Electronic supplementary information

A Substrate-Binding-State Mimic of H_2O_2-Dependent Cytochrome P450 Produced by One-point Mutagenesis and Peroxygenation of Non-native Substrates

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EXPERINENTAL SECTION

Materials. All chemical reagents were purchased from commercial sources and used without further purification. δ-aminolevulinic acid hydrochloride and DNase I (20 U/μL) were purchased from Cosmo Bio Co., Ltd (Tokyo, Japan) and Roche Diagnostics K. K. (Basel, Switzerland), respectively. 2-(*N*-morpholino)ethanesulfonic acid (MES) was purchased from Dojindo Laboratories (Kumamoto, Japan). 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), (±)-2-methyl-2,4-pentanediol (MPD), thrombin, were purchased from Sigma-Aldrich Co., (St. Louis, MO). H_2O_2, dithiothreitol (DTT), ethylene glycol, sodium dodecyl sulfate (SDS), cholic acid, isopropyl-β-D-thiogalactopyranoside (IPTG), kanamycin sulfate, and reduced glutathione were obtained from WAKO Pure Chemical Industries, Ltd (Osaka, Japan). The following chemicals were purchased from Nacalai Tesque Inc. (Kyoto, Japan): glycerol, potassium chloride, tris(hydroxymethyl)aminomethane, urea, hydrochloric acid, phosphoric acid, K_2HPO_4, ampicillin sodium salt, lysozyme, and phenylmethylsulfonyl fluoride.

Preparation of mutants. Site-directed mutagenesis of $P450_{SP\alpha}$ was performed according to the instruction manual of the QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA).[1] The mutagenic primers were 5′-tcgcccgaccgtcgagatcgcagtgtacatc-3′ (sense) and 5′-gatgtacactgcgatctcgacggtcgggcga-3′ (antisense) for A245E, 5′-tcgcccgaccgtcgacatcgcagtgtacatc-3′ (sense) and 5′-gatgtacactgcgatgtcgacggtcgggcga-3′ (antisense) for A245D, and 5′-tcgcccgaccgtccacatcgcagtgtacatc-3′ (sense) and 5′-gatgtacactgcgatgtggacggtcgggcga-3′ (antisense) for A245H, and 5′-gtaaatgtccttgaaccgaccgtcgcgatcg-3′ (sense) and 5′-gatcgcgacggtcggttcaaggacatttacc-3′ (antisense) for R241E. In the same manner to the mutants of $P450_{SP\alpha}$, the mutants of P450BM3, $P450_{cam}$, and CYP119 were prepared. The mutagenic primers were 5′-gcgggacacgaagaaacaagtggtc-3′ (sense) and 5′-gaccacttgtttcttcgtcccgc-3′ (antisense) for T268E of P450BM3, 5′-ggaaattgaccacctcatccaggccgg-3′ (sense) and 5′-gcggcctggatgaggtggtcaatttcc-3′ (antisense) for T252E of $P450_{cam}$, 5′-ccacgaggaaacggcctcgatgacctccctca-3′ (sense) and 5′- tcatcgaggccgtttcctcgtggccggcgatga -3′ (antisense) for T213E of CYP119.

Expression and purification of P450s.

$P450_{SP\alpha}$. A recombinant form of $P450_{SP\alpha}$ and mutants were expressed in *Escherichia coli* BL21 and purified by column chromatography as previously reported. [1, 2]

P450BM3.

The gene of P450BM3 inserted into pT7 was modified. The codon for Gly456 was replaced with a stop codon by mutagenesis with the primers 5′-CCGCTTTAGGGTATTCCTTCA-3′ (forward) and 5′-ACCCTAAAGCGGAATTTTTTTGGATTT-3′ (reverse). The transformed *Escherichia coli* BL21(DE3) were cultivated in LB medium supplemented with 100 μg/ml ampicillin at 37 °C. At log phase, δ-aminolevulinic acid was added at OD_{600} = 0.5 to a final concentration of 0.5 mM. IPTG was added to a final concentration of 1 mM at OD_{600} = 0.8 and the cultivation proceeded at 20 °C for 10 h. The harvested cells were collected by centrifugation and stored at –80 °C. The frozen cells were suspended in 20 mM Tris-HCl buffer (pH 7.4) containing 1 mM DTT, 1 mM EDTA. After sonication of the suspended cells, the supernatant was collected by centrifugation. The solution was loaded on an anion exchange column (DE52, Whatman) and the column was washed with 20 mM Tris-HCl buffer (pH 7.4) containing 1 mM DTT, 1 mM EDTA, and 50 mM KCl. A recombinant form of P450BM3

was eluted with 20 mM Tris-HCl buffer (pH 7.4) containing 1 mM DTT, 1 mM EDTA, and 50-500 mM KCl. The fractions were collected and concentrated to less than 5 ml by centrifugation using Amicon Ultra filter units (Millipore, Co., Cork, Ireland). The concentrated sample was loaded onto an S-200 Sephacryl HR column (GE Healthcare UK Ltd) for size exclusion chromatography using BioAssist eZ (TOSOH Corp.). The purity of P450BM3 was examined using SDS-PAGE (Fig. S1) and the concentration of P450BM3 was determined by pyridine hemochromogen method.[3] T268E mutant was prepared by the same procedure to the wild-type.

P450cam.

