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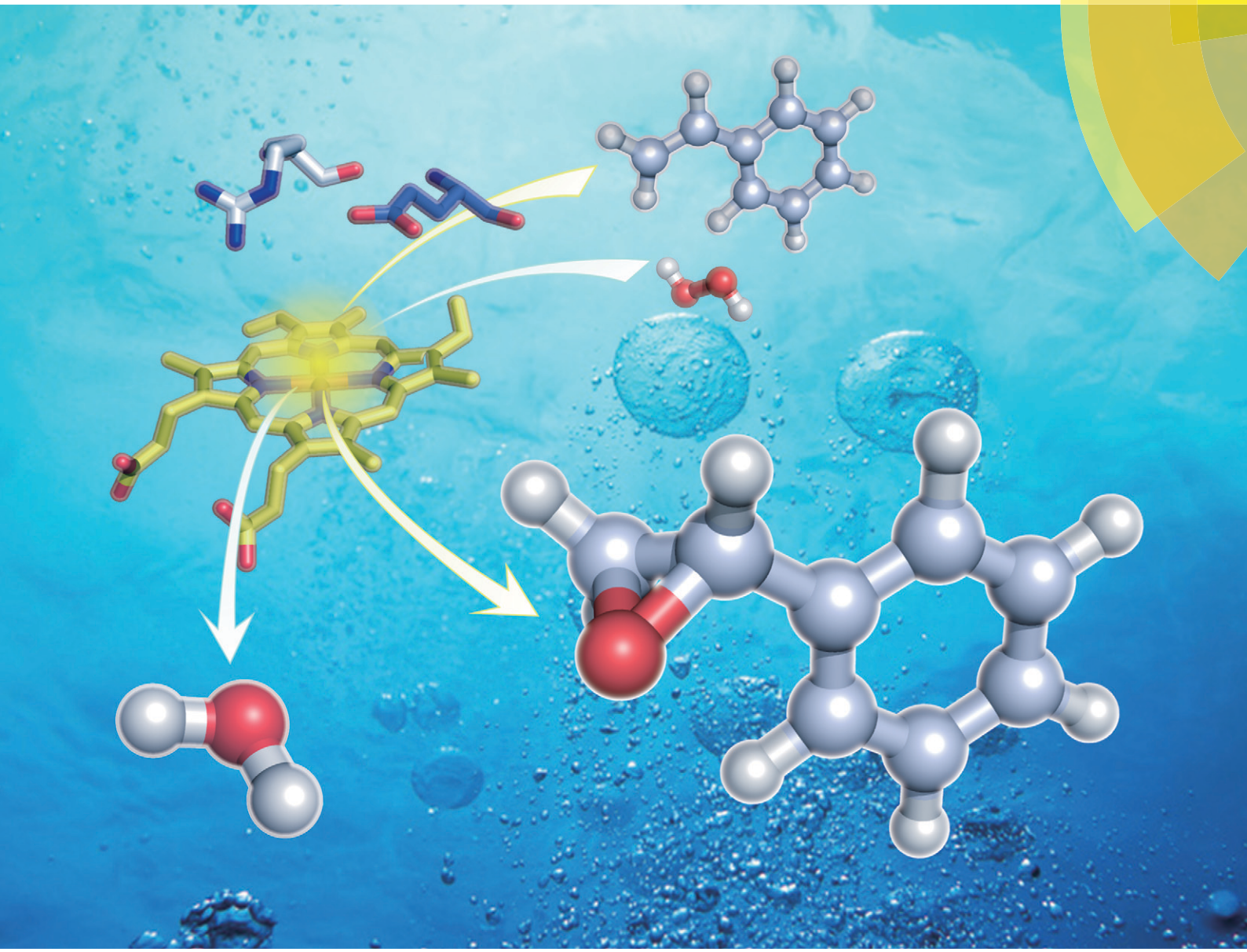

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A substrate-binding-state mimic of H_2O_2-dependent cytochrome P450 produced by one-point mutagenesis and peroxygenation of non-native substrates†

