J Biol Inorg Chem (2010) 15:1109–1115
DOI 10.1007/s00775-010-0671-9

ORIGINAL PAPER

Aromatic C–H bond hydroxylation by P450 peroxygenases: a facile colorimetric assay for monooxygenation activities of enzymes based on Russig's blue formation

Osami Shoji · Christian Wiese · Takashi Fujishiro · Chikako Shirataki · Bernhard Wünsch · Yoshihito Watanabe

Received: 19 December 2009 / Accepted: 27 April 2010 / Published online: 19 May 2010
© SBIC 2010

Abstract Aromatic C–H bond hydroxylation of 1-methoxynaphthalene was efficiently catalyzed by the substrate misrecognition system of the hydrogen peroxide dependent cytochrome $P450_{BS\beta}$ (CYP152A1), which usually catalyzes hydroxylation of long-alkyl-chain fatty acids. Very importantly, the hydroxylation of 1-methoxynaphthalene can be monitored by a color change since the formation of 4-methoxy-1-naphthol was immediately followed by its further oxidation to yield Russig's blue. Russig's blue formation allows us to estimate the peroxygenation activity of enzymes without the use of high performance liquid chromatography, gas chromatography, and nuclear magnetic resonance measurements.

Keywords Cytochrome P450 · Hydroxylation · Hydrogen peroxide · Colorimetric assay · Enzyme catalysis

Electronic supplementary material The online version of this article (doi:10.1007/s00775-010-0671-9) contains supplementary material, which is available to authorized users.

O. Shoji · T. Fujishiro · C. Shirataki
Department of Pharmaceutical and Medicinal Chemistry, Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464-8602, Japan

Y. Watanabe (✉)
Research Center for Materials Science, Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464-8602, Japan
e-mail: yoshi@nucc.cc.nagoya-u.ac.jp

C. Wiese · B. Wünsch
Department of Pharmaceutical and Medicinal Chemistry, University of Münster, Hittorfstr. 58-62, 48149 Münster, Germany

Abbreviations

P450	Cytochrome P450
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
MALDI	Matrix-assisted laser deposition ionization
TOF	Time-of-flight

Introduction

Aromatic C–H bond hydroxylation is one of the key oxidation reactions in organic synthesis. Hydroxylation reactions of aromatic rings, however, tend to be accompanied by side reactions and often require harsh conditions [1, 2]. By contrast, in metabolic processes in organisms, many enzymes such as Rieske iron–sulfur non-heme dioxygenases [3, 4], copper-containing oxidases [5], pterin-dependent hydroxylases [6], and cytochromes P450 (P450s) [7, 8] catalyze aromatic hydroxylations with high catalytic activity and regioselectivity under mild conditions. Methane monooxygenase also catalyzes aromatic hydroxylation through the peroxide shunt pathway [9]. P450s are a family of hemoproteins that catalyze the insertion of an oxygen atom (monooxygenation) into a wide variety of substrates related to drug metabolism, detoxification of xenobiotics, and biosynthesis of steroids [7, 8]. P450s are promising enzymes with enormous potential in drug discovery, bioremediation, and chemical synthesis [10, 11]. One of the limitations for the application of P450s to biocatalysts is that the most of P450s consume a stoichiometric amount of expensive cofactors, NADH or NADPH, in the oxidation reactions. Even though the hydrogen peroxide shunt pathway is a way to avoid the consumption of these expensive