E. coli BL21(DE3) transformed with pET-11a containing the gene of P450cam WT were cultivated in LB medium supplemented with 100 μg/ml ampicillin at 37 °C. At log phase, δ-aminolevulinic acid was added at OD_{600} = 0.6 to a final concentration of 0.5 mM. IPTG was added to a final concentration of 1 mM at OD_{600} = 0.8 and the cultivation proceeded at 25 °C for 12 h. The harvested cells were collected by centrifugation and stored at -80 °C. The frozen cells were suspended in 50 mM potassium phosphate buffer (pH 6.0) containing 1mM camphor. After sonication of the suspended cells, the supernatant was collected by centrifugation. The solution was loaded on an anion exchange column (DE52, Whatman) and the column was washed with 50 mM potassium phosphate buffer (pH 6.0) containing 1mM camphor and 50 mM KCl. A recombinant form of P450cam was eluted with 50 mM potassium phosphate buffer (pH 6.0) containing 1mM camphor and 300 mM KCl. The fractions were collected and concentrated to less than 5 ml by centrifugation using Amicon Ultra filter units (Millipore, Co., Cork, Ireland). The concentrated sample was loaded onto DEAE 650S (TOSOH) and the column was washed with 50 mM potassium phosphate buffer (pH 6.0) containing 1mM camphor and 50 mM KCl and eluted with 50 mM potassium phosphate buffer (pH 6.0) containing 1mM camphor and 300 mM KCl. The concentrated sample was loaded onto an S-200 Sephacryl HR column (GE Healthcare UK Ltd) for size exclusion chromatography using BioAssist eZ (TOSOH Corp.). The purity of P450cam was examined using SDS-PAGE (Fig. S1) and the concentration of P450cam was determined by pyridine hemochromogen method. T252E mutant was prepared by the same procedure to wild-type.

CYP119.

The synthetic gene (codon optimized for expression in *E. coli.*) obtained from Eurofins was cloned into pACYCDuet-1 vector. *E. coli* BL21(DE3) transformed with pACYCDuet-1 harboring CYP119 gene was cultivated in LB medium supplemented with 25 μg/ml chloramphenicol) at 37 °C. At log phase, δ-aminolevulinic acid and IPTG (final concentration of 1 mM) was added at OD_{600} = 0.9 and the cultivation proceeded at 30 °C for 47 h. The harvested cells were collected by centrifugation and stored at -80 °C. The frozen cells were suspended in 20 mM Tris-HCl buffer (pH 7.4) containing 1 mM DTT, 1 mM EDTA. After sonication of the suspended cells, the supernatant was collected by centrifugation. The solution was loaded on an anion exchange column (DE52, Whatman) and the column was washed with 50 mM potassium phosphate buffer (pH 6.0) containing 1mM camphor and 50 mM KCl. A recombinant form of P450cam was eluted with 50 mM potassium phosphate buffer (pH 6.0) containing 1mM camphor and 200 mM KCl. The concentrated sample was loaded onto Super Q (TOSOH) and the column was washed with 50 mM potassium phosphate buffer (pH 6.0) containing 1mM camphor and 50 mM KCl and eluted with 50 mM potassium phosphate buffer (pH 6.0) containing 1mM camphor and 300 mM KCl. The fractions were collected and concentrated to less than 5 ml by centrifugation using Amicon Ultra filter units (Millipore, Co., Cork, Ireland). The concentrated sample was loaded onto an S-200 Sephacryl HR column (GE Healthcare UK Ltd) for size exclusion chromatography using BioAssist eZ (TOSOH Corp.). The purity of CYP119 was examined using SDS-PAGE (Fig. S1) and the concentration of CYP119 was determined by pyridine hemochromogen method. T213E mutant was prepared by the same procedure to wild-type.

The sequence of synthetic gene (CYP119) optimized for expression in *E. coli.*

ATGTATGATTGGTTCTCCGAAATGCGCAAGAAAGATCCAGTGTATTATGATGGCAACATTTGGCAGGTGTTCAGTTAT
CGGTATACCAAGGAGGTGTTAAATAACTTTTCGAAATTCTCGAGTGATCTTACTGGTTATCACGAGCGGCTTGAAGAT
CTCCGCAACGGCAAAATTCGGTTTGATATTCCTACACGCTACACGATGTTAACCTCAGACCCACCTCTGCACGATGAAC
TCCGTAGCATGAGCGCGGACATCTTTTCTCCGCAAAAATTACAAACCTTGGAGACATTCATTCGTGAGACTACCCGTTC
CTTGTTAGATTCGATTGACCCACGTGAGGACGACATCGTAAAGAAATTAGCGGTACCGCTGCCGATCATTGTTATCTC
GAAAATCCTTGGGTTACCGATCGAGGATAAAGAGAAGTTCAAGGAATGGTCTGACCTTGTTGCTTTTCGCCTCGGGAA
ACCGGGCGAAATTTTTGAACTCGGAAAGAAATACCTGGAACTGATTGGATACGTGAAAGATCACTTGAATAGCGGCAC
TGAAGTCGTGAGTCGCGTTGTTAACTCCAACCTGAGTGATATCGAAAAACTGGGTTATATTATCCTGTTGCTCATCGC
AGGCAATGAAACGACCACGAATTTGATCAGCAATTCTGTTATCGACTTCACGCGCTTCAATTTGTGGCAGCGCATTCGT

GAAGAAAATCTGTACCTGAAAGCGATCGAAGAAGCGCTGCGCTATTCACCGCCGGTCATGCGCACCGTGCGCAAAACG
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GATGAAGAGGTGTTTCATGATGGCGAAAAATTTATTCCGGATCGCAACCCGAACCCGCATCTGTCATTTGGTTCTGGC
ATTCATCTGTGCCTGGGTGCACCGCTGGCCCGCCTGGAAGCCCGTATTGCCATTGAGGAATTTAGCAAACGTTTTCGTC
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GCAATGAATAA

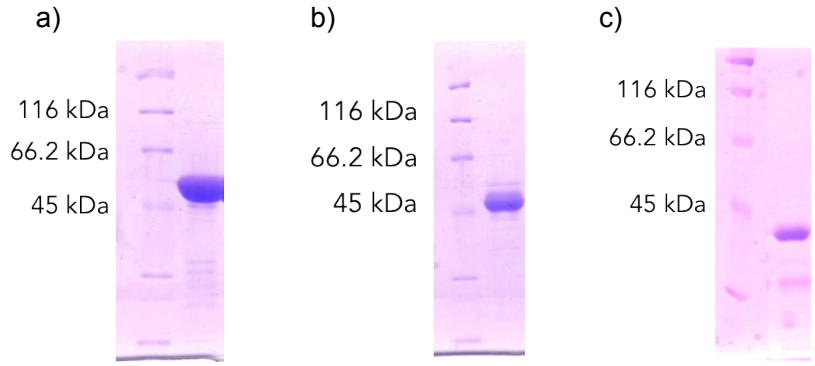


Fig. S1 SDS-Page of P450BM3 T268E (a), P450cam T252E (b), and CYP119 T213E(c) after purification.

