Supplemental data

Crystal Structure of H_2O_2-Dependent Cytochrome $P450_{SP\alpha}$ with Its Bound Fatty Acid Substrate: Insight into the Regioselective Hydroxylation of Fatty Acids at the α-Position

Takashi Fujishiro, Osami Shoji, Hiroshi Sugimoto, Shingo Nagano, Yoshitsugu Shiro, Yoshihito Watanabe

MATERIALS AND METHODS

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. *N*, *O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and 9-anthryldiazomethane (ADAM) were obtained from Tokyo Chemical Industry Co. (Tokyo, Japan) and Funakoshi Co., Ltd (Tokyo, Japan), 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, 12-hydroxydodecanoic acid (12-OH C_{12}), myristic acid (C_{14}), α-hydroxymyristic acid (α-OH C_{14}), and β-hydroxymyristic acid (β-OH C_{14}) 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): trimethylchlorosilane (TMCS), glycerol, potassium chloride, tris(hydroxymethyl)aminomethane, urea, hydrochloric acid, phosphoric acid, K_2HPO_4, ampicillin sodium salt, lysozyme, and phenylmethylsulfonyl fluoride.

Preparation of recombinant forms of $P450_{SP\alpha}$.

A recombinant enzyme of $P450_{SP\alpha}$ (S1, S2) was expressed in *Escherichia coli* BL21 as previously reported with some modification. The transformed *E. coli* cells were cultivated in LB medium supplemented with 100 mg/ml ampicillin at 27 °C. At log phase, δ-aminolevulinic acid was added at OD_{600} = 0.7–0.8 to a final concentration of 0.5 mM. For expression of the recombinant enzyme of $P450_{SP\alpha}$, IPTG was added to a final concentration of 0.1 mM at OD_{600} = 1.0–1.2 and the cultivation proceeded at 20 °C for 20 h. The harvested cells were collected by centrifugation and stored at –80 °C. The frozen cells were resuspended in 0.1 M Tris-HCl buffer (pH 7.5) containing 20%(v/v) ethylene glycol, 1 mM DTT, 1%(w/v) cholic acid, 0.1%(w/v) SDS, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride containing 1 mg/ml lysozyme, and 140 U/100 ml DNase I. After sonication of the suspended cells, the supernatant was collected by centrifugation and diluted in two volumes of 0.1 M Tris-HCl buffer (pH 7.5) containing 20%(v/v) ethylene glycol and 1 mM DTT. The

diluted solution was loaded on a glutathione–sepharose column (GE Healthcare UK Ltd, Little Chalfont, UK) and the column was washed with 0.1 M Tris-HCl buffer (pH 7.5) containing 20%(v/v) ethylene glycol, 1 mM DTT, 0.3%(w/v) cholic acid, and 0.03%(w/v) SDS. Glutathione *S*-transferase (GST)-fusion $P450_{SP\alpha}$ was eluted with 0.1 M Tris-HCl buffer (pH 7.5) containing 20%(v/v) ethylene glycol, 1 mM DTT, 0.3%(w/v) cholic acid, 0.03%(w/v) SDS, 1.5 M urea, and 5 mM reduced glutathione. Thrombin cleavage of GST-fusion $P450_{SP\alpha}$ was performed during dialysis against 0.1 M potassium phosphate buffer (pH 7.0) containing 0.3 M KCl and 20%(v/v) glycerol at 4 °C for 15 h. The reaction mixture was passed through a glutathione Sepharose column and a benzamidine Sepharose column (GE Healthcare UK Ltd) to separate GST and thrombin from $P450_{SP\alpha}$, respectively. 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. The purity of $P450_{SP\alpha}$ was estimated using SDS-PAGE and the concentration of $P450_{SP\alpha}$ was determined using CO difference spectra (S3, S4). All mutants were prepared according to the procedure used for the WT. The substrate free-form of $P450_{SP\alpha}$ was prepared by removing fatty acids originating from *E. coli* cells. $P450_{SP\alpha}$ was passed thorough Hydroxyalkoxypropyl-Dextran, Type VI (Sigma-Aldrich, Co., USA) column equilibrated with 0.1 M potassium phosphate buffer (pH 7.0) at room temperature, followed by dialysis against 0.1 M potassium phosphate buffer (pH 7.0) containing 0.3 M KCl and 20%(v/v) glycerol. The amount of fatty acids after the column chromatography was estimated to be less than 3% ([Fatty acid]/[$P450_{SP\alpha}$] × 100) by gas chromatography according to the previously reported procedure. (S5)

