in 50 mM potassium phosphate buffer, pH 7.4, containing 10% glycerol. The reactions were performed at 65 °C for 2 min. Closed circles and solid line, 175FR; open circles and solid line, 175RF; open triangles and dotted line, CYP175A1 (non-fused protein). (B) Effect of exogenous Fdx on β-carotene hydroxylation activity. The reaction mixture contained β-carotene (80 μM), NADPH (1 mM), Fdx (60–480 nM), and 175FR (30 nM) or 175RF (30 nM) in 50 mM potassium phosphate buffer, pH 7.4, containing 10% glycerol. The reactions were performed at 65 °C for 2 min. Closed circles, 175FR; open circles, 175RF. (C) Thermostability of 175RF. 175RF (60 nM) was incubated at various temperatures (50–90 °C) for 10 min in 50 mM potassium phosphate buffer, pH 7.4, containing 10% glycerol. The heat treatment was stopped by placement of the sample on ice for 5 min. Then, the residual β-carotene hydroxylation activity was measured in 50 mM potassium phosphate buffer, pH 7.4, containing 10% glycerol, β-carotene (80 μM), and NADPH (1 mM) at 50 °C for 2 min. The values represent means ± SD of triplicate experiments.

electrons are transferred within the molecule in both 175FR and 175RF. Furthermore, we measured β-carotene hydroxylation activity in the presence of exogenous CYP175A1, Fdx, and FNR to characterize the interaction between the three components (Fig. 2B). Addition of either purified FNR or CYP175A1 to 175FR and 175RF did not stimulate the hydroxylation of β-carotene (data not shown). However, addition of purified Fdx to 175FR significantly enhanced the activity, indicating that the intramolecular interaction between the FNR domain and the Fdx domain and/or between the Fdx domain and the CYP175A1 domain is suboptimal in 175FR. On the other hand, addition of purified Fdx to 175RF only slightly enhanced the activity, indicating that the intramolecular interaction between the three components in 175RF is nearly optimal.

Thermostability of 175RF

The thermostability of 175RF was evaluated by measuring the residual β-carotene hydroxylation activity after 10 min at various temperatures (Fig. 2C). 175RF maintained 100% residual activity at 70 °C and 50% residual activity even at 80 °C, indicating that 175RF is an extremely thermostable protein.

Table 1
Hydroxylation activity of testosterone and β-carotene by 175RF and mutants.

Name of construct	Activity (nmol/min/nmol of P450)				
	Testosterone[a]				β-carotene[b]
	2β	6β	16α	Total	
175RF	ND[c]	ND[c]	ND[c]	ND[c]	14.4 ± 0.3
175RFm1	0.002 ± 0.001	ND[c]	ND[c]	0.002 ± 0.001	0.032 ± 0.001
175RFm2	0.17 ± 0.01	0.018 ± 0.001	0.019 ± 0.000	0.207 ± 0.013	0.007 ± 0.001
175RFm3	0.068 ± 0.002	0.008 ± 0.001	0.002 ± 0.000	0.078 ± 0.002	ND[c]

[a] Testosterone hydroxylation activities were performed as described under Materials and methods. The values represent means ± SD of triplicate experiments.
[b] In the case of WT, β-carotene hydroxylation activity was performed as described in Fig. 1B (β-carotene concentration, 80 μM). In the case of mutants, 1 μM enzymes and 80 μM β-carotene were used, and other reaction conditions were the same as those of WT.
[c] ND, not detected.

