A substrate-binding-state mimic of H2O2-dependent cytochrome P450 produced by one-point mutagenesis and peroxygenation of non-native substrates
A substrate-binding-state mimic of H$_2$O$_2$-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$_2$O$_2$-dependent cytochrome P450 that is able to catalyze monoxygenation of non-native substrates was constructed by one-point mutagenesis of P450$_{sp}$ (CYP152B1). P450$_{sp}$, 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$_2$O$_2$. 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$_{sp}$ 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.

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Introduction

Construction of biocatalysts based on cytochrome P450s (P450s) has attracted much attention because P450s can efficiently catalyze monoxygenation 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 mutagenesis¹⁻¹¹ and modification.¹²⁻²⁰ Most P450s, however, consume a stoichiometric amount of a costly cofactor (NAD(P)H) for the activation of molecular oxygen. Alternatively, H$_2$O$_2$ can be used as an oxidant for the generation of the active species (H$_2$O$_2$-shunt reaction). The H$_2$O$_2$-shunt reaction is an attractive candidate for monoxygenation 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$_2$O$_2$ allows the reaction to be performed on an industrial scale. However, in general, the H$_2$O$_2$-shunt reaction is inefficient because the catalytic activities of P450s for the H$_2$O$_2$-shunt reaction are lower than those when molecular oxygen is used. In contrast to most P450s which use molecular oxygen, H$_2$O$_2$-dependent P450s, such as P450$_{s_{Pv}}$₂²⁻₂⁴ P450$_{bs}$₂⁵ and P450$_{c_{la}}$₂⁶ efficiently utilize H$_2$O$_2$ for the hydroxylation of less reactive alkanes. H$_2$O$_2$-dependent P450s have thus been considered as excellent candidates for practical biocatalysts. Unfortunately, these H$_2$O$_2$-dependent P450s exclusively catalyze the hydroxylation of long-alkyl-chain fatty acids, and their substrate specificity is very high. The crystal structures of P450$_{s_{Pv}}$ (Fig. 1, PDB code: 3AWM)²⁷ and P450$_{bs}$ (PDB code: 1IZO)²⁸ in the palmitic acid-bound forms reveal that P450$_{s_{Pv}}$ and P450$_{bs}$ 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$_{s_{Pv}}$ and P450$_{bs}$ never oxidize substrates other than fatty acids.
Nevertheless, we succeeded in oxidizing non-native substrates other than fatty acids by P450spα (ref. 17) and P450bsβ (ref. 29–31) by employing a series of short-alkyl-chain carboxylic acids as decoy molecules (inert dummy substrates). In these reaction systems, P450spα and P450bsβ 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 P450spα and P450bsβ 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 peroxigenation of non-native substrates by P450spα 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 P450spα. The crystal structure of P450spα (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 family34 (Thr-268 of P450BM3 (Fig. S3†) and Thr-252 of P450cam), which is critical for oxygen activation.35–37 Herein, we report a substrate-binding-state mimic of P450spα 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 H2O2-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 (CcP).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
cause 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\(^{-1}\), \(K_m = 3.5 \text{mM}\)), 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\(_{osp}\) in the presence of acetic acid gave the (R)-isomer,\(^{13}\) 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 AaeAP0 (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 peroxigenase activities\(^{17}\) of a series of H64D myoglobin mutants in which the distance between the

![Fig. 3 UV-visible absorption spectra of P450\(_{osp}\) 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\(^{III}\)–CO state (red line).](Image 30x90 to 214x127)

### Table 1 Catalytic activity (\(k_{cat}\)) for styrene oxidation by P450\(_{osp}\) and mutants evaluated by Michaelis–Menten kinetics\(^a\)

|          | \(k_{cat}/\text{min}^{-1}\) | \(K_m/\text{mM}\) | \(k_{cat}/K_m/\text{M}^{-1} \text{s}^{-1}\) | 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\(_2\)O\(_2\) and 1 \(\mu\)M P450\(_{osp}\) 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.
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.

The active site structures of P450$_{cam}$ mutants and AaeAPO (PDB code 2YP1). a) A245E, b) A245H, c) R241E, and d) AaeAPO. Two alternative conformations of His-245 of A245H are shown.

Fig. 4

The active site structures of P450$_{cam}$, mutations and AaeAPO (PDB code 2YP1). a) A245E, b) A245H, c) R241E, and d) AaeAPO. Two alternative conformations of His-245 of A245H are shown.

The active site structures of P450$_{cam}$, mutations and AaeAPO (PDB code 2YP1). a) A245E, b) A245H, c) R241E, and d) AaeAPO. Two alternative conformations of His-245 of A245H are shown.

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

As pointed out, the location of Ala-245 in P450$_{bhp}$ corresponds to the conserved threonine in the P450 family34 (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 ($\text{H}_2\text{O}_2$ production).35–37 To examine whether the mutation of the conserved threonine to glutamic acid effectively introduces peroxynasenase 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 adducts of the mutants exhibited absorption at around 450 nm, which indicated that the proteins were folded correctly. The catalytic activity for $\text{H}_2\text{O}_2$-dependent styrene oxidation by these mutants was evaluated using Michaelis–Menten kinetics, and the $k_{\text{cat}}$ and $K_m$ values were estimated by using 60 mM $\text{H}_2\text{O}_2$ (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 $k_{\text{cat}}$ (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 $\text{H}_2\text{O}_2$: 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 $\text{H}_2\text{O}_2$-dependent oxidation, the $k_{\text{cat}}$ of the T252E mutant of P450$_{cam}$
was estimated to be 3.1 min⁻¹ (Km = 5.4 mM). The kcat of the T213E mutant of CYP119 (16 min⁻¹, Km = 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 H2O2). Natural substrates were also oxidized by these mutants, whereas the wild type of these P450s shows almost no catalytic activity upon the use of H2O2. For example, the catalytic activities for myristic acid hydroxylisation 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 α-camphor hydroxylation by the T252E mutant of P450cam 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 H2O2-dependent P450s by single-point mutagenesis of the highly conserved threonine.

## Conclusions

The A245E mutant of P450spv 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 prepared⁶,⁵¹ 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 H2O2-dependent oxidation. Given that Ala-245 in P450SP is a carboxylate in the distal side of the heme that accelerates catalytic activity (Fig. S13)⁴ and considering that threonine is also conserved threonine.

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