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A substrate-binding-state mimic of H_2O_2-dependent cytochrome P450 that is able to catalyze monooxygenation of non-native substrates was constructed by one-point mutagenesis of $P450_{SP\alpha}$ (CYP152B1). $P450_{SP\alpha}$, a long-alkyl-chain fatty acid hydroxylase, lacks any general acid–base residue around the heme. The carboxylate group of a fatty acid is thus indispensable for the generation of active species using H_2O_2. We prepared an A245E mutant to mimic a substrate-binding state by placing a carboxylate group at the active site. The active site structure of the A245E mutant is similar to that of the fatty-acid-bound state of $P450_{SP\alpha}$ and catalyzes styrene oxidation at a rate of 280 min^{-1} (k_{cat}), whereas the wild-type enzyme does not show any catalytic activity. More importantly, the same mutation, *i.e.* the mutation of the highly conserved threonine in P450s to glutamic acid, was also effective in introducing peroxygenase activity into P450BM3, $P450_{cam}$, and CYP119. These results indicate that a variety of peroxygenases based on P450s can be constructed by one-point mutagenesis.

Introduction

Construction of biocatalysts based on cytochrome P450s (P450s) has attracted much attention because P450s can efficiently catalyze monooxygenation of less reactive organic compounds under mild conditions.[1–3] A wide variety of engineered P450s for the oxidation of inert alkanes and aromatic compounds have thus been constructed by mutagenesis[4–13] and modification.[14–20] Most P450s, however, consume a stoichiometric amount of a costly cofactor (NAD(P)H) for the activation of molecular oxygen. Alternatively, H_2O_2 can be used as an oxidant for the generation of the active species (H_2O_2-shunt reaction). The H_2O_2-shunt reaction is an attractive candidate for monooxygenation reactions catalyzed by P450s,[21] because i) electron transfer partners, such as P450 reductase, are not required for the generation of an active species, and ii) the low cost of H_2O_2 allows the reaction to be performed on an industrial scale. However, in general, the H_2O_2-shunt reaction is inefficient because the catalytic activities of P450s for the H_2O_2-shunt reaction are lower than those when molecular oxygen is used. In contrast to most P450s which use molecular oxygen, H_2O_2-dependent P450s, such as $P450_{SP\alpha}$,[22–24] $P450_{BS\beta}$,[25] and $P450_{CLA}$,[26] efficiently utilize H_2O_2 for the hydroxylation of less reactive alkanes. H_2O_2-dependent P450s have thus been considered as excellent candidates for practical biocatalysts. Unfortunately, these H_2O_2-dependent P450s exclusively catalyze the hydroxylation of long-alkyl-chain fatty acids, and their substrate specificity is very high. The crystal structures of $P450_{SP\alpha}$ (Fig. 1, PDB code: 3AWM)[27] and $P450_{BS\beta}$ (PDB code: 1IZO)[28] in the palmitic acid-bound forms reveal that $P450_{SP\alpha}$ and $P450_{BS\beta}$ lack any general acid–base residue around the distal side of the heme, whereas the carboxylate group of palmitic acid interacts with the arginine located at the distal side of the heme. Thus, salt bridge formation between the arginine residue and the carboxylate group in the substrate is expected to be crucial for the formation of compound I (Fig. 2a).[28] This substrate-assisted reaction mechanism also contributes to the high substrate specificity of the enzymes; thus, $P450_{SP\alpha}$ and $P450_{BS\beta}$ never oxidize substrates other than fatty acids.

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† Electronic supplementary information (ESI) available: Experimental section including expression and purification of P450s, additional figures of crystal structures, UV-vis spectra, plots of the initial turnover rate of oxidation reactions, and GC-MS analysis. Table of data collection and refinement statistics of crystal structures (PDB codes: 3VOO, 3VTJ, and 3VNO). See DOI: 10.1039/c6cy00630b