Preparation of $P450_{SP\alpha}$ and $P450_{BS\beta}$ 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) using pGEX-AX2 plasmids containing the gene of $P450_{SP\alpha}$ WT as templates (S2). The mutagenic primers were (mutated codons are underlined and capitalized) 5′-cagaagacactgTTTggccaaggcgg-3′ (sense) and 5′-cgccgccttggccAAAcagtgtcttc-3′ (antisense) for $P450_{SP\alpha}$ L78F, 5′-gcgctgttcgacgccTTCggctc-3′ (sense) and 5′-ctcgccgagccGAAggcgtcg-3′ (antisense) for $P450_{SP\alpha}$ A172F, and 5′-attctatcccttcGGTcccgctgtggt-3′ (sense) and 5′-caccacagcgggACCgaagggatagaa-3′ (antisense) for $P450_{SP\alpha}$ F288G. Expression and purification of the $P450_{SP\alpha}$ mutants were performed as described above. Site-directed mutagenesis of $P450_{BS\beta}$ was performed in the same manner for the $P450_{SP\alpha}$ mutants using pQE30tBSβ WT (S6) and $P450_{BS\beta}$ F79L (S7) plasmids as templates. The mutagenic primers for the G290F mutant of $P450_{BS\beta}$ were 5′-ccgcagatattatccgttcTTCccgtttttagg-3′ (sense) and 5′-gcgcccctaaaaacggGAAgaacgg-3′ (antisense). Expression and purification of the $P450_{BS\beta}$ mutants were performed as previously reported.

References

S1. Matsunaga, I., Yokotani, N., Gotoh, O., Kusunose, E., Yamada, M., and Ichihara, K. (1997) *J. Biol. Chem.* **272**, 23592–23596

S2. Matsunaga, I., Sumimoto, T., Kusunose, E., and Ichihara, K. (1998) *Lipids* **33**, 1213–1216

S3. Omura, T. and Sato, R. (1964) *J. Biol. Chem.* **239**, 2370–2378

S4. Gunsalus, I. C., Wagner, G. C., (1978) *Methods Enzymol.* **52**, 166–188.

S5. Shoji, O., Fujishiro, T., Nagano, S., Tanaka, S., Hirose, T., Shiro, Y., and Watanabe, Y. (2010) *J. Biol. Inorg. Chem.* **15**, 1331-1339

S6. Matsunaga, I., Ueda, A., Sumimoto, T., Ichihara, K., Ayata, M., and Ogura, H. (2001) *Arch. Biochem. Biophys.* **394**, 45–53

S7. Lee, D.-S., Yamada, A., Sugimoto, H., Matsugana, I., Ogura, H., Ichihara, K., Adachi, S.-i., Park, S.-Y., and Shiro, Y. (2003) *J. Biol. Chem.* **278**, 9761–9767