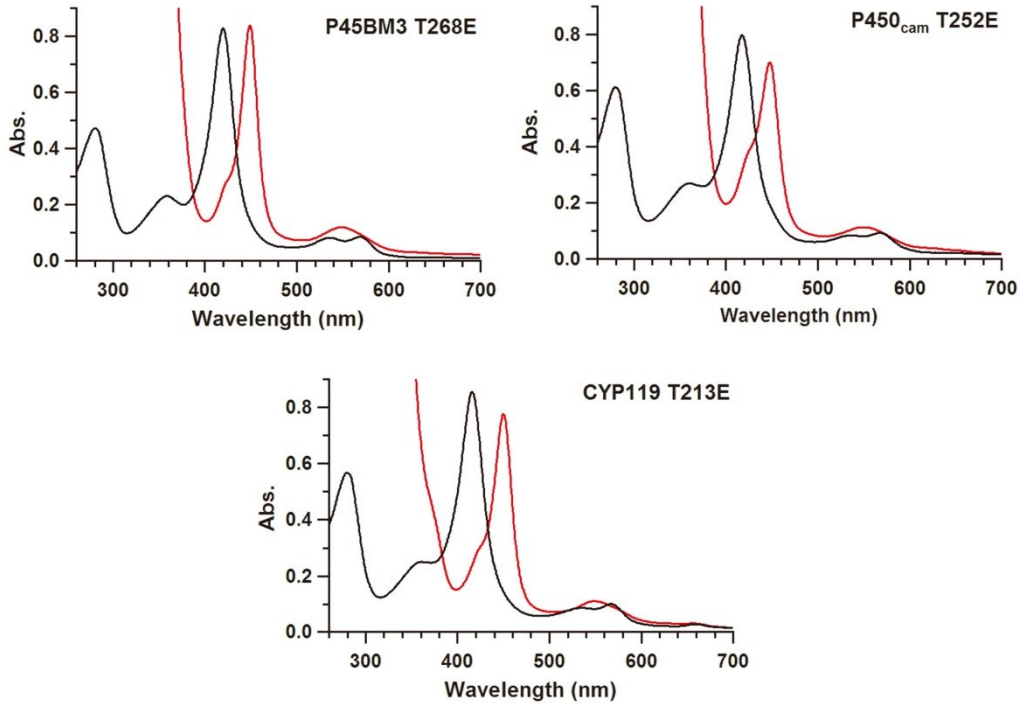


Fig. S2 UV-visible absorption spectra of mutants. T268E mutant of P450BM3 in 50 mM Tris-HCl (pH 7.4), T252E mutant of $P450_{cam}$ in 50 mM potassium phosphate buffer (pH 7.4), and T213E mutant of CYP119 in 50 mM Tris-HCl (pH 7.4). The resting state (black line) and Fe^{2+}-CO state (red line).

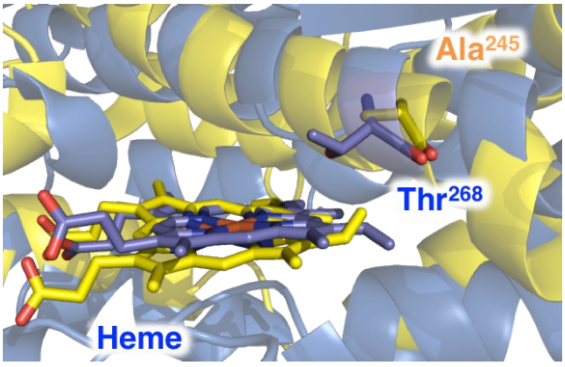


Fig. S3 The active site structures of $P450_{SP\alpha}$ (yellow) superposed on that of P450BM3 (blue). The heme, Ala-245 of $P450_{SP\alpha}$, and Thr-268 of P450BM3 were shown as stick models.

Styrene oxidation. Styrene was purified by aluminum column chromatography to remove a polymerization inhibitor before measurements and used immediately after the purification.[4] Epoxidation of styrene was performed as follows. $P450_{SP\alpha}$: 0.5-3 mM styrene, 4 mM H_2O_2 and 1μM $P450_{SP\alpha}$ in 0.1 M potassium phosphate buffer (pH 7.0) at 25 °C for 1 min. P450BM3, P450cam, and CYP119: 1-15 mM styrene, 60 mM H_2O_2 and 5μM P450 in 0.1 M potassium phosphate buffer (pH 7.0) at 25 °C (at 70 °C in the case of CYP119) for 1 min. Dichloromethane was added immediately to the reaction mixture for quenching and methyl phenylacetate was added as an internal standard. The extract was evaporated and the resulting solution was analyzed by gas chromatography (Shimadzu GC-2014) equipped with a cyclosil-β column (Agilent Technologies, Inc.). The absolute conFiguration of styrene oxide was determined by using authentic (*R*)-styrene oxide. GC analytical conditions were as follows: injector temperature: 200 °C, detector temperature: 250 °C oven temperature: 90 °C (30 min)-10 °C/min-190 °C (20 min), carrier gas: He. The retention times of styrene products and the internal standard were as follows: styrene (3.6 min), (R)-styrene oxide (13.0 min), (S)-styrene oxide (13.7 min), phenylacetaldehyde (15.2 min), methyl phenylacetate (28.2 min). All initial turnover rates are the average of at least three measurements and evaluated by Michaelis–Menten kinetics.