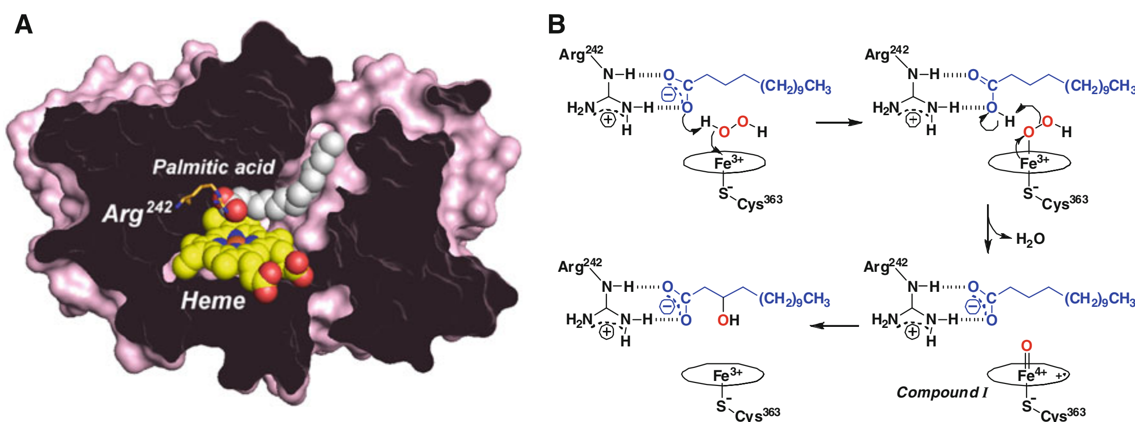
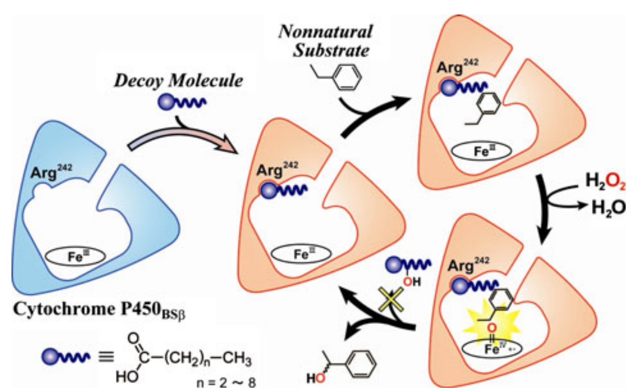


Fig. 1 The active-site structure of the palmitic acid bound form of cytochrome $P450_{BS\beta}$ ($P450_{BS\beta}$) (**a**) (Protein Data Bank code 1IZO) and a plausible mechanism for the catalytic hydroxylation reaction for myristic acid (**b**)

cofactors, this pathway is inefficient for typical P450s [12, 13]. In contrast to most P450s, $P450_{BS\beta}$ (CYP152A1) isolated from *Bacillus subtilis* exclusively utilizes hydrogen peroxide to catalyze the hydroxylation (peroxygenation) of long-alkyl-chain fatty acids [14]. The crystal structure of a palmitic acid bound form of $P450_{BS\beta}$ and a plausible reaction mechanism for the hydroxylation of long-alkyl-chain fatty acids have been reported (Fig. 1) [15]. In the hydroxylation of long-alkyl-chain fatty acids, the electrostatic interaction between the carboxyl group of the fatty acid and Arg-242 is indispensable for the generation of the active species (compound I) using hydrogen peroxide. According to this reaction mechanism, $P450_{BS\beta}$ exclusively catalyzes the hydroxylation of long-alkyl-chain fatty acids. Recently, we have reported that $P450_{BS\beta}$ can catalyze the peroxygenation of a variety of nonnatural substrates, such as epoxidation of styrene and hydroxylation of ethylbenzene, by employing a simple substrate trick; a series of short-alkyl-chain carboxylic acids are misrecognized as substrates and $P450_{BS\beta}$ starts the peroxygenation of nonnatural substrates [16]. We refer to a series of short-alkyl-chain carboxylic acids and this reaction system as a “decoy molecule” and as a “substrate misrecognition system,” respectively (Scheme 1). The catalytic activity and the enantioselectivity of the substrate misrecognition system can be manipulated by changing the decoy molecule. Indeed, the hydroxylation reaction of ethylbenzene in the presence of heptanoic acid gave the maximum turnover and the highest enantioselectivity among a series of short-alkyl-chain carboxylic acids [16]. During the screening of various substrates, we found that aromatic ring hydroxylation of 1-methoxynaphthalene is catalyzed by the substrate misrecognition system. Moreover, we found that the progress of this hydroxylation can be monitored by a color change of the reaction mixture. Herein we report the hydroxylation of an aromatic ring by



Scheme 1 The substrate misrecognition system of cytochrome $P450_{BS\beta}$

hydrogen peroxide dependent P450 and offer a useful colorimetric assay for detecting the hydroxylation activity.