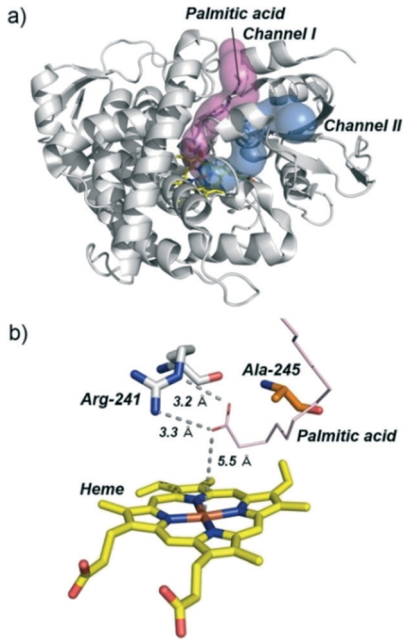


Fig. 1 a) Overall structure of cytochrome $P450_{SP\alpha}$ with palmitic acid. The two channels are represented as pink (channel I) and light-blue (channel II) surfaces. b) Structure of the active site around the heme group. The heme, Arg-241, and Ala-245 are represented as stick models. The palmitic acid of the productive conformation that afforded the α-hydroxy fatty acid is shown as a line drawing.

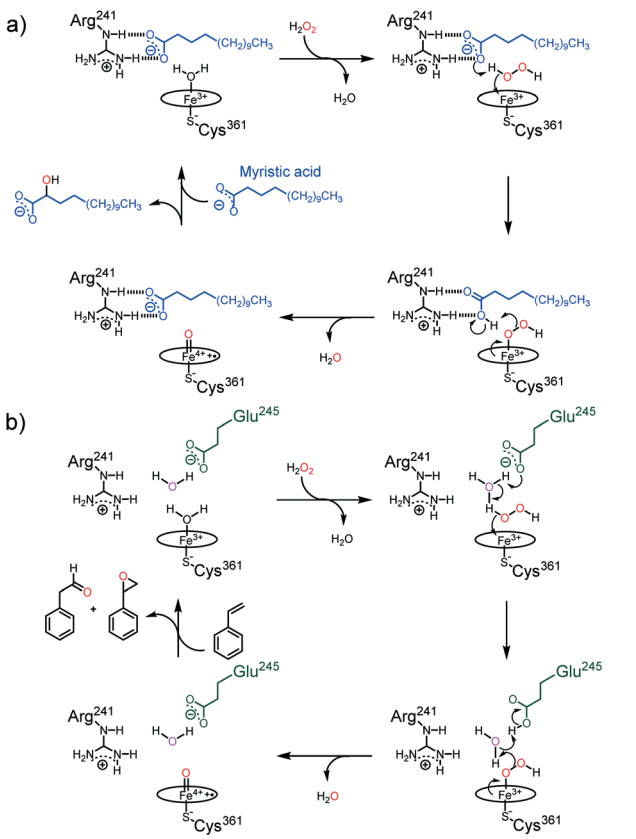


Fig. 2 Proposed catalytic reaction mechanisms for hydroxylation of long-alkyl-chain fatty acids by wild-type $P450_{SP\alpha}$ (a) and for oxidation of styrene by A245E (b).

Nevertheless, we succeeded in oxidizing non-native substrates other than fatty acids by $P450_{SP\alpha}$ (ref. 17) and $P450_{BS\beta}$ (ref. 29–31) by employing a series of short-alkyl-chain carboxylic acids as decoy molecules (inert dummy substrates). In these reaction systems, $P450_{SP\alpha}$ and $P450_{BS\beta}$ misrecognize the decoy molecules as substrates and the carboxylate group of the decoy molecules serves as an acid–base catalyst for the generation of compound **I**.[32] Very recently, we have reported that $P450_{SP\alpha}$ and $P450_{BS\beta}$ can catalyze the oxidation of non-native substrates in the presence of a high concentration of the acetate anion, which also serves as a general acid–base catalyst.[33] Although the addition of external carboxylic acids (decoy molecules) is effective for the oxidation of non-native substrates, the use of decoy molecules would not be suitable for the enzyme reaction *in vivo* in *Escherichia coli*. To perform peroxygenation of non-native substrates by $P450_{SP\alpha}$ without the use of external carboxylic acids, we introduced an amino acid bearing a carboxylate side chain to mimic the fatty-acid-binding state of $P450_{SP\alpha}$. The crystal structure of $P450_{SP\alpha}$ (ref. 27) suggests that Ala-245, in the distal I helix, is a candidate that is suitable for placing a carboxylate group close to the heme by mutagenesis (Fig. 1b). Interestingly, the location of Ala-245 corresponds to a highly conserved threonine in the P450 family[34] (Thr-268 of P450BM3 (Fig. S3†) and Thr-252 of $P450_{cam}$), which is critical for oxygen activation.[35–37] Herein, we report a substrate-binding-state mimic of $P450_{SP\alpha}$ prepared by one-point mutagenesis that oxidizes non-native substrates without requiring a decoy molecule. Similar mutation of other P450s that utilize molecular oxygen was also attempted to convert them into H_2O_2-dependent P450s.