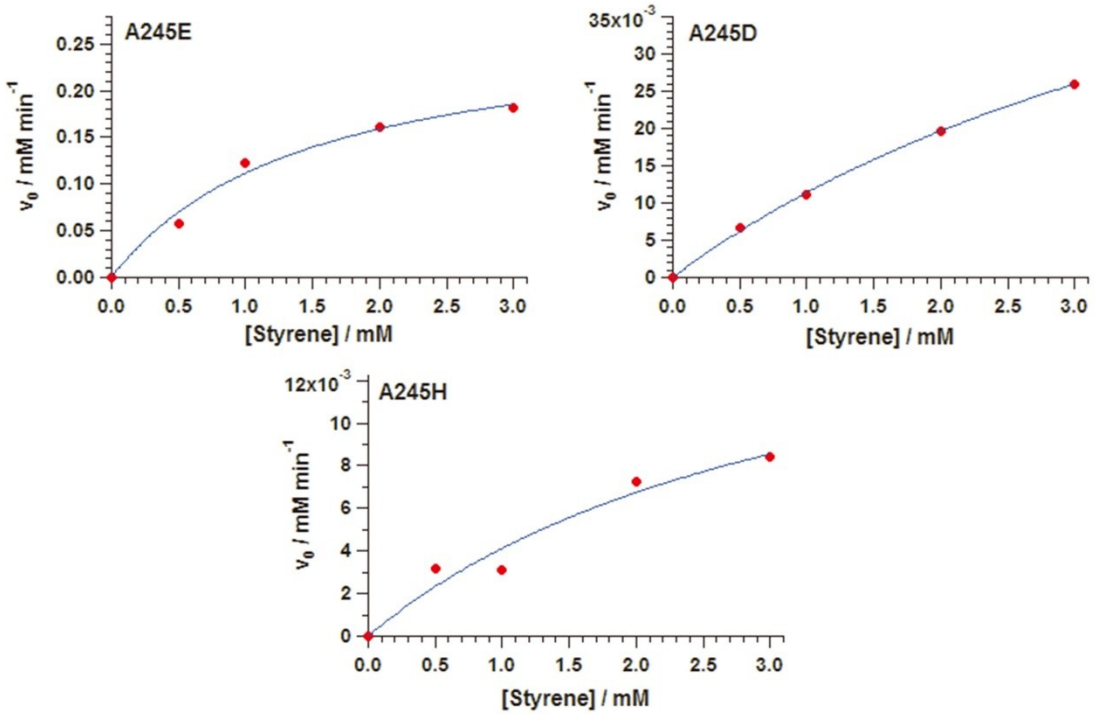


Fig. S4 Plots of the initial rate (v_0) of styrene oxidation as a function of styrene concentration.

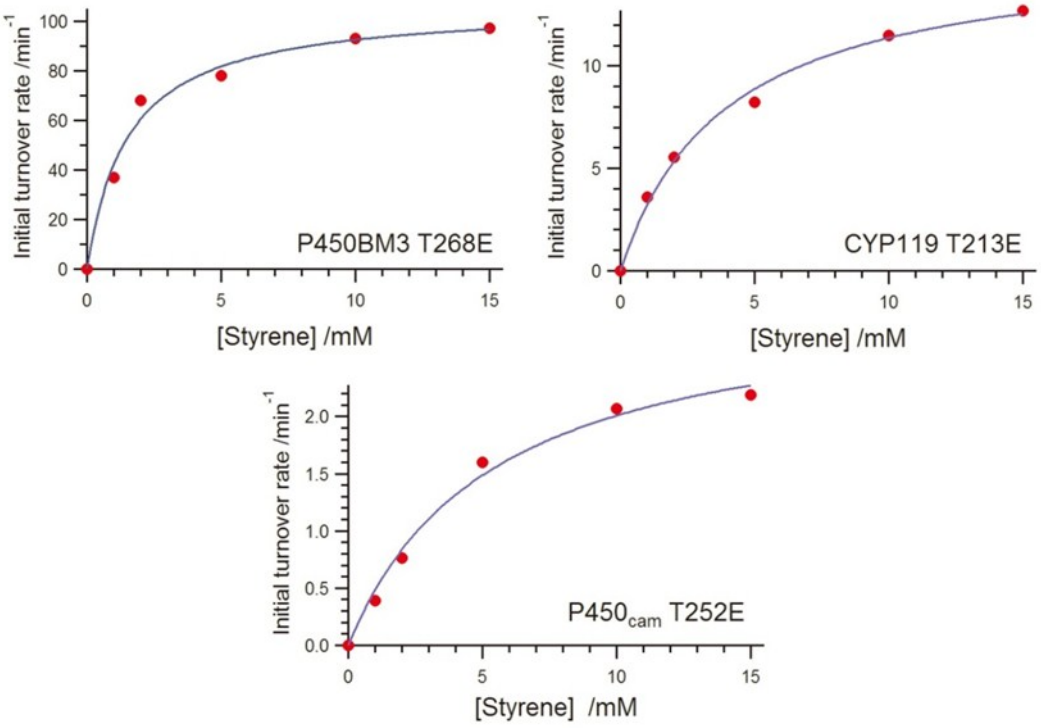


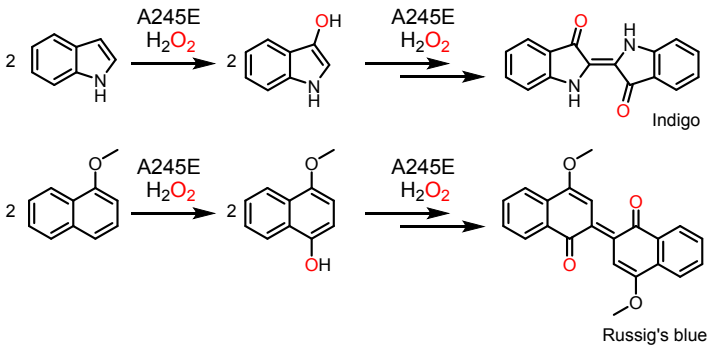
Fig. S5 Plots of the initial turnover rate of styrene oxidation as a function of styrene concentration.

1-Methoxynaphthalene oxidation

The reaction was carried out in 100 mM potassium phosphate buffer (pH 7.0) with 0.5 µM of A245E mutant of $P450_{SP\alpha}$, 0.5 mM of 1-methoxynaphthalene, and 0.5mM of H_2O_2 for 1 min at 25 °C. After the reaction, the reaction mixture was extracted with chloroform. The extract was filtrated, transferred into a volumetric flask, and diluted to 5 mL. The catalytic activities for 1-methoxynaphthalene were determined by monitoring the absorption at 634.5 nm with a molar absorption coefficient of 18,900 $M^{-1}cm^{-1}$ in chloroform. The initial turnover rates are averages of at least three measurements and are expressed in terms of micromoles of product per minute per micromole of P450.