Hydroxylation of testosterone and β-carotene by 175RF mutants

We engineered the CYP175A1 domain of 175RF for the hydroxylation of unnatural substrates. The active site of CYP175A1 is better suited to a linear aliphatic chain of the natural substrate, β-carotene, resulting in a narrow active site. In particular, Q67, Y68, W269, and I270 have large side chains (Fig. S4), which may prevent larger molecules such as testosterone from binding to the active site. In order to allow the entry of large molecules into the active site, we constructed three mutants: 175RFm1, W269P/I270A; 175RFm2, Q67G/Y68I; 175RFm3, Q67G/Y68I/W269P/I270A. The mutants were expressed in *E. coli* and purified. The reduced CO-difference spectrum of 175RFm1 showed an absorption peak at 450 nm and did not show a peak at 420 nm, whereas the reduced CO-difference spectra of 175RFm2 and 175RFm3 had a minor peak at 420 nm in addition to a major peak at 450 nm (data not shown). Although 175RF did not show any hydroxylation of testosterone, all mutants could hydroxylate testosterone (Table 1). In particular, 175RFm2 had efficient catalytic activity toward testosterone and produced 2β-hydroxytestosterone as a major metabolite (Fig. 3). Furthermore, we evaluated the effect of these mutations of the active site on the hydroxylation of β-carotene (Table 1). The β-carotene hydroxylation activity of 175RFm1 and 175RFm2 was significantly lower (450- and 2000-fold, respectively) than that of 175RF. Furthermore, 175RFm3 completely abolished the β-carotene hydroxylation activity.

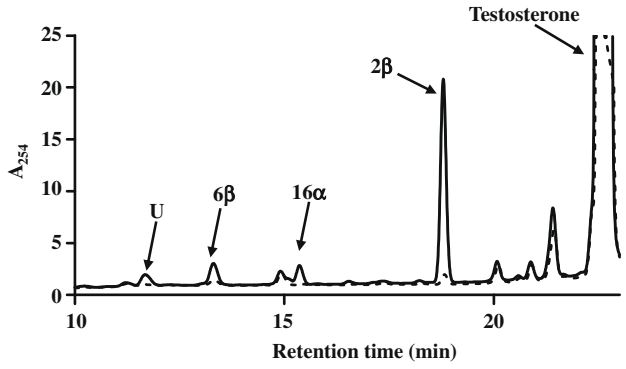

Fig. 3. HPLC profiles of testosterone metabolites produced by 175RF and 175RFm2. Testosterone hydroxylation activity was measured as described under Materials and methods. The chromatography was done with a linear gradient from H_2O–methanol–acetonitrile (62:36:2) to H_2O–methanol–acetonitrile (30:64:6) for 20 min at a flow rate of 1 ml/min. Solid line, 175RFm2; dotted line, 175RF. 6β, 16α, and 2β indicate the hydroxylated position of testosterone. U indicates an unidentified metabolite.

Discussion

We have constructed and characterized artificial fusion proteins consisting of CYP175A1, Fdx, and FNR. The β-carotene hydroxylation activity of 175RF was significantly greater than that of 175FR, although the ferricyanide reduction activity of 175FR was comparable with that of 175RF. The high level of activity by 175RF is probably due to both the intramolecular electron transfer and nearly optimal interaction between the three components. Furthermore, 175RF retained 50% residual activity even at 80 °C. Eiben et al. constructed an artificial thermostable self-sufficient P450 chimera using CYP102A1 (P450 BM3) and CYP102A3, but its activity decreased 50% at 56 °C [24]. Thus, this is the first report of an extremely thermostable self-sufficient P450.

We engineered the CYP175A1 domain of 175RF for the oxidation of unnatural substrates. 175RF did not hydroxylate testosterone, whereas all the mutants hydroxylate testosterone. In particular, 175RFm2 in which Q67 and Y68 were substituted with Gly and Ile, respectively, showed the strongest testosterone hydroxylation activity. This is the first report of the engineering of a thermophilic cytochrome P450 for the oxidation of an unnatural substrate. Furthermore, all mutants showed significantly less β-carotene hydroxylation activity than 175RF, indicating that the large residues targeted for mutagenesis have a pivotal role in the binding of the natural substrate, β-carotene, to the active site. Yano et al. reported that the active site of CYP175A1 is nearly identical to that of P450BM3 [6], whose mutant (R47L/F87V/L188Q) can metabolize several drug-like compounds including testosterone [25,26]. Therefore, 175RFm2 may also metabolize drug-like compounds other than testosterone.

Finally, 175RF, which had strong catalytic activity and was extremely thermostable, could be useful for studying the interaction between the three components and the mechanism of electron transfer from NADPH via FNR and Fdx to P450. Furthermore, 175RFm2, which was active toward testosterone, could lead to the biotechnological exploitation of CYP175A1.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.04.064.

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