Results and discussion

Design and preparation of mutants

Based on the crystal structure analysis shown in Fig. 1, an A245E mutant in which to place a carboxylate group close to the heme was prepared. An A245D mutant was also prepared to examine the influence of the side-chain length. Furthermore, Ala-245 was replaced with histidine, which serves as a general acid–base catalyst in peroxidases such as horseradish peroxidase (HRP)[38] and cytochrome *c* peroxidase (C*c*P).[39] Because Arg-241 is also close to the heme, this residue was replaced with glutamic acid to examine whether the introduction of a carboxylate at a position other than that of Ala-245 is effective for the generation of compound **I**. Accordingly, A245E, A245D, A245H, and R241E mutants were prepared under the same conditions used for the wild-type enzyme and they were purified by using several chromatographic columns.[27] The UV-vis spectrum of purified A245E showed an absorption maximum at 417 nm that was identical to that observed for the wild-type enzyme (Fig. 3). The reduced form of A245E, which was formed upon treatment with sodium dithionite under CO atmosphere, absorbed at 444 nm. A245D and A245H also showed essentially the same absorption as observed for the wild-type enzyme; however, the small

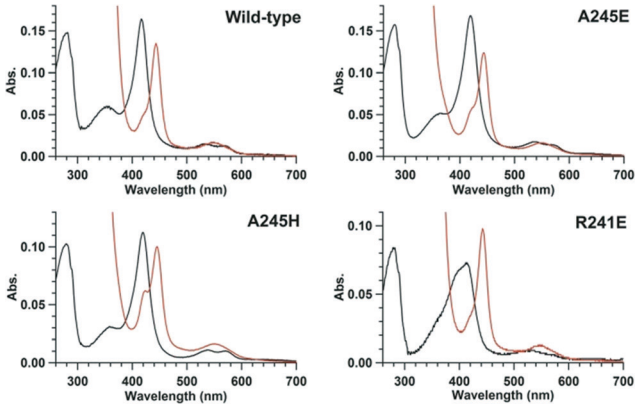


Fig. 3 UV-visible absorption spectra of $P450_{SP\alpha}$ in 0.1 M potassium phosphate buffer (pH 7.0) containing 0.3 M KCl and 20% (v/v) glycerol. The resting state (black line) and the Fe^{2+}–CO state (red line).

shoulder peak at around 420 nm indicated a slightly different mode of cysteine ligation.[40] The resting state of R241E gave a broad Soret band with a peak that was shifted to shorter wavelength, which resembles that of the fatty-acid-bound form of P450BM3.[41] In the case of P450BM3,[42] a water molecule coordinated to the heme iron moves upon fatty acid binding, leading to a low- to high-spin shift of iron(III). Indeed, a slight shift of the water molecule above the heme was observed in the crystal structure of R241E (Fig. S9†).