Indole oxidation

The reaction was carried out in 100 mM potassium phosphate buffer (pH 7.4) containing 0.5 mM indole, 0.5 µM of A245E mutant of $P450_{SP\alpha}$, 0.5mM of H_2O_2 for 1 min at 25 °C. After the reaction, the mixture is extracted with chloroform to extract indigo from aqueous phase. The catalytic activity was determined by the absorbance of indigo at 605 nm with a molar absorption coefficient of 11,000 $M^{-1}cm^{-1}$ and the turnover numbers were averaged of least three measurements and expressed in terms of micromolar of product per micromolar of P450 per minute.



Scheme S1 The hydroxylation reaction of indole and 1-methoxynaphtalen catalyzed by A245E mutant of $P450_{SP\alpha}$ giving indigo and Russig's blue, respectively.

Myristic acid oxidation

The reaction mixture contained 0.1 M potassium phosphate (pH 7.4), 100 μM myristic acid, 5μM P450BM3 or CYP119, and 20 mM H_2O_2 in a total volume of 1 mL. The reaction mixture was incubated at 70 °C for 1 min and then the reaction was quenched by adding 1 mL of dichloromethane. After the addition of palmitic acid as an internal standard, the products were extracted with dichloromethane. For derivatization of the extract, 50 μL of *N, O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylsilylchlorosilane (TMCS) was added and the mixture was incubated in the dark at room temperature for 1 h. The derivatized products were analyzed using a GCMS-QP2010 SE (Shimadzu Corp., Kyoto, Japan) equipped with DB-624 capillary column (30 m × 0.32 mm; Agilent Technologies, Inc., Santa Clara, CA) to identify the products. The GC/MS analytical conditions were as follows: column temperature, 80 °C (2 min) to 10 °C/min (17 min) to 250 °C (15 min); injection temperature, 250 °C; interface temperature, 250 °C; ditecter temperature, 250 °C; carrier gas, He; flow rate, 1.9 mL/min, mode, splitless mode. The amount of products was estimated based on decrease of myristic acid. All initial turnover rates are the average of at least three measurements.

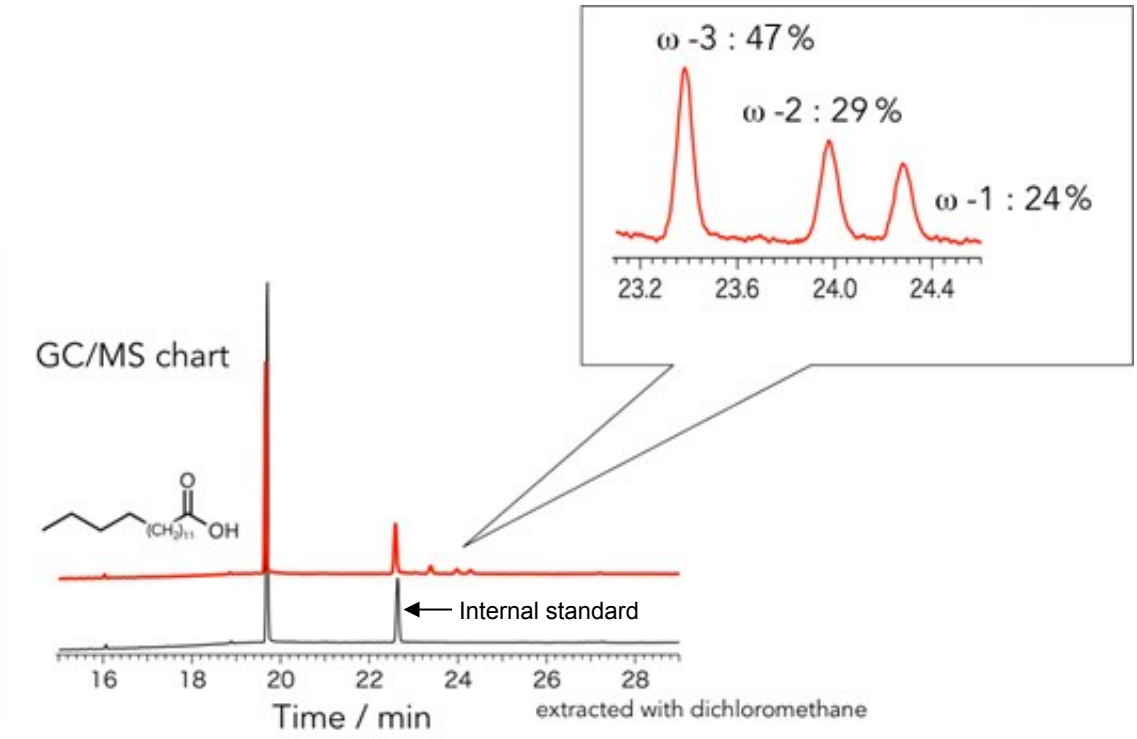


Fig. S6 GC-mass analysis of the H_2O_2-dependent hydroxylation reaction of myristic acid catalyzed by wild-type (black line) and T268E mutant (red line) of P450BM3.

