Residue size at position 87 of cytochrome P450 BM-3 determines its stereoselectivity in propylbenzene and 3-chlorostyrene oxidation

Qing-Shan Li[a], Jun Ogawa[a], Rolf D. Schmid[b], Sakayu Shimizu[a,*]

[a] *Division of Applied Life Science, Graduate School of Agriculture, Kyoto University, Kitashirakawa-oiwakecho, Sakyo-ku, Kyoto 606-8502, Japan*
[b] *Institut für Technische Biochemie, Universität Stuttgart, Allmandring 31, D-70459 Stuttgart, Germany*

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Abstract We report here oxidation of propylbenzene and 3-chlorostyrene by wild-type cytochrome P450 BM-3 with high turnover (479 nmol 1-phenyl-1-propanol/min/nmol P450 and 300 nmol 3-chlorostyrene oxide/min/nmol P450). Furthermore, the residue size at position 87 of P450 BM-3 was found to play critical roles in determining stereoselectivity in oxidation of propylbenzene and 3-chlorostyrene. Replacement of Phe87 with Val, Ala and Gly resulted in decreases in optical purity of produced (*R*)-(+)-1-phenyl-1-propanol from 90.0 to 37.4, 26.0 and −15.6% *e.e.*, respectively, and in increases in those of produced (*R*)-(+)-3-chlorostyrene oxide from −61.0 to −38.0, 67.0 and 94.6% *e.e.*, respectively. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Cytochrome P450; Cytochrome P450 BM-3; Oxidation; Stereoselectivity; Propylbenzene; 3-Chlorostyrene

1. Introduction

Cytochrome P450 (P450) BM-3 from *Bacillus megaterium*, which catalyzes the hydroxylation and epoxidation of long-chain saturated and unsaturated fatty acids, respectively, is one of the best-characterized P450 enzymes [1,2]. This soluble bacterial P450 is a catalytically self-sufficient P450, which is a single polypeptide naturally consisting of a P450 and a diflavin NADPH-P450 reductase. It is highly active with catalytic turnover of several thousands per minute for fatty acid oxidation, and catalyzes the oxidation of fatty acids with high stereoselectivity [3–5]. For instance, 98% of 15- and 14-hydroxypalmitic acids produced through palmitic acid hydroxylation by P450 BM-3 are in the *R* configuration [5]. P450 BM-3 oxidizes eicosapentaenoic acid stereoselectively to 17(*S*),18(*R*)-epoxyeicosatetraenoic acid with an optical purity of 94% *e.e.* [4].

Here, we demonstrated for the first time that P450 BM-3 shows high activities towards propylbenzene and 3-chlorostyrene with considerable stereoselectivity. Furthermore, reaction analysis using its mutants revealed that the stereoselectivity is under the control of the size of the residue at position 87 of this enzyme. Phe87 is one of the most studied residues in P450 BM-3 with respect to its activity and selectivity [3,10,11]. The replacement of Phe87 by other amino acids, such as Ala, Gly, Tyr and Val, affected the activity and regio- and/or stereoselectivity toward its original substrates, long-chain fatty acids such as laurate [10,11], myristate [10] and arachidonic acid [3]. Our findings as to artificial substrates, propylbenzene and 3-chlorostyrene, provide new clues for understanding the ways in which P450 BM-3 recognizes substrate structures and Phe87 of the P450 controls spatial coordination of these substrates in the active site. These results indicate the high potential of P450 BM-3 and its mutants for the preparation of useful chiral compounds.

2. Materials and methods

2.1. Chemicals

(*R*)-(+)-1-Phenyl-1-propanol, (*S*)-(−)-1-phenyl-1-propanol and (*R*)-(+)-3-chlorostyrene oxide were purchased from Aldrich (Milwaukee, WI, USA). 1-Phenyl-2-propanol and 3-phenyl-1-propanol were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). All other chemicals used in this study were of reagent grade.