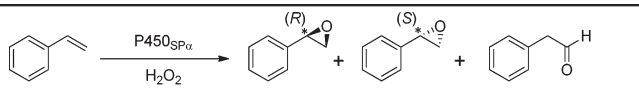
Styrene epoxidation

In contrast to the wild-type $P450_{SP\alpha}$, which does not catalyze styrene oxidation, A245E catalyzes this reaction with k_{cat} and K_m estimated to be 280 min^{-1} and 1.5 mM, respectively, based on Michaelis–Menten kinetics analysis (Table 1). The catalytic activity is comparable to that of chloroperoxidase (288 min^{-1}),[43] whereas it is lower than that of the *Agrocybe aegerita* aromatic peroxygenase (*Aae*APO, 790 min^{-1})[44] which is one of the most efficient hydrogen peroxide-dependent biocatalysts known. In contrast to A245E, A245D showed a lower catalytic activity (72 min^{-1}, K_m = 5.3 mM), presumably because the carboxylate of A245D is located too far from the heme iron to serve as a general acid–base catalyst. Although the side-chain length of histidine is not very different from that of glutamic acid, the catalytic activity of A245H was much lower (18 min^{-1}, K_m = 3.5 mM), suggesting that the location and orientation of histidine-245 of A245H are not suitable for serving as a general acid–base catalyst. That no catalytic activity was observed with R241E indicates that the carboxylate of R241E is also too far from the heme iron. The stereoselectivity of styrene epoxidation by mutants was (*S*)-configuration, whereas the wild-type $P450_{SP\alpha}$ in the presence of acetic acid gave the (*R*)-isomer,[33] suggesting that the replacement of Ala-245 affects the styrene orientation in the active site. We also examined the hydroxylation of indole to give indigo[45] as well as the hydroxylation of 1-methoxynaphthalene to give Russig's blue[31] (Scheme S1†) and found that the A245E mutant also catalyzes these reactions. The catalytic activities for the hydroxylation of indole and 1-methoxynaphthalene were estimated to be 20 and 75 min^{-1}, respectively, indicating that the A245E mutant catalyzes the hydroxylation of various substrates bearing structures that differ from those of fatty acids.

X-ray crystal structure analysis

X-ray crystal structure analysis of A245E, A245H, and R241E allows us to evaluate the location and orientation of the mutated amino acids in their active sites. The overall structures of the mutants, which were obtained in substrate-free forms, were found to be essentially the same as that of the wild type (Fig. S10†). The crystal structure of A245E showed that the carboxylate group of Glu-245 is located above the heme (Fig. 4a). The distance between the nearest carboxylate oxygen atom of Glu-245 and the heme iron is estimated to be 6.9 Å, which is 1.7 Å longer than that of palmitic acid (5.2 Å). A triad structure (Arg–Glu–Heme) in the A245E mutant is similar to that of *Aae*APO (Fig. 4d).[46] The longer distance between the carboxylate group of Glu-245 and the heme iron suggests that this carboxylate group does not directly serve as a general acid–base catalyst. This observation is reminiscent of the high peroxidase and peroxygenase activities[47] of a series of H64D myoglobin mutants in which the distance between the

Table 1 Catalytic activity (k_{cat}) for styrene oxidation by $P450_{SP\alpha}$ and mutants evaluated by Michaelis–Menten kinetics[a]



	k_{cat}/min^{-1}	K_m/mM	k_{cat}/K_m/M^{-1} s^{-1}	SO : PAA[b]	%ee[b]
Wild-type	n.d.				
A245E	280 ± 40	1.5 ± 0.4	190	70 : 30	20 (*S*)
A245D	72 ± 6	5.3 ± 0.6	14	81 : 19	41 (*S*)
A245H	18 ± 8	3.5 ± 2.5	5.3	79 : 21	0.5 (*S*)
R241E	n.d.				

[a] Reaction conditions: 0.5–3 mM styrene, 4 mM H_2O_2 and 1 μM $P450_{SP\alpha}$ in 0.1 M potassium phosphate buffer (pH 7.0) at 25 °C for 1 min. [b] The values under the conditions of 3 mM styrene. n.d. = not detected.

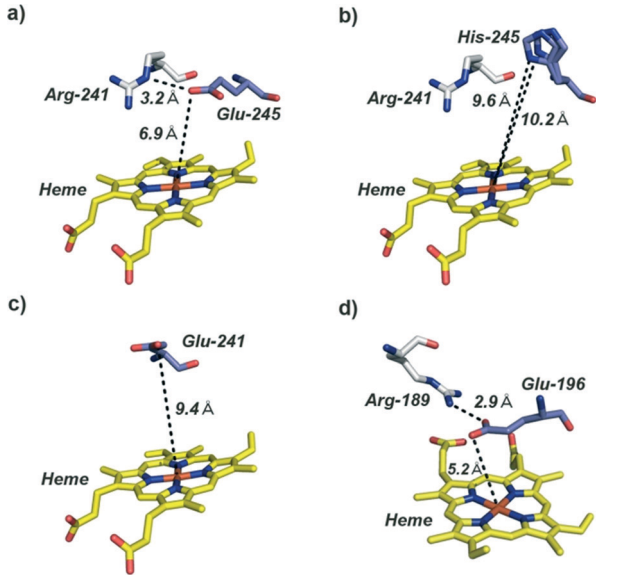


Fig. 4 The active site structures of $P450_{SP\alpha}$ mutants and *Aae*APO (PDB code 2YP1). a) A245E, b) A245H, c) R241E, and d) *Aae*APO. Two alternative conformations of His-245 of A245H are shown.