Materials and methods

Chemicals

All chemicals were purchased from commercial sources and used without further purification. The following reagents were obtained from Nacalai Tesque (Kyoto Japan): acetic acid, propionic acid, butanoic acid, hexanoic acid, octanoic acid, 3,3-dimethylglutaric acid, *n*-hexane, and 2-propanol. Heptanoic acid, hydrogen peroxide, 1-methoxynaphthalene, and 1-naphthol were obtained from Wako Pure Chemical Industries (Osaka, Japan). Pentanoic acid, suberic acid, and 4-methoxy-1-naphthol were obtained from Tokyo Chemical Industry (Tokyo, Japan). Benzoic acid, trimethylacetic acid (pivalic acid), monomethyl glutarate, mono-*tert*-butyl succinate, cyclohexanecarboxylic acid, dithranol, and horseradish peroxidase

(HRP) were obtained from Sigma–Aldrich (USA). $H_2{}^{18}O_2$ was purchased from ICON (Isotope) Services (Summit, NJ, USA). The recombinant $P450_{BS\beta}$ with a 6× histidine tag at its N-terminal was expressed in *Escherichia coli* M15 (pREP4) and purified by nickel chelate affinity chromatography (HiTrap, GE Healthcare Bio-Sciences, USA) using BioAssist eZ (TOSOH, Japan) or ÄKTA FPLC (GE Healthcare Bio-Sciences, USA) according to a reported procedure [15, 17]. V170F and F79L mutants were expressed and purified by the same procedure. Reduced P450 carbon monoxide difference spectra were measured to determine the P450 concentration of these enzymes according to a reported method [18]. The recombinant sperm whale myoglobin and its mutants were expressed in *E. coli* TB1 and purified according to a reported procedure [19].

Instruments

UV–vis spectra were recorded with a Shimadzu UV-2400 PC spectrophotometer. Matrix-assisted laser deposition ionization (MALDI) time-of-flight (TOF) mass specrometry was performed using a Bruker Daltonics Ultraflex III MALDI-TOF/TOF mass spectrometer. ^{1}H NMR spectra were recorded with a JEOL ECA600 spectrometer. The optical density of the wells was recorded using a model 680 microplate reader (Bio-Rad, Philadelphia, PA, USA) equipped with a 595-nm wavelength filter.

Catalytic activity assay

The reaction was carried out in 0.1 M potassium phosphate buffer (pH 7.0) in the presence of 0.67 μM $P450_{BS\beta}$, 0.67 mM substrate, and 6.7 mM carboxylic acid. The substrates and carboxylic acids were added as an ethanol solution. When 1-methoxynaphthalene was added, the reaction mixture became slightly clouded owing to its low solubility. Reactions were initiated by the addition of 20 mM hydrogen peroxide (final concentration of 1.3 mM). The total volume of the reaction mixture was 3 mL including 10% of ethanol. The reaction was performed at 25 °C for 1 min. After the reaction, the reaction mixture was extracted with 2.5 mL of chloroform. The extract was filtrated, transferred into a volumetric flask, and diluted to 5 mL. The catalytic activities for 1-methoxynaphthalene and 4-methoxy-1-naphthol were determined by monitoring the absorption at 634.5 nm with a molar absorption coefficient of $\varepsilon = 1.8 \times 10^4\ M^{-1}\ cm^{-1}$ in chloroform. The molar absorption coefficient of Russig's blue was determined using the purified sample. 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.

The catalytic activity for 1-methoxynaphthalene hydroxylation by myoglobin mutants was determined in a similar manner as that of $P450_{BS\beta}$. A reaction mixture containing potassium phosphate buffer (50 mM, pH 7.0), 3.6 μM myoglobin, and 0.5 mM 1-methoxynaphthalene was prepared. The reaction was initiated by the addition of 100 mM hydrogen peroxide (final concentration of 1.0 mM). The reaction was repeated at least three times and the average of the absorbance is summarized in Fig. 4.