2.2. Preparation of P450 BM-3 and its mutant enzymes

The P450 BM-3 mutants were obtained by replacement of Phe87 with Val (F87V), Ala (F87A) or Gly (F87G) as described previously [6]. The expression and purification of P450s and determination of the concentration of P450s were carried out as described previously [6].

2.3. Enzyme activity assay

Throughout the reaction, the concentration of the substrates was maintained enough higher than their K_d values (Tables 2 and 3) to get a maximum reaction rate. To measure the NADPH consumption rate, 60 μl of a 1.5 μM P450 sample was mixed with 480 μl of 0.1 M Tris–HCl buffer (pH 7.4) containing 6 μl of 1 M propylbenzene or 3-chlorostyrene in dimethyl sulfoxide (DMSO). The mixture was allowed to stand for 2 min at 25°C, then 60 μl of 3.5 mM NADPH was added to start the reaction. The final volume of the reaction mixture was 600 μl and concentrations of the components at the beginning of the reaction were as follows: P450 (0.15 μM), Tris–HCl (80 mM), substrate (10 mM), and NADPH (0.35 mM). NADPH consumption was monitored at 340 nm with a Shimadzu MultiSpec-1500 spectrophotometer (Kyoto, Japan) at 25°C for 20 s. The NADPH concentration was calculated using $\varepsilon_M = 6200\ M^{-1}\ cm^{-1}$ [7].

The substrate oxidation activity was calculated by multiplying the NADPH consumption rate by the coupling efficiency. To measure the coupling efficiency, 100 μl of a 25 μM P450 sample was mixed with 4800 μl of 0.1 M Tris–HCl buffer (pH 7.4) containing 80 U of catalase and 50 μl of 1 M propylbenzene or 3-chlorostyrene in DMSO. After standing for 2 min, 100 μl of 20.0 mM NADPH was added, and the reaction mixture was incubated for 20 min at 25°C to allow all NADPH to be consumed. The final volume of the reaction mixture was 5000 μl and concentrations of the components at the beginning of the reaction were as follows: P450 (0.5 μM), Tris–HCl (96 mM), catalase (160 U/ml), substrate (10 mM), and NADPH (0.4 mM). Control experiments were carried out as described above except for the absence of NADPH. After incubation, 50 μl of 200 mM 4-phenyl-

*Corresponding author. Fax: (81)-75-753 6128.
E-mail address: sim@kais.kyoto-u.ac.jp (S. Shimizu).

Abbreviations: P450, cytochrome P450; WT, wild-type P450 BM-3; F87V, F87A, and F87G, P450 BM-3 mutants

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Table 1
Product distribution of propylbenzene hydroxylated by P450 BM-3 and its mutants[a]

	Products ratio (%)			
	WT	F87V	F87A	F87G
1-Phenyl-1-propanol	99.2 ± 0.4	80.0 ± 1.1	34.5 ± 1.4	67.8 ± 1.8
1-Phenyl-2-propanol	0.8 ± 0.4	19.0 ± 0.8	54.0 ± 1.4	25.9 ± 1.2
3-Phenyl-1-propanol	0	1.0 ± 0.6	11.5 ± 1.1	6.3 ± 1.1

[a]All results are given as means ± S.D. for three individual experiments.

1-butanol in DMSO was added to the reaction mixtures as an internal standard, and the reaction mixtures were extracted twice with chloroform (6 ml in total). The extracts were evaporated to about 100 μl and then used for gas–liquid chromatography (GC) and GC-mass spectrometry (MS) analyses. The amount of product was calculated with a standard calibration curve obtained using authentic samples.

2.4. GC and GC-MS analyses

GC analysis was performed with a Shimadzu GC-17A gas chromatograph equipped with a flame ionization detector and a split injection system, and fitted with a capillary column DB-17 (30 m × 0.554 mm i.d.; J&W Scientific, Folsom, CA, USA). The column temperature was initially 100°C, and was then raised to 280°C at a rate of 5°C/min and maintained at that temperature for 20 min. The injector and detector were operated at 280°C. Helium was used as the carrier gas at 30 kPa/cm^2.