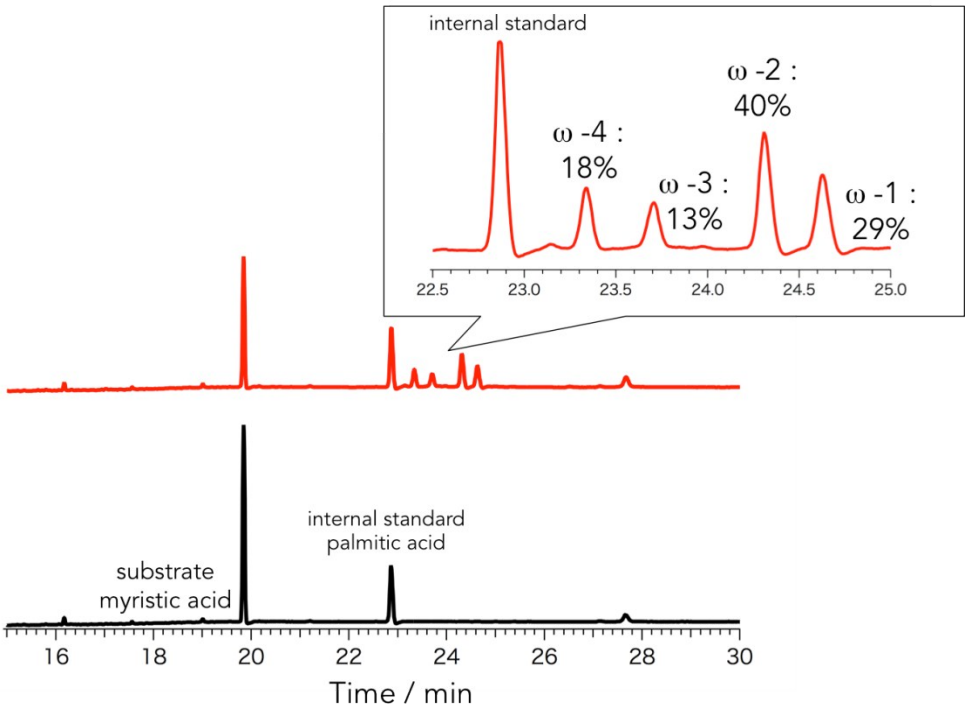


Fig. S7 GC-mass analysis of the the H_2O_2-dependent hydroxylation reaction of myristic acid catalyzed by wild-type (black line) and T213E mutant (red line) of CYP119.

Camphor oxidation

The reaction mixture contained 100 mM potassium phosphate (pH 7.4), 100 μM camphor, 10μM of $P450_{cam}$, and 20 mM H_2O_2 in a total volume of 0.5 mL. The reaction mixture was incubated at 25 °C for 1 min and then the reaction was quenched by adding 0.5 mL of ethyl acetate. After the addition of 1-nonanol as an internal standard, the products were extracted with ethyl acetate. The products were analyzed using a GCMS-QP2010 SE (Shimadzu Corp., Kyoto, Japan) equipped with DB-624 capillary column (30 m × 0.32 mm; Agilent Technologies, Inc., Santa Clara, CA) to identify the products. The GC/MS analytical conditions were as follows: column temperature, 80 °C (2 min) to 25 °C/min (6.8 min) to 250 °C (3 min); injection temperature, 250 °C; interface temperature, 250 °C; ditecter temperature, 250 °C; carrier gas, He; flow rate, 1.9 mL/min, mode, splitless mode. The total amount of products was estimated based on decrease of camphor. All initial turnover rates are the average of at least three measurements.

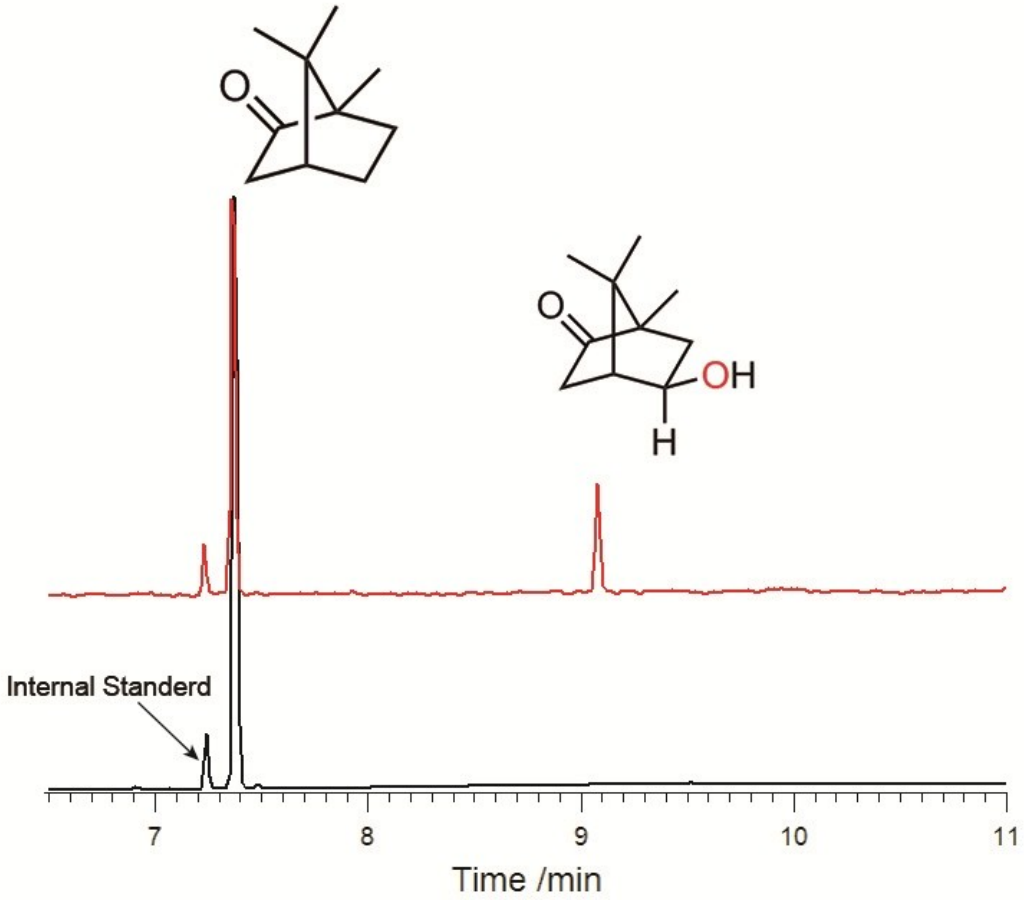


Fig. S8 GC-mass analysis of the the H_2O_2-dependent hydroxylation reaction of *d*-camphor catalyzed by wild-type (black line) and T252E mutant (red line) of P450cam.

Crystallization of $P450_{SP\alpha}$.

The A245E of $450_{SP\alpha}$ was concentrated to 10 mg/ml in 50 mM MES (pH 7.0) containing 20%(v/v) glycerol by centrifugation using Amicon Ultra filter units (Millipore, Co.). A 2 μL aliquot of the concentrated $P450_{SP\alpha}$ solution was mixed with 2 μL of a reservoir solution composed of 0.1 M HEPES (pH 7.0) and 35%(v/v) MPD. Crystals of $P450_{SP\alpha}$ were grown by a sitting-drop vapor diffusion method at 20 °C for 6 days. The A245D and R241E were crystallized under the same conditions used for the A245E. The A245H was crystalized at 4 °C.