Analysis of the reaction mixture after the oxidation reaction

The reaction mixture was extracted with chloroform and analyzed by MALDI-TOF mass spectrometry using dithranol (1,8,9-anthracenetriol) as a matrix. Formaldehyde formation owing to the O-demethylation reaction was confirmed by the tryptophan–sulfuric acid–iron reaction according to a reported method [20, 21].

Screening of the combination of $P450_{BS\beta}$ mutants and the decoy molecules

A reaction mixture containing potassium phosphate buffer (0.1 M, pH 7.0), 0.67 μM wild-type P450 or mutants, 0.67 mM 1-methoxynaphthalene, and 6.7 mM carboxylic acid was prepared in a Costar® 96-well enzyme-linked immunoassay/radioimmunoassay plate (Corning, NY, USA) with different carboxylic acids. The reaction was initiated by the addition of 20 mM hydrogen peroxide (final concentration of 1.3 mM) and the mixture was incubated for 90 min at 25 °C. The UV absorption at 595 nm was recorded with the microplate reader. The reaction was repeated three times and the average of the absorbance is summarized in Fig. 4. For the screening, P450 mutants with a His-tag were used.

Results and discussion

When 1-methoxynaphthalene was oxidized by $P450_{BS\beta}$ in the presence of heptanoic acid as a decoy molecule, the color of the reaction mixture turned to blue. The UV–vis spectra of the reaction mixture after the addition of hydrogen peroxide show a increase of absorption around 600 nm (Fig. 2). The blue product had absorption maxima at 634.5 nm in chloroform and the MALDI-TOF mass spectrum showed a peak at *m/z* 345.13 $[M + H]^+$, suggesting the blue product was 4,4′-dimethoxy-[2,2′]-binaphthalenylidene-1,1′-dione (calcd. exact mass for $C_{22}H_{16}O_4$ 344.11), so-called Russig's blue. [22, 23] Indeed, the structure of the blue product after the purification by silica gel column chromatography was confirmed

by the ^{1}H NMR spectrum to be identical to that of Russig's blue (see the electronic supplementary material) [24]. High performance liquid chromatography (HPLC) analysis of the extract of the reaction mixture monitored at 614 nm showed a single peak, suggesting no isomer of Russig's blue was formed (see the electronic supplementary material).

A plausible reaction mechanism for Russig's blue formation shown in Scheme 2 begins with the hydroxylation of 1-methoxynaphthalene (**1**) at its 4-position, followed by one-electron-oxidation reactions of 4-methoxy-1-naphthol (**2**) (peroxidase reaction) and the radical coupling reaction. Experiments with ^{18}O-labeled hydrogen peroxide further support the proposed reaction mechanism. The MALDI-TOF mass spectrum of Russig's blue obtained with $H_2{}^{18}O_2$ showed a peak at *m/z* 349.11 $[M + H]^+$ (calcd. exact mass for $C_{22}H_{16}{}^{18}O_2{}^{16}O_2$ 348.11), indicating two ^{18}O atoms are inserted into Russig's blue. No peaks corresponding to $C_{22}H_{16}{}^{18}O_1{}^{16}O_3$ and $C_{22}H_{16}{}^{16}O_4$ were observed. No formaldehyde formation due to the O-demethylation reaction of 1-methoxynaphthalene was confirmed by the tryptophan–sulfuric acid–iron reaction [20, 21], even though the O-dealkylation has been frequently observed in human and rat hepatic P450s [25, 26]. In fact, HPLC analysis (monitored at 280 nm) of the extract of the reaction mixture showed no peak corresponding to 1-naphthol, whereas peaks related to other side reactions were observed (see the electronic supplementary material). Oxidation of 1-methoxynaphthalene and 1-naphthol by HRP did not result in a color change, whereas Russig's blue was obtained by HRP-catalyzed oxidation when 4-methoxy-1-naphthol was used as a starting substrate, suggesting that 4-methoxy-1-naphthol is the intermediate and its one-electron oxidation is expected to be the initial step for the formation of Russig's blue. Apparently, it is hard for HRP to oxidize 1-methoxynaphthalene even though HRP forms compound I. The initial turnover rate for Russig's blue formation by $P450_{BS\beta}$ in the presence of heptanoic acid was 56 ± 1 min^{-1}. Since 2 mol of 1-methoxynaphthalene is required for the formation of 1 mol of Russig's blue, the hydroxylation rate is twice as high, e.g., 112 min^{-1}. This is the first example of efficient aromatic C–H bond hydroxylation catalyzed by a hydrogen peroxide dependent P450 system. Although the hydroxylation of 9-methylanthracene catalyzed by $P450_{BS\beta}$ without the decoy molecule has been reported, the specific activity was less than 1 min^{-1} [27]. The initial turnover rate of the oxidative dimerization of 4-methoxy-1-naphthol was determined to be 282 ± 3 min^{-1}; that is more than twice as fast as the hydroxylation of 1-methoxynaphthalene, indicating the rate-determining step for Russig's blue formation is the hydroxylation of the aromatic C–H bond. Thus, the catalytic activity of the hydroxylation reaction can be estimated from the rate of Russig's blue formation. These results allow us to evaluate the catalytic activity of $P450_{BS\beta}$ indirectly by observing the color change of the reaction mixture.