GC-MS analysis was performed on a GC-MS QP5050 (Shimadzu) with a GC-17A gas chromatograph equipped with a capillary column (HR-1, 25 m × 0.25 mm i.d.; Shinwa Kako, Kyoto, Japan) at 150°C. Helium was used as the carrier gas at 225 kPa/cm^2. MS was performed in the electron impact mode at 70 eV with a source temperature of 250°C. Split injection was employed with the injection port at 250°C.

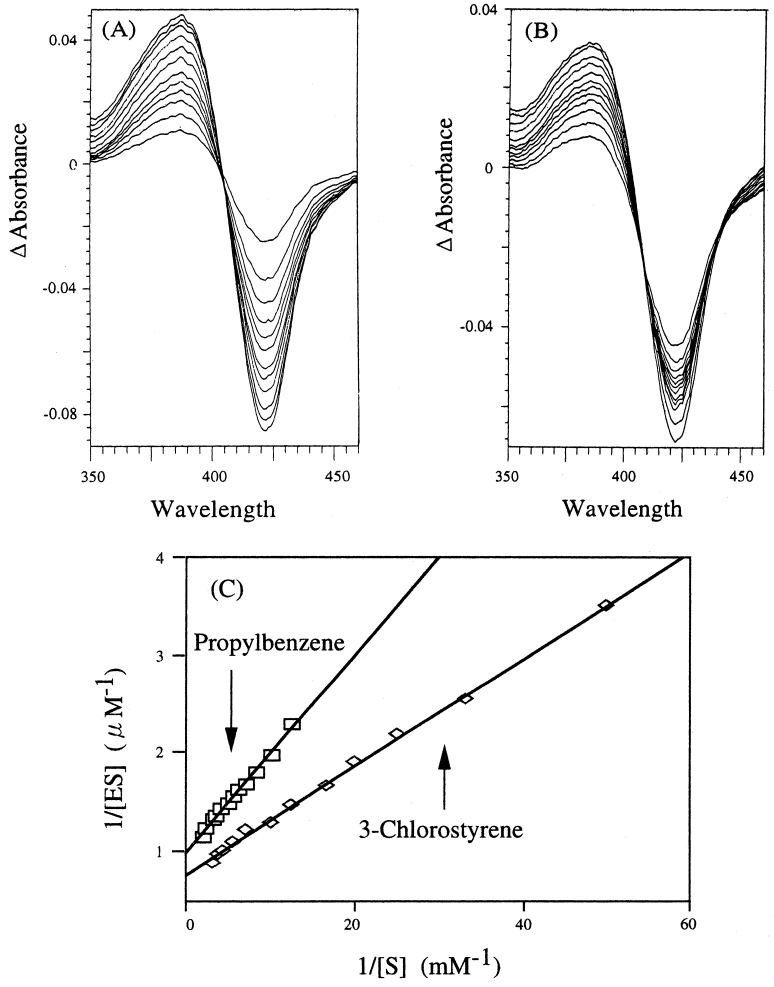

Fig. 1. Absorbance spectral changes on propylbenzene (A) and 3-chlorostyrene (B) binding to P450 BM-3. P450 BM-3 (3.8 μM) in Tris–HCl buffer (100 mM, pH 7.4) was titrated with a freshly prepared solution of propylbenzene or 3-chlorostyrene and the absorbance changes were recorded following each addition: for propylbenzene, final concentrations of 0.02, 0.03, 0.04, 0.05, 0.06, 0.08, 0.1, 0.14, 0.18, 0.22, 0.26 and 0.32 mM; for 3-chlorostyrene, final concentrations of 0.08, 0.1, 0.12, 0.14, 0.16, 0.18, 0.2, 0.24, 0.28, 0.32, 0.42 and 0.52 mM. A plot of $1/[ES_i]$ versus $1/[S_i]$ for each substrate (C), where $[ES_i]$ was calculated from the change in the absorbance (ΔA_i) using the equation $[ES_i] = \Delta A_i \times [P450]/\Delta A_{max} \times \Delta A_i$ was determined by subtracting the absorbance at 422 nm (trough) from that at 388 nm (peak) and ΔA_{max} as the maximum ΔA_i obtained from palmitic acid binding to P450 BM-3; $[S_i] = [S_{total}] - [ES_i]$.