Data collection and refinement.

Crystals were flash-cooled in liquid nitrogen. X-ray diffraction data sets were collected on beam line BL26B1 equipped with RIGAKU Saturn A200 CCD detector at the RIKEN SPring-8 (Hyogo, Japan) with a 1.0 Å wavelength at 100 K. HKL2000 program[5] was used for integration of the diffraction intensities and scaling. The structure of mutants were solved by a molecular replacement using MolRep.[6] The model building and refinement were performed using COOT[7] and REFMAC5.[8] TLS refinement [9] was performed at the final stage of the refinement, defining each chain in the asymmetric unit as a separate TLS group. Structure validation was performed using PROCHECK. [10] The final refinement statistics are summarized in Table S1.

Table S1
Data collection and refinement statistics

$P450_{SPa}$ mutant (PDB code)	A245E (3VOO)	A245H (3VTJ)	R241E (3VNO)
Data collection			
Wavelength (Å)	1	1	1
Space group	$P3_121$	$P3_121$	$P3_121$
Cell dimensions			
a, *b*, *c* (Å)	94.426, 94.426, 113.260	94.558, 94.558, 114.183	94.54, 94.54, 113.004
a, b, g (°)	90.000, 90.000, 120.000	90.000, 90.000, 120.000	90.000, 90.000,120.000
Resolution (Å)	50.00–2.34 (2.42-2.34)	50.0–2.56 (2.56–2.65)	20.0–2.17 (2.25–2.17)
No. of total observed reflections	266694	203334	328897
No. of unique reflections	25136	19489	31376
R_{merge} [a, b] (%)	5.8 (36.4)	11.0 (35.0)	4.8 (33.9)
Completeness [a] (%)	99.9 (100)	100.0 (100.0)	99.9 (100.0)
$I/\mathrm{s}(I)$ [a]	44.2 (6.5)	22.3 (7.2)	47.0 (7.8)
Redundancy [a]	10.6 (11.0)	10.4 (10.8)	10.5 (10.9)
Refinement statistics			
Resolution range (Å)	19.64–2.34	19.94–2.56	19.79–2.17
No. of monomer/asymmetric unit	1	1	1
$R_{\mathrm{work}}/R_{\mathrm{free}}$ [c, d] (%)	19.6/23.4	18.8/23.4	19.9/22.7
RMSD bond length [e] (Å)	0.007	0.010	0.012
RMSD bond angles [e] (º)	1.269	1.612	1.250
No. of atoms	3374	3331	3359
Average *B*-factor (Å^2)	19.0	24.4	18.2

[a] The values in parentheses are for the highest resolution shell.

[b] $R_{\mathrm{merge}} = \Sigma_{hkl}\Sigma_i|I_i(hkl) - \langle I(hkl)\rangle| / \Sigma_{hkl}\Sigma_i I_i(hkl)$, where $\langle I(hkl)\rangle$ is the average intensity of the i observations.

[c] $R_{\mathrm{work}} = \Sigma_{hkl}|F_{obs}(hkl) - F_{calc}(hkl)|\Sigma_{hkl}F_{obs}(hkl)$, where F_{obs} and F_{calc} are the observed and calculated structure factors, respectively.

[d] R_{free} was calculated with 5% of the reflections that were not included in the refinement.

[e] r. m. s. d. = root mean square deviation

Structural analysis of P450$_{SP\alpha}$. The accessible channels were calculated using CAVER.[11] All protein Figures were depicted using PyMOL.[12]

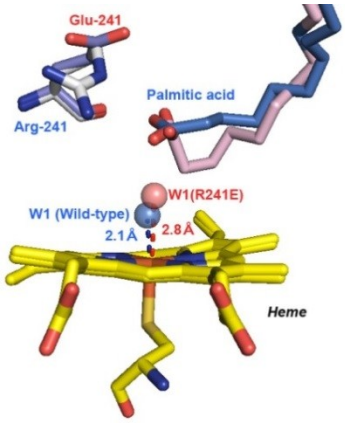


Fig. S9 The active site structures of R241E superposed on that of wild-type. The water molecule in wild-type coordinated to the heme iron is shown in blue sphere and the water molecule above the heme in R241E is shown in red sphere.

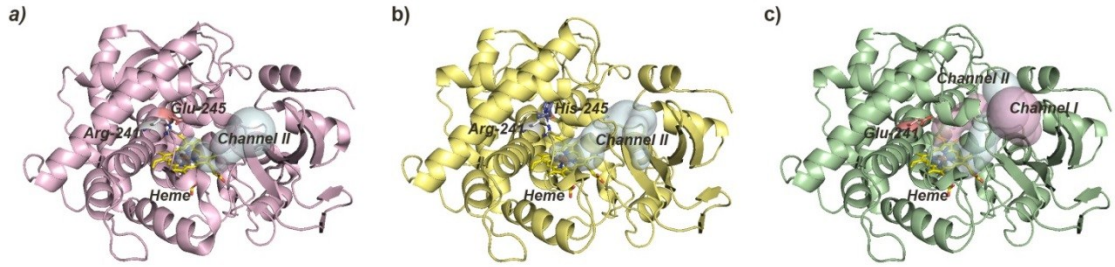


Fig. S10 a) Overall structure of A245E (a), A245H (b), and R241E (c). The two channels were represented as pink (Channel I) and light-blue surfaces (Channel II). Heme, Arg-241, Ala-245, His-245, Glu-241 are represented as stick models.