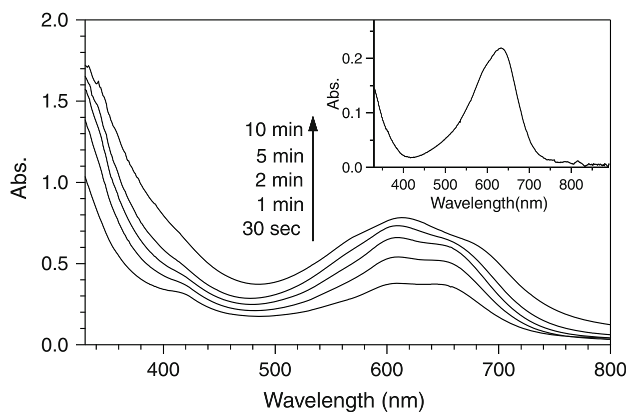
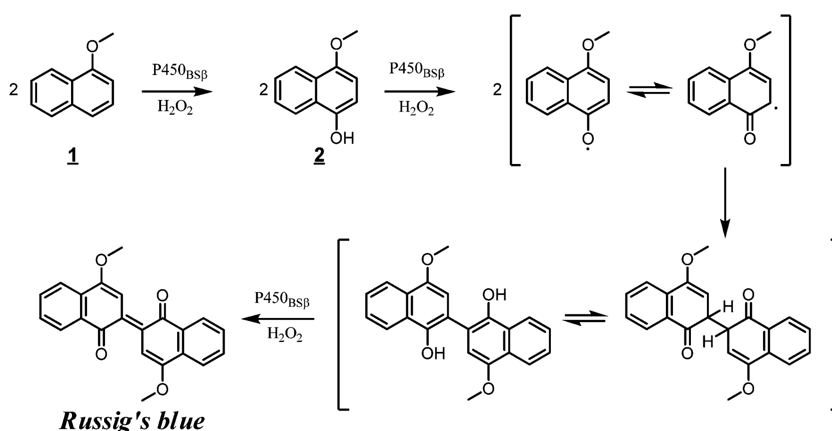


Fig. 2 Time course of UV–vis spectral changes during the reaction. A reaction mixture containing potassium phosphate buffer (0.1 M, pH 7.0), 0.67 μM $P450_{BS\beta}$, 0.67 mM 1-methoxynaphthalene, 6.7 mM carboxylic acid, and 1.3 mM hydrogen peroxide was incubated at 25 °C. The *inset* shows the UV–vis spectrum of the blue product in chloroform