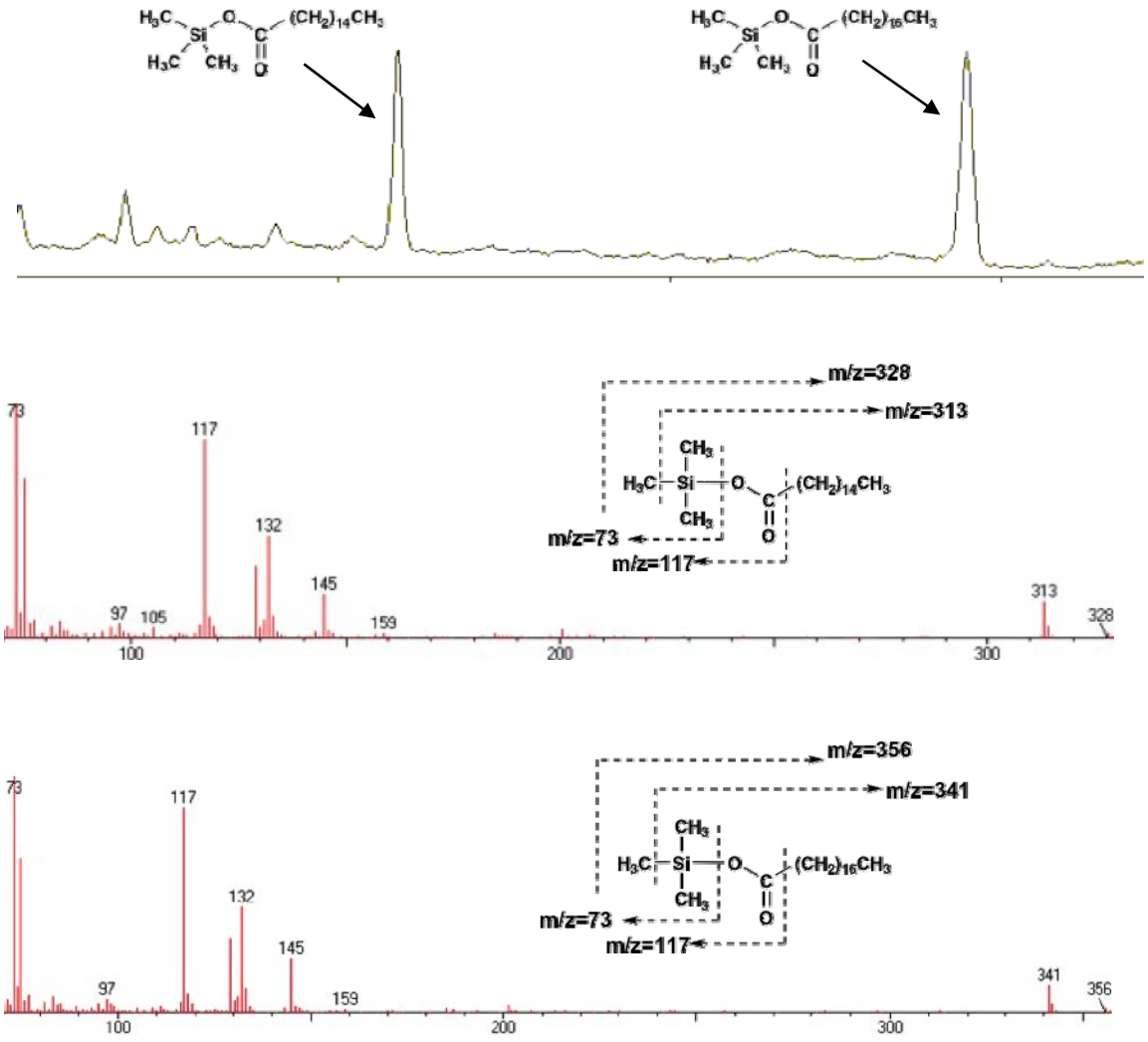

Figure S1. GC/MS analysis of the extracts of P450$_{SP\alpha}$ WT. *A*, GC chromatogram. The peaks of the extracted palmitic acid and stearic acid derivatives are indicated by arrows with their chemical structures. *B*, Mass spectrum of the palmitic acid derivative. *C*, Mass spectrum of the stearic acid derivative.