carboxylate oxygen atom of Asp-64 and the heme iron is 7.7 Å (PDB code: 1LUE).[48,49] It was suggested that Asp-64 is indirectly associated with the generation of compound **I** and that the arrangement of distal water molecules affects the catalytic activity of myoglobin mutants. In the crystal structure of A245E, a water molecule interacting with Glu-245 (W4 in Fig. 5b) is observed. The distance between this water molecule and the heme iron is 4.9 Å, which is suitable for the general acid–base function. Interestingly, the location of this water molecule is virtually identical to that of the carboxylate oxygen atom of the fatty acid observed in the wild type (Fig. 5a), suggesting that this water molecule works together with Glu-245 and Arg-241 to serve as a general acid–base catalyst (Fig. 2b). In the case of the carboxylate group of the palmitic acid in the crystal structure of the wild-type enzyme, a salt bridge between Arg-241 and Glu-245 was observed. This salt bridge seems to be important for the fixation of Glu-245 and the water molecule (W4 in Fig. 5b). In the crystal structure of A245E, channel I, which allows fatty acids access to the active site, is blocked because Glu-245 and Phe-288 (one of dual conformations) occupy this channel (Fig. S10 and S11†); however, channel II is still accessible for additional foreign substrates, thus styrene is able to access the heme cavity through channel II. In contrast to A245E, the carboxylate of R241E is positioned far from the heme iron (9.4 Å, Fig. 4c), and no water molecule interacts with Glu-241 (Fig. 5c). This suggests that R241E cannot generate the active species. The crystal structure of A245H reveals that the side chain of His-245 flipped away from the heme iron (Fig. 4b), and this was accompanied by side-chain flipping of Phe-169 toward channel I (Fig. S11b†). Accordingly, the nitrogen atom (N_δ) of His-245 is far from the heme iron (9.6–10.2 Å), and thus, this residue cannot serve as an efficient general acid–base catalyst.

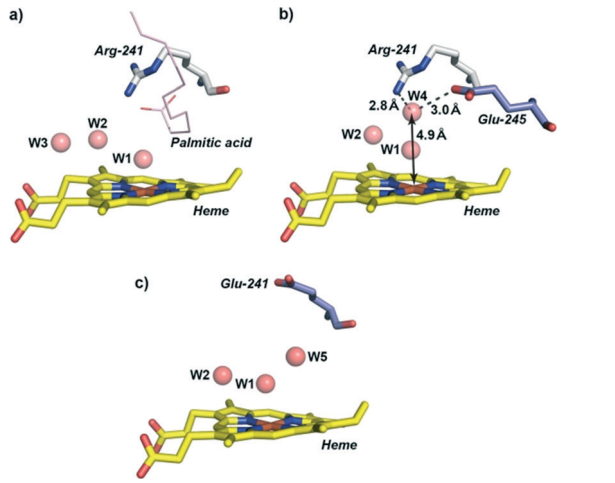


Fig. 5 The active site structures of $P450_{SP\alpha}$: a) wild type, b) A245E, and c) R241E. The water molecule in A245E (W4) would serve as a general acid–base catalyst.

Construction of cytochrome P450 peroxygenase by the replacement of the highly conserved threonine with glutamic acid