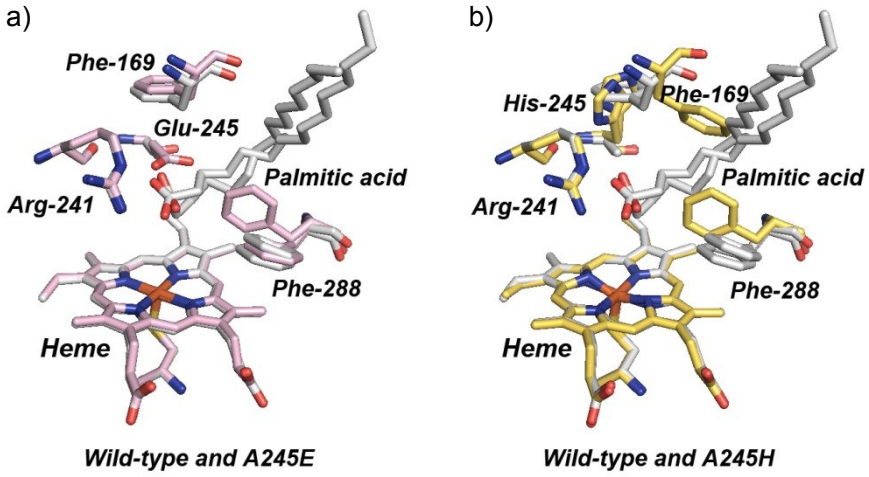


Fig. S11 The active site structures of A245E shown as pink stick models (a) and A245H shown as yellow stick models (b) superposed on that of wild-type represented as white stick models. Two alternative conformations of palmitic acid (wild-type and A245E), Phe-288 (wild-type and A245E), His-245 (A245H) are shown.

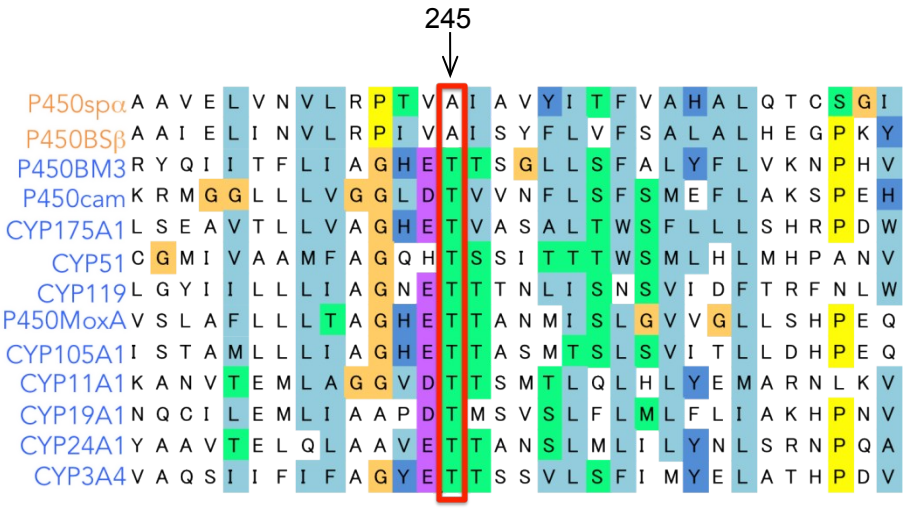


Fig. S12 Clustal W alignment of amino acid sequence of P450s. The sequence number of CYP152A1 is shown on the top of amino acid sequence.

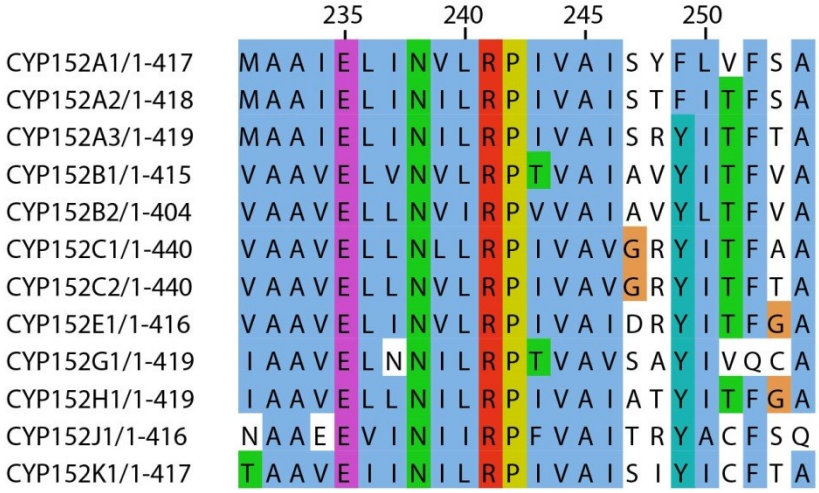


Fig. S13 A clustal W amino acid sequence alignment of CYP152 family. The sequence number of CYP152A1 is shown on the top of amino acid sequence.

References

1. I. Matsunaga, T. Sumimoto, E. Kusunose and K. Ichihara, *Lipids*, 1998, **33**, 1213-1216.
2. I. Matsunaga, N. Yokotani, O. Gotoh, E. Kusunose, M. Yamada and K. Ichihara, *J. Biol. Chem.*, 1997, **272**, 23592-23596.
3. E. B. Flink and C. J. Watson, *J. Biol. Chem.*, 1942, **146**, 171-178.
4. W. L. F. Armarego and D. D. Perrin, *Purification of Laboratory Chemicals, 4th Ed.*, Butterworth-Heinemann, Oxford, 1996.
5. Z. Otwinowski, D. Borek, W. Majewski and W. Minor, *Acta Crystallogr. Sect. A: Found. Crystallogr.*, 2003, **59**, 228-234.
6. A. Vagin and A. Teplyakov, *J. Appl. Crystallogr.*, 1997, **30**, 1022-1025.
7. P. Emsley and K. Cowtan, *Acta Crystallogr. Sect. D. Biol. Crystallogr.*, 2004, **60**, 2126-2132.
8. M. D. Winn, G. N. Murshudov and M. Z. Papiz, *Methods Enzymol.*, 2003, **374**, 300-321.
9. M. D. Winn, M. N. Isupov and G. N. Murshudov, *Acta Crystallogr. Sect. D. Biol. Crystallogr.*, 2001, **57**, 122-133.
10. R. A. Laskowski, M. W. Macarthur, D. S. Moss and J. M. Thornton, *J. Appl. Crystallogr.*, 1993, **26**, 283-291.
11. M. Petrek, M. Otyepka, P. Banas, P. Kosinova, J. Koca and J. Damborsky, *BMC Bioinformatics*, 2006, **7**.
12. W. L. DeLano, *The PyMOL Molecular Graphics System (2002) on World Wide Web* http://www.pymol.org., 2002.