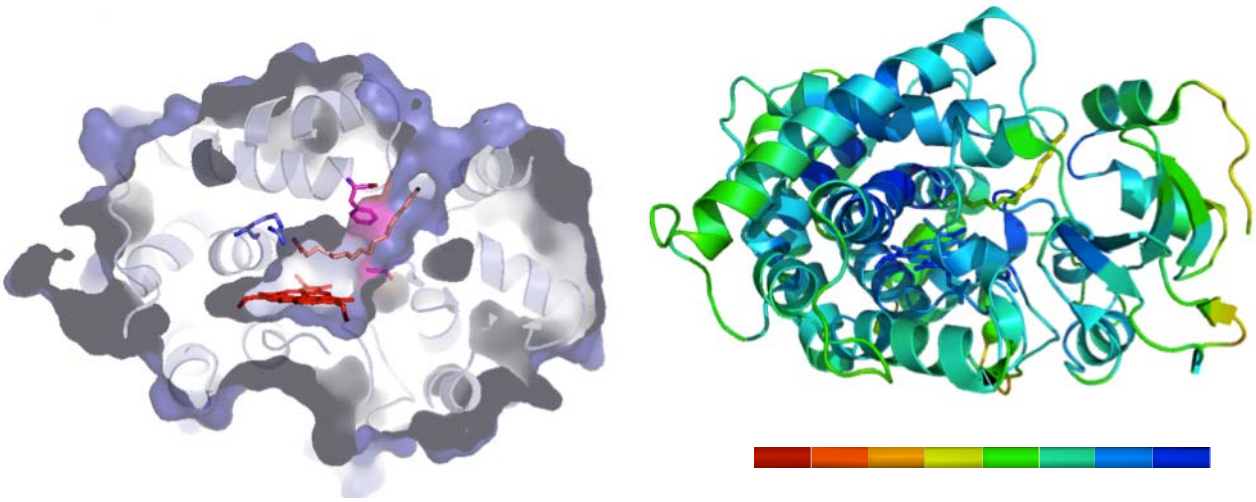

Figure S2. The substrate access channel of $P450_{BS\beta}$. *A*, The substrate access channel of $P450_{BS\beta}$. Heme, palmitic acid, Phe-173, and Gly-290 are represented as *stick models*. *B*, *B*-factor of $P450_{BS\beta}$.

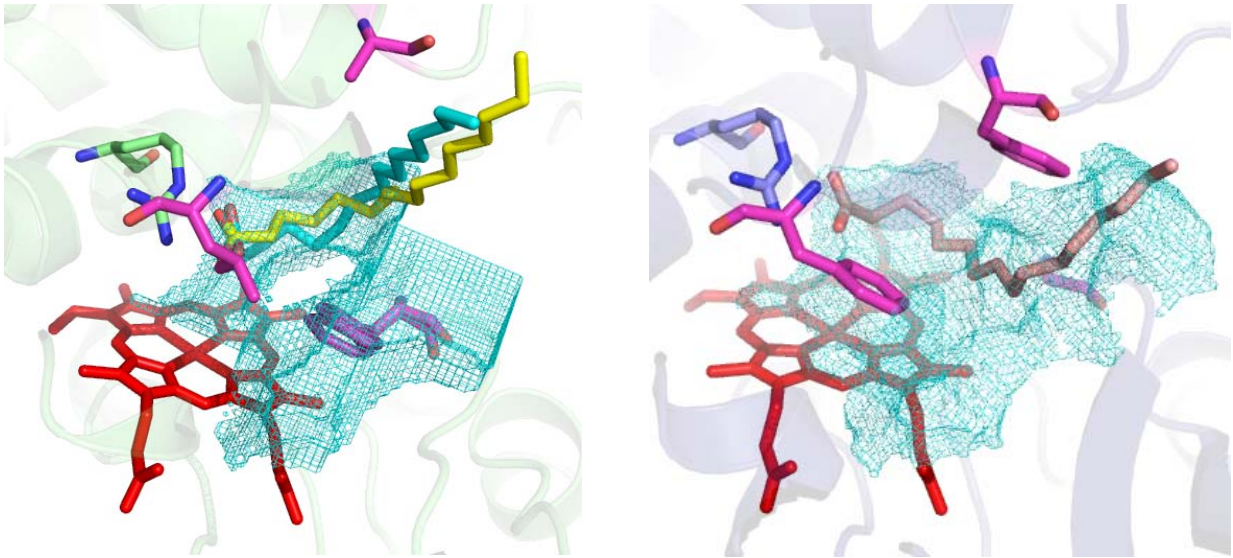

Figure S3. The active site structures of $P450_{SP\alpha}$ (*A*) and $P450_{BS\beta}$ (*B*). Palmitic acid, heme, Arg-241 of $P450_{SP\alpha}$, and Arg-242 of $P450_{BS\beta}$ are represented as *red*, *cyan* and *yellow*, *pink*, *green*, and *blue* stick models, respectively. Leu-78, Ala-172, and Phe-288 of $P450_{SP\alpha}$ and Phe-79, Phe-173, and Gly-290 of $P450_{BS\beta}$ are represented as *magenta* stick models. The active site cavities were calculated using VOIDOO and are shown in *light-blue* mesh.