Scheme 2 A plausible reaction mechanism for Russig's blue formation



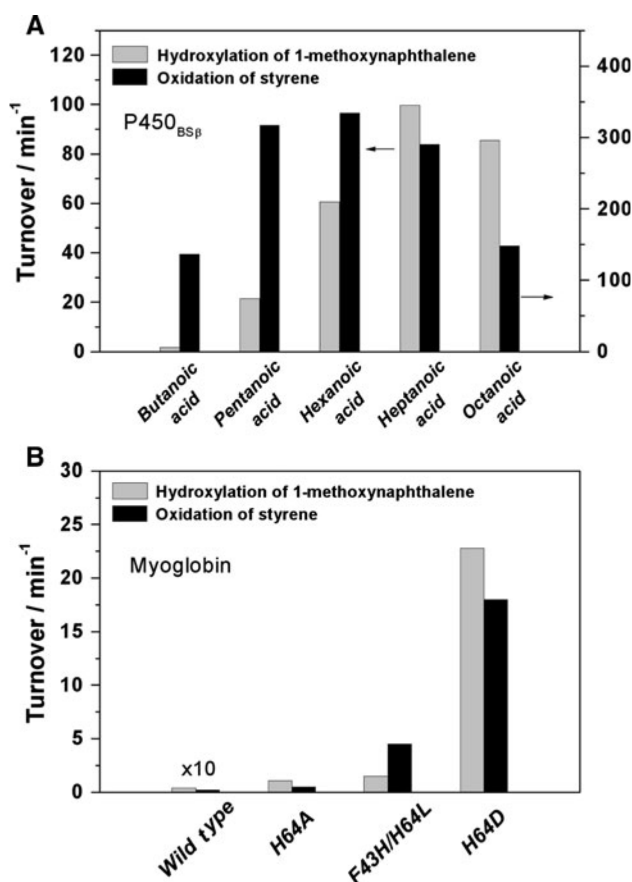


Fig. 3 Comparison of the activities of Russig's blue formation and those of styrene oxidation catalyzed by $P450_{BS\beta}$ in the presence of decoy molecules (**a**) and by myoglobin mutants (**b**). In the case of the F43H/H64L mutant, the initial turnover rate of the styrene epoxidation is shown [28, 29]

We have already reported that the peroxygenase activities of $P450_{BS\beta}$ in styrene oxidation are dependent on the structure of the decoy molecule. Therefore, we compared the oxidation activities of styrene and 1-methoxynaphthalene in the presence of a series of decoy molecules (Fig. 3a). The effects of the decoy molecule on both activities are similar, suggesting that the oxidation activity of heme enzyme can be roughly estimated by monitoring the Russig's blue formation. The effect of the structure of the decoy molecule on the activity of ethylbenzene hydroxylation reported previously accords with that of 1-methoxynaphthalene hydroxylation, indicating that the activity of ethylbenzene hydroxylation also correlates well with Russig's blue formation.

We have developed a series of myoglobin mutants which show high peroxygenase activities in sulfoxidation and epoxidation [28, 29]. Thus, we compared the activities of Russig's blue formation and styrene oxidation catalyzed by myoglobin mutants. Among the myoglobin mutants, H64D myoglobin is known to show the highest oxidation activity. As summarized in Fig. 3b, the peroxygenase activities of myoglobins in 1-methoxynaphthalene and styrene correlate quite well, whereas the catalytic activities of myoglobin mutants are much smaller than the catalytic activity of $P450_{BS\beta}$.

Encouraged by the correlation between the color change and the hydroxylation activity of 1-methoxynaphthalene as well as styrene oxidation by $P450_{BS\beta}$, we decided to screen the combination of $P450_{BS\beta}$ mutants and decoy molecules to determine higher aromatic C–H bond hydroxylation conditions. The catalytic activities of V170F, F79L, and wild-type $P450_{BS\beta}$ were evaluated by changing the decoy molecule. Val-170 and Phe-79 interacting with the bound palmitic acid (Fig. 4d) are key residues for fatty acid binding [15]. The catalytic activity of V170F, F79L, and wild-type $P450_{BS\beta}$ for the hydroxylation of myristic acid have been reported to be 113 ± 28, 175 ± 5, and $365 \pm 19\ min^{-1}$, respectively [15, 17]. The reaction mixtures were prepared in a 96-well plate with different carboxylic acids and were incubated for 90 min at 25 °C (Fig. 4a). The decoy molecules used in this study and the result of colorimetric screening are shown in Fig. 4c. From the result of colorimetric screening (Fig. 4b), wild-type $P450_{BS\beta}$ and pentanoic acid is the optimum combination for the reaction under the conditions examined (90-min reaction). Interestingly, several carboxylic acids other than *n*-alkyl carboxylic acids such as mono-*tert*-butyl succinate also serve as good decoy molecules. In addition, even though the hydroxylation activity of V170F for myristic acid is about one third of that of the wild type, its catalytic activities for aromatic hydroxylation in the presence of octanoic acid or nonanoic acid are comparable to the catalytic activity of the wild type with pentanoic acid.