As pointed out, the location of Ala-245 in $P450_{SP\alpha}$ corresponds to the conserved threonine in the P450 family[34] (Fig. S3 and S12†), and this residue is crucial for the reductive activation of molecular oxygen as a proton donor. Thus, the mutation of the conserved threonine to alanine or valine induced the uncoupling reaction (H_2O_2 production).[35–37] To examine whether the mutation of the conserved threonine to glutamic acid effectively introduces peroxygenase activity into P450s in general, we prepared the corresponding mutants of P450BM3, $P450_{cam}$, and CYP119 (T268E of P450BM3, T252E of $P450_{cam}$, and T213E of CYP119). The UV-vis spectra of the purified mutants were essentially identical to those of the wild type in spite of the introduction of glutamic acid close to the heme (Fig. S2†). The CO adduct of the mutants exhibited absorption at around 450 nm, which indicated that the proteins were folded correctly. The catalytic activity for H_2O_2-dependent styrene oxidation by these mutants was evaluated by Michaelis–Menten kinetics, and the k_{cat} and K_m values were estimated by using 60 mM H_2O_2 (final concentration). In contrast to the wild-type P450s, which show very low or no catalytic activity, all the mutants showed catalytic activity for styrene oxidation (Table 2). Among the mutants examined, the T268E mutant of P450BM3 gave the highest k_{cat} (110 min^{-1}, K_m = 1.5 mM). It is interesting to note here that the catalytic turnover rate of the T268E mutant is comparable to that of 21B3 (initial rate using 10 mM H_2O_2: 56 min^{-1}), which is a mutant of P450BM3 (ref. 50) having 10 mutations prepared by random mutagenesis. Whereas the wild-type $P450_{cam}$ did not show any catalytic activity for the H_2O_2-dependent oxidation, the k_{cat} of the T252E mutant of $P450_{cam}$

Table 2 Catalytic activity (k_{cat}) for styrene oxidation by mutants of P450BM3, $P450_{cam}$, and CYP119[a]

	k_{cat}/min^{-1}	K_m/mM	k_{cat}/K_m/M^{-1} s^{-1}	SO : PAA[b]	%ee[b]
P450BM3 T268E	110 ± 5	1.5 ± 0.3	72	85 : 15	22 (*R*)
$P450_{cam}$ T252E	3.1 ± 0.2	5.4 ± 1.0	0.57	78 : 22	70 (*S*)
CYP119 T213E	16 ± 0.7	3.9 ± 0.5	4.1	88 : 12	29 (*S*)

[a] Reaction conditions: 0–15 mM styrene, 60 mM H_2O_2 and 5 μM P450 in 0.1 M potassium phosphate buffer (pH 7.0) at 25 °C for 1 min. [b] The values under the conditions of 5 mM styrene. The initial turnover rate at 70 °C is shown for CYP119.

was estimated to be 3.1 min^{-1} (K_m = 5.4 mM). The k_{cat} of the T213E mutant of CYP119 (16 min^{-1}, K_m = 3.9 mM) was also improved compared with that of the CYP119 wild type (initial turnover rate: 1.6 min^{-1}/P450, 10 mM styrene, and 60 mM H_2O_2). Natural substrates were also oxidized by these mutants, whereas the wild type of these P450s shows almost no catalytic activity upon the use of H_2O_2. For example, the catalytic activities for myristic acid hydroxylation by the T268E mutant of P450BM3 and by the T213E mutant of CYP119 were estimated to be 4.9 and 8.3 min^{-1}/P450, respectively. The total turnover numbers for a 30 min reaction were 18 and 76/P450. The catalytic activity for D-camphor hydroxylation by the T252E mutant of $P450_{cam}$ was estimated to be 28 min^{-1}/P450, and the total turnover number for a 30 min reaction was 40/P450. These results clearly show that even P450s that utilize molecular oxygen can be converted into H_2O_2-dependent P450s by single-point mutagenesis of the highly conserved threonine.

Conclusions

The A245E mutant of $P450_{SP\alpha}$ essentially mimics the substrate-bound state and facilitates oxidation of non-native substrates. It is important to mention that the corresponding mutation has never been found in mutants prepared[6,51] by random mutagenesis, possibly because the replacement of threonine with glutamic acid requires at least 2 base-pair changes, *e.g.* ACG (Thr) to GAG (Glu). Furthermore, to our knowledge, this is the first example of a P450 mutant having a carboxylate in the distal side of the heme that accelerates H_2O_2-dependent oxidation. Given that Ala-245 in $P450_{SP\alpha}$ is highly conserved among H_2O_2-dependent P450s (CYP152 family, Fig. S13†)[33,52] and considering that threonine is also highly conserved among P450s, we expect that most P450s can be converted into versatile peroxygenases by similar one-point mutagenesis. This mutation is anticipated to become one of the key mutations that can lead to further development of biocatalysts by point mutagenesis as well as through directed evolution. These findings also further contribute to our understanding of the reaction mechanism of H_2O_2-dependent monooxygenation.

Acknowledgements

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