Table 2
Binding and oxidation of propylbenzene by P450 BM-3 and its mutants[a]

	WT	F87V	F87A	F87G
Optical purity of produced (*R*)-(+)-1-phenyl-1-propanol (% *e.e.*)[b]	90.0	37.4	26.0	−15.6
Activity (nmol phenylpropanol/min/nmol P450)	479 ± 29	546 ± 34	439 ± 21	661 ± 37
Coupling efficiency (%)	60.0 ± 4.7	38.0 ± 4.2	35.5 ± 3.8	44.7 ± 5.1
High-spin heme (%)	35.0 ± 5.1	81.1 ± 6.2	41.4 ± 3.9	48.2 ± 4.3
Dissociation constant (K_d, μM)	73.5 ± 5.4	44.9 ± 4.7	34.7 ± 4.2	20.1 ± 2.6

[a]All results are given as means ± S.D. for three individual experiments.
[b]S.D. was within 0.8%.

2.5. Resolution of 1-phenyl-1-propanol and 3-chlorostyrene oxide enantiomers

The propylbenzene reaction sample was extracted twice with chloroform (6 ml in total), evaporated to dryness, and then dissolved in 5% (v/v) of 2-propanol in *n*-hexane for HPLC analysis. HPLC analysis was carried out on a Chiralcel OJ column (4.6 mm i.d.×250 mm; Daicel Chemical Industries, Osaka, Japan), at a flow rate of 0.5 ml/min with *n*-hexane/2-propanol (95:5, by volume) as the eluent. The eluent was monitored at 254 nm.

The enantiomers of 3-chlorostyrene oxide were analyzed by GC-MS essentially under the same conditions as described above on a Cyclodex-B chirality-sensitive GC column (30 m×0.25 mm i.d.; Chrompack, Middelburg, The Netherlands) using a 100°C isotherm.

2.6. Spectral binding studies

The substrate-induced spectral shifts of P450 BM-3 enzymes were recorded with a Shimadzu MultiSpec-1500 spectrophotometer at 25°C. Propylbenzene and 3-chlorostyrene dissolved in DMSO at concentrations of 20 mM were freshly prepared for the analysis. For each substrate, the substrate solution was added to the enzyme solution (600 μl) containing 3.8 μM P450 in 0.1 M Tris–HCl (pH 7.4) in a cuvette, and the same volume of DMSO was added to the reference cuvette containing the enzyme solution. For each substrate for all enzymes, triplicate binding assays with 12 variations of substrate concentration were performed. The change in absorbance (ΔA) was determined by subtracting the absorbance at 422 nm (trough) from that at 388 nm (peak). The maximum heme spin-state shift of wild-type binding to palmitic acid was taken as 100% heme spin-state shift. Data analysis and calculations were carried out according to the methods described elsewhere [7].

3. Results

3.1. Hydroxylation of propylbenzene

Wild-type P450 BM-3 hydroxylated propylbenzene mainly to 1-phenyl-1-propanol (99.2% of the total product) with co-production of a small amount of 1-phenyl-2-propanol (0.8% of the total product). These products were identified on the basis of co-elution with authentic samples on GC (retention time for 1-phenyl-1-propanol 12.8 min, for 1-phenyl-2-propanol 12.3 min) and also the results of GC-MS. P450 BM-3 mutants F87V, F87A and F87G all hydroxylated propylbenzene to a mixture of 1-phenyl-1-propanol, 1-phenyl-2-propanol and 3-phenyl-1-propanol (identified on the basis of the co-elution with the authentic sample on GC and the results of GC-MS, retention time on GC 16.0 min). The product distribution was significantly influenced by the size of the residue at position 87 (Table 1).