In conclusion, we have reported here that the aromatic hydroxylation of 1-methoxynaphthalene is efficiently catalyzed by $P450_{BS\beta}$ in the presence of decoy molecules and its activity is simply monitored by a color change due to Russig's blue formation. The initial turnover rate was $112\ min^{-1}$ in the presence of heptanoic acid. This is the first example of efficient aromatic C–H bond hydroxylation catalyzed by P450 using hydrogen peroxide as an oxidant. Furthermore, we have demonstrated that 1-methoxynaphthalene is a very useful substrate for estimating the per-

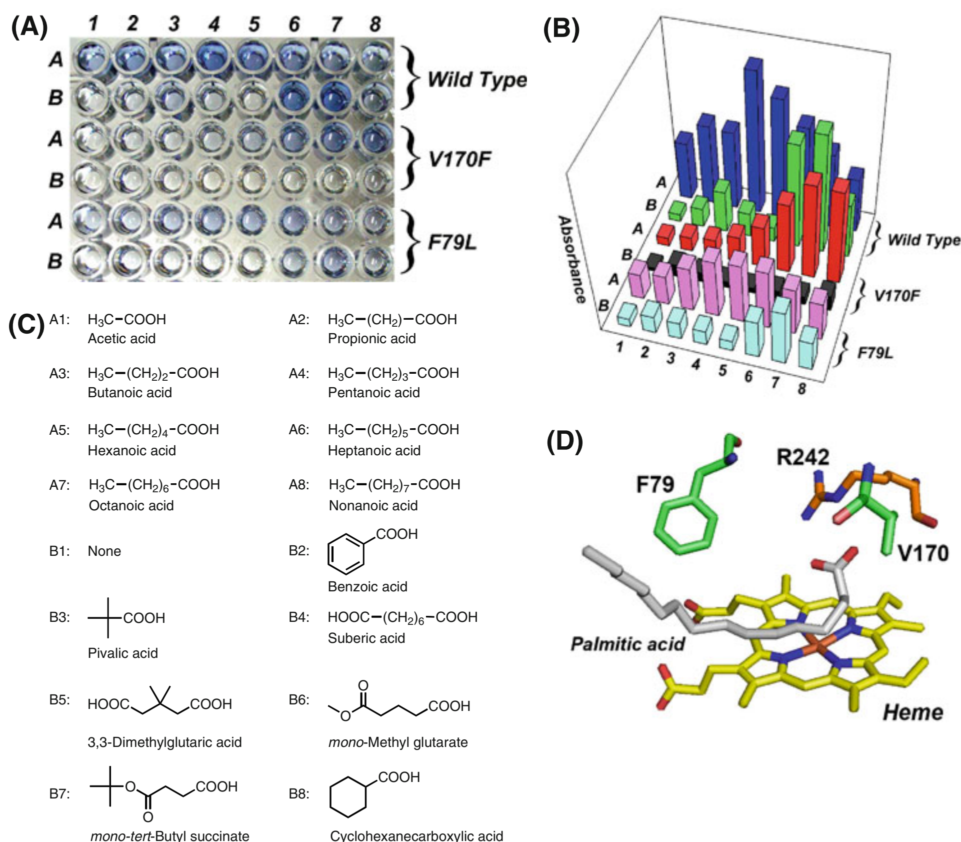


Fig. 4 Screening of the combination of $P450_{BS\beta}$ and decoy molecules. **a** A reaction mixture containing potassium phosphate buffer (0.1 M, pH 7.0), 0.67 μM wild-type $P450_{BS\beta}$ or mutants, 0.67 mM 1-methoxynaphthalene, 6.7 mM carboxylic acid, and 1.3 mM hydrogen peroxide was prepared in 96-well plates with different carboxylic acids, and was incubated for 90 min at 25 °C. **b** The absorption of the solution in the well at 595 nm. **c** Decoy molecules used in the screening. **d** The active-site structure of the palmitic acid-bound form showing the location of Phe-79 and Val-170

oxygenation activity of enzymes without the use of HPLC, gas chromatography, and NMR measurements.