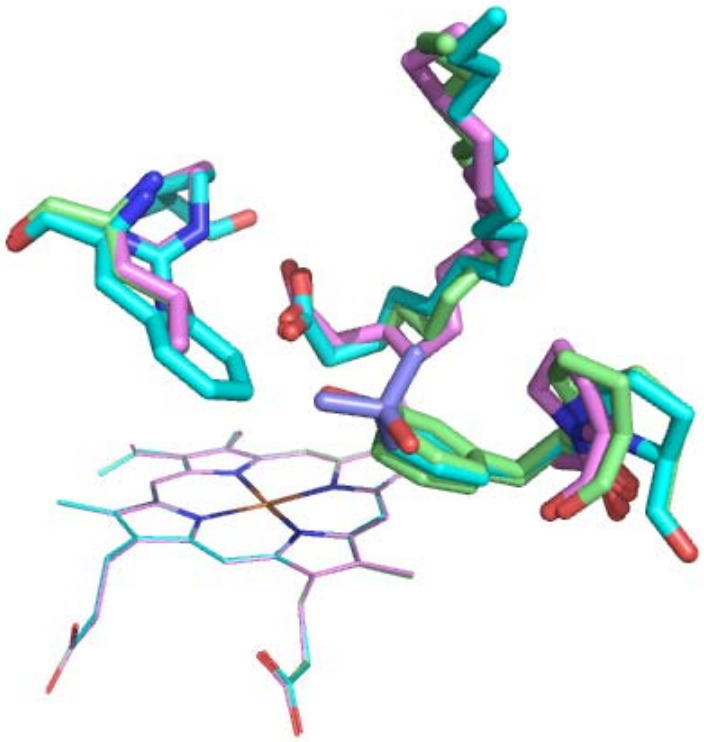

Figure S4. Comparison of WT (green), L78F (purple), and F288G (cyan). Superimposition of two mutants and WT $P450_{SP\alpha}$. *A*, Side view from the propionate of the heme. Heme is represented as a line. MPD in F288G is represented as a blue stick model.

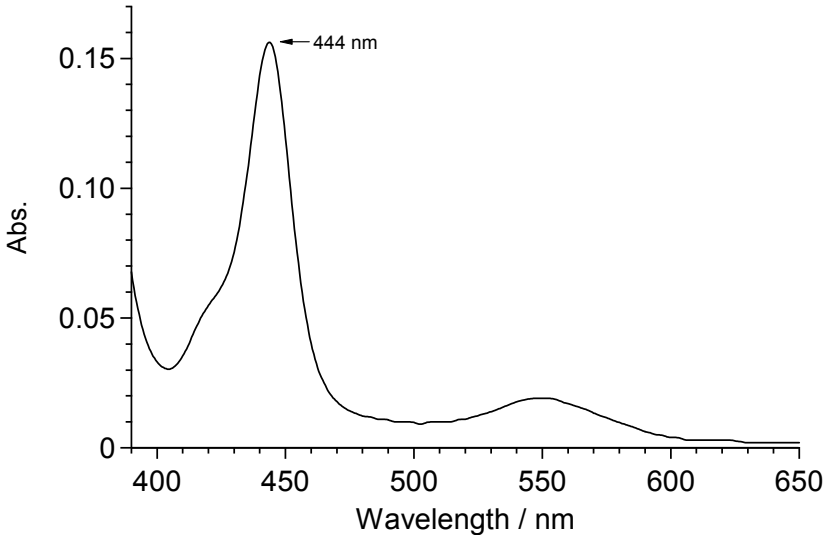

Fig. S5. UV–visible absorption spectra of the ferrous-CO bound form of $P450_{SP\alpha}$ in 0.1 M potassium phosphate buffer (pH 7.0) containing 0.3 M KCl, 20%(v/v) glycerol, and sodium dithionite. The buffer was saturated with carbon monoxide (CO). The concentration of $P450_{SP\alpha}$ was 1.3 μM.