Identification of the rate-limiting step of the peroxygenase reactions catalyzed by the thermophilic cytochrome P450 from *Sulfolobus tokodaii* strain 7

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ABSTRACT

Cytochrome P450 from the thermoacidophilic crenarchaeon *Sulfolobus tokodaii* strain 7 (P450st) is a thermophilic cytochrome P450 that shows high tolerance for harsh conditions and is capable of catalyzing some peroxygenase reactions. Here, we investigated the pH dependence of the peroxygenase reactions catalyzed by wild-type P450st and a mutant, in which the residues located close to the proximal heme ligand are mutated. Both hydrogen peroxide-driven ethylbenzene hydroxylation and styrene epoxidation by wild-type P450st were found to be activated in weak-acidic and weak-basic solutions. Under these conditions, the Michaelis constant for hydrogen peroxide (K_m^{H2O2}) was decreased. The turnover rate (k_{cat}) of ethylbenzene hydroxylation was increased and followed an S-shaped curve with an increase in the pH

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value. The apparent acid dissociation constant (pK_a^{app}) of the k_{cat} was 7.0, which suggests that the rate-limiting step of this reaction is the deprotonation of the Fe^{III}-H_2O_2 complex. By introducing a double mutation around the proximal heme ligand, the peroxygenase activity was increased over a wide pH range and was dramatically increased at pH 5. The spectroscopic properties of this F310A/A320Q mutant indicated that the Lewis acidity of the heme was increased by this mutation. Kinetic investigations showed that the rise in the Lewis acidity of the heme facilitates the rate-limiting step of peroxygenase reactions and decreases the K_m^{H2O2} value. Differences in the pH dependence of the k_{cat} value between wild-type P450st and the mutant suggest that the rate-limiting step switches to the protonation of the ferric-hydroperoxo species (compound 0) under alkaline conditions.

INTRODUCTION

Cytochrome P450s (P450s) [1] are a family of heme-thiolate protein that are involved in various metabolic and biosynthetic pathways in ubiquitous organisms. P450s catalyze the monooxygenation of hydrophobic substrates by activating dioxygen for insertion into inactive hydrogen-carbon bonds. Their ability to catalyze various oxidation reactions (e.g., hydroxylation, epoxidation, dealkylation, and C-C bond cleavage) with high regio- and stereoselectivity potentially expands the application of P450s to industrial chemistry.

In general, monooxygenation by P450s proceeds along a reaction cycle that requires a dioxygen, two electrons from NAD(P)H, and two protons [1]. However, some P450s and P450 variants catalyze peroxide driven-monooxygenation reactions via the peroxide shunt pathway [2-8]. The overall reaction equation of this "peroxygenase reaction" is as follows:

$$RH + H_2O_2 \rightarrow ROH + H_2O$$

In this reaction, a molecule of peroxide (e.g., hydrogen peroxide, cumene hydroperoxide, *t*-butyl hydroperoxide, or *m*-chloroperbenzoic acid (*m*-CPBA)) accesses the heme and directly forms active oxidants. This reaction is especially useful for industrial applications because the P450 electron transport system and NAD(P)H, which is expensive, are not required. However, the peroxygenase activities of P450s are generally low. Although some P450 mutants [9, 10] and P450 derivatives (several fungal peroxygenases [8, 11-13]) have been reported to catalyze peroxygenase reactions efficiently, there is still not enough knowledge of the peroxygenase reaction properties.

In recent years, several thermophilic P450s from various thermophilic archaea and bacteria have been reported [14-18]. These P450s possess a rigid structure and maintain their enzymatic activities even under extreme conditions. For example, CYP119 from the acidothermophilic archaeon *Sulfolobus acidocaldarius* was the first reported thermophilic P450. CYP119 exhibits a much higher melting temperature (T_m = 91°C) than typical mesophilic P450s, such as $P450_{cam}$ (T_m = 54°C) [19, 20]. The high structural stability of thermophilic P450s is adaptable not only for industrial applications but also for elucidating the biochemical properties of P450s under a wide range of conditions.

We previously isolated a thermophilic P450 from the thermoacidophilic crenarchaeon *Sulfolobus tokodaii* strain 7 (P450st). P450st exhibits high tolerance for heat (does not denature even at 80°C), acid, and alkaline (maintains its local structure around the heme at pH 1.5-11) [16, 21-23]. We also revealed the

capability of P450st to catalyze peroxygenase reactions (the k_{cat} value of styrene epoxidation is 8.7×10^{-3} s^{-1} at pH 7.5) [21]. Therefore, P450st is an appropriate model for the elucidation of the peroxygenase properties of P450s under various conditions. In this paper, we report the pH dependence of the peroxygenase reactions catalyzed by P450st. The rate-limiting step of these reactions was identified by kinetic investigations over the wide pH range. We also developed a more active mutant by mutating the residues around the proximal heme ligand and determined that the Lewis acidity of the heme controls the rate of this rate-limiting step.

RESULTS

pH dependence of the peroxygenase reactions catalyzed by wild-type P450st. The specific activities of several peroxygenase reactions catalyzed by wild-type P450st were investigated at various pH values. The endogenous substrates for P450st are unknown. However, long-chain saturated fatty acids (especially lauric acid) have been experimentally shown to bind to P450st with high affinity, inducing a type I spectral change. In this study, ethylbenzene and styrene were selected as model substrates because they are commonly utilized and are easy to detect by gas chromatography. However, the binding of these substrates to P450st does not induce a type I spectral change. After incubation with wild-type P450st and hydrogen peroxide, ethylbenzene was converted into two products. The major product was identified as 1-phenylethanol by direct chromatographic comparison with an authentic standard, while the minor product was unidentified. The peak area ratio of the minor product to the major product was 1:15 and was relatively unchanged when the pH value was varied. Styrene was converted into a sole product, styrene oxide, as reported previously [21]. Under all pH conditions, the identities of the products were unchanged, and no products were detected in the absence of P450st.

The pH dependences of the specific activities of ethylbenzene hydroxylation and styrene epoxidation by wild-type P450st via the peroxide shunt pathway are shown in Figure 1. At all pH values, the ethylbenzene hydroxylation activity was higher than the styrene epoxidation activity. This result could be due to a difference in the binding affinity of the two substrates. Notably, the pH dependences of these reactions were in good agreement with each other although their substrate oxidation mechanisms are different. This result suggests that the rate-limiting steps of ethylbenzene hydroxylation and styrene epoxidation are the same and could be any step before the formation of the active oxidant.

To provide further insight into hydrogen peroxide-driven ethylbenzene hydroxylation at each pH value, steady-state kinetic measurements of this reaction