Acknowledgements This work was supported by a Grant-in-Aid for Scientific Research (S) to Y.W. from the Ministry of Education, Culture, Sports, Science, and Technology (Japan) and the International Research Training Group (DFG and JSPS) "Complex Functional Systems in Chemistry" (Münster and Nagoya). We thank Prof. Isamu Matsunaga for his kind gift of the expression system of $P450_{BS\beta}$ and its mutants. T.F. is supported by JSPS Research Fellowships for Young Scientists.

References

1. Olah GA, Fung AP, Keumi T (1981) J Org Chem 46:4305–4306
2. Olah GA, Keumi T, Lecoq JC, Fung AP, Olah JA (1991) J Org Chem 56:6148–6151
3. Wackett LP (2002) Enzyme Microb Technol 31:577–587
4. Ferraro DJ, Gakhar L, Ramaswamy S (2005) Biochem Biophys Res Commun 338:175–190
5. McGuirl MA, Dooley DM (1999) Curr Opin Chem Biol 3:138–144
6. Kappock TJ, Caradonna JP (1996) Chem Rev 96:2659–2756
7. Denisov IG, Makris TM, Sligar SG, Schlichting I (2005) Chem Rev 105:2253–2278
8. Ortiz de Montellano PR (2005) Cytochrome P450: structure, mechanism, and biochemistry, 3rd edn. Plenum Press, New York
9. Andersson KK, Froland WA, Lee SK, Lipscomb JD (1991) New J Chem 15:411–415
10. Urlacher VB, Eiben S (2006) Trends Biotechnol 24:324–330
11. Yun C-H, Kim K-H, Kim D-H, Jung H-C, Pan J-G (2007) Trends Biotechnol 25:289–298
12. Nordblom GD, White RE, Coon MJ (1976) Arch Biochem Biophys 175:524–533
13. Hrycay EG, Gustafsson J-a, Ingelman-Sundberg M, Ernster L (1975) Biochem Biophys Res Commun 66:209–216
14. Matsunaga I, Ueda A, Fujiwara N, Sumimoto T, Ichihara K (1999) Lipids 34:841–846
15. Lee D-S, Yamada A, Sugimoto H, Matsunaga I, Ogura H, Ichihara K, Adachi S-i, Park S-Y, Shiro Y (2003) J Biol Chem 278:9761–9767
16. Shoji O, Fujishiro T, Nakajima H, Kim M, Nagano S, Shiro Y, Watanabe Y (2007) Angew Chem Int Ed 46:3656–3659
17. Matsunaga I, Ueda A, Sumimoto T, Ichihara K, Ayata M, Ogura H (2001) Arch Biochem Biophys 394:45–53
18. Gunsalus IC, Wagner GC (1978) Methods Enzymol 52:166–188
19. Springer BA, Egeberg KD, Sligar SG, Rohlfs RJ, Mathews AJ, Olson JS (1989) J Biol Chem 264:3057–3060
20. Chrastil J, Wilson JT (1975) Anal Biochem 63:202–207
21. Kleeberg U, Klinger W (1982) J Pharm Methods 8:19–31
22. Russig F (1900) J Prakt Chem 62:30–60
23. Goldschmidt S, Wessbecher H (1928) Ber Dtsch Chem Ges 61:372–377
24. Takeya T, Doi H, Ogata T, Otsuka T, Okamoto I, Kotani E (2004) Tetrahedron 60:6295–6310
25. Burke MD, Thompson S, Weaver RJ, Wolf CR, Mayers RT (1994) Biochem Pharmacol 48:923–936
26. Li AP, Kaminski DL, Rasmussen A (1995) Toxicology 104:1–8

27. Torres E, Hayen H, Niemeyer CM (2007) Biochem Biophys Res Commun 355:286–293
28. Matsui T, Ozaki S, Liong E, Phillips GN Jr, Watanabe Y (1999) J Biol Chem 274:2838–2844
29. Ozaki S, Yang H-J, Matsui T, Goto Y, Watanabe Y (1999) Tetrahedron Asymmetr 10:183–192