The resolution of the two enantiomers of 1-phenyl-1-propanol was carried out on a Chiralcel OJ HPLC column. The retention times for (*R*)-(+)-1-phenyl-1-propanol and (*S*)-(−)-1-phenyl-1-propanol were 20.1 and 19.1 min, respectively. Wild-type produced (*R*)-(+)-1-phenyl-1-propanol with 90% *e.e.*, but the optical purity of the produced (*R*)-(+)-1-phenyl-1-propanol was decreased to 37.4, 26.0 or −15.6% *e.e.* by replacement of Phe87 with Val, Ala or Gly, respectively (Table 2). These observations indicated that the size of the residue at position 87 of P450 BM-3 determined the proportion of the two enantiomers.

The activity of P450 BM-3 towards propylbenzene did not change significantly with the replacement of Phe87 by these small residues, but the coupling efficiency decreased by 30–40% (Table 2).

Substrate binding to P450 usually triggers a heme spin-state shift from low to high, and it can be monitored as changes in P450 absorbance spectra. Propylbenzene binds to wild-type with a maximum heme spin-state shift of 35.0% and a dissociation constant of 73.5 μM (Fig. 1 and Table 2). The maximum heme spin-state shift of P450 BM-3 triggered by the binding of propylbenzene was increased by replacement of Phe87 with small amino acid residues (Table 2). The replacement of Phe87 with small amino acid residues also increased the affinity of P450 BM-3 towards propylbenzene, reflected in a reduction of the dissociation constants (Table 2).

3.2. Epoxidation of 3-chlorostyrene

The wild-type and its mutants, F87V, F87A and F87G, catalyzed the epoxidation of 3-chlorostyrene to 3-chlorostyrene oxide, which was identified on the basis of the co-elution with authentic sample on GC-MS and also the results of MS. No other products were detected.

The 3-chlorostyrene oxide produced was resolved into two peaks with retention times of 26.6 and 27.8 min on the chirality-sensitive GC column. The peak with the retention time of 26.6 min was identified as (*R*)-(+)-3-chlorostyrene oxide on

Table 3
Oxidation and binding of 3-chlorostyrene by P450 BM-3 and its mutants[a]

	WT	F87V	F87A	F87G
Optical purity of produced (*R*)-(+)-chlorostyrene oxide (% *e.e.*)[b]	−61.0	−38.0	67.0	94.6
Activity (nmol 3-chlorostyrene oxide/min/nmol P450)	300 ± 33	769 ± 38	196 ± 24	900 ± 41
Coupling efficiency (%)	46.6 ± 5.2	52.6 ± 4.9	47.9 ± 5.1	52.3 ± 5.7
High-spin heme (%)	29.5 ± 4.8	56.6 ± 4.9	54.3 ± 4.2	54.1 ± 5.6
Dissociation constant (K_d, μM)	107 ± 10	90.8 ± 6.1	72.7 ± 5.5	66.3 ± 4.5

[a]All results are given as means ± S.D. for three individual experiments.
[b]S.D. was within 0.6%.

the basis of co-elution with an authentic sample on the chirality-sensitive GC column and also the results of MS. The peak with the retention time of 27.8 min was identified as (*S*)-(−)-3-chlorostyrene oxide on the basis of the results of MS, which indicates that the two peaks resolved on the chirality-sensitive GC column were the same compound. It was of interest that the optical purity of the produced (*R*)-(+)-3-chlorostyrene oxide was increased from −61.0 to −38.0, 67.0 and 94.6% *e.e.* by replacement of Phe87 with Val, Ala and Gly, respectively (Table 3). Thus, the size of the residue at position 87 of P450 BM-3 determines the proportion of the two enantiomers in 3-chlorostyrene oxidation as well as in propylbenzene oxidation.

The coupling efficiencies of wild-type and the three mutants are similar to each other, but the 3-chlorostyrene epoxidation activity of P450 BM-3 was increased two- or three-fold by replacement of Phe87 with Val or Gly, respectively (Table 3). However, mutation of F87A decreased the 3-chlorostyrene epoxidation activity by 35% (Table 3).

3-Chlorostyrene binds to the wild-type with a maximum heme spin-state shift of 29.5% and a dissociation constant of 107 μM (Fig. 1 and Table 3). The maximum heme spin-state shift of P450 BM-3 triggered by binding of 3-chlorostyrene was significantly increased by replacement of Phe87 with small amino acid residues (Table 3). The replacement of Phe87 with small amino acid residues also increased the affinity of P450 BM-3 towards 3-chlorostyrene, reflected in the reduction of the dissociation constants (Table 3).

4. Discussion

P450 BM-3 has been crystallized and its structural data are available. The substrate binding site of P450 BM-3 is a long, narrow channel [8], which seems suitable for binding of long-chain fatty acids. However, the results of the present study indicate that propylbenzene and 3-chlorostyrene with structures different from long-chain fatty acids can also efficiently bind to P450 BM-3, and that P450 BM-3 also shows high activity towards these two compounds.

Due to the flexibility of long-chain fatty acids in the substrate binding site of P450 BM-3, the enzyme hydroxylates the substrates at ω-1, ω-2, ω-3 carbons [5,8–10]. Wild-type P450 BM-3 hydroxylates propylbenzene at ω-2 carbons (99.2%) indicating that propylbenzene in the binding site of P450 BM-3, unlike long-chain fatty acids, may lack sufficient flexibility resulting in ω-1 or terminal hydroxylation. However, replacement of Phe87 with Ala (F87A) greatly increases the hydroxylation at ω-1 and the terminal carbons of propylbenzene. This indicates that the flexibility of propylbenzene in the binding site of F87A was significantly increased due to the removal of the Phe87 side chain and the increment of the space of the binding site around the heme iron. Wild-type BM-3 hydroxylated propylbenzene with high stereoselectivity. However, the stereoselectivity was decreased by replacement of Phe87 with small amino acid residues. This also may have been because of increased free space in the binding site due to removal of the large residue.

The epoxidation of 3-chlorostyrene to 3-chlorostyrene oxide by P450 BM-3 also showed stereoselectivity. Although the stereoselectivity of the wild-type is not so strict, the proportion of (*R*)-(−)-3-chlorostyrene oxide was gradually increased by replacement of Phe87 with large to small amino acid residues. The production of (*S*)-(+)-3-chlorostyrene oxide almost disappeared when Phe87 was replaced with the smallest residue, Gly, and (*R*)-(+)-3-chlorostyrene oxide of high optical purity (96.4% *e.e.*) was obtained with F87G. In this case, increasing the free space in the substrate binding site created a superior stereoselective catalyst.

The effects of position 87 in P450 BM-3 on its activities, regio- and/or stereoselective oxidation of long-chain fatty acids have been extensively studied [3,10,11]. F87A hydroxylates laurate and myristate on terminal carbon [10], unlike wild-type on subterminal carbons, and F87V oxidizes arachidonic acid and eicosapentaenoic acid with high stereoselectivity [3]. In this work, we observed that residue size at position 87 of P450 BM-3 also determines its stereoselectivity in propylbenzene and 3-chlorostyrene oxidation. These interesting results together with those of previous studies on modification of substrate specificity by site-directed mutagenesis [12–16] may be useful for engineering of P450 BM-3 to synthesize other useful chiral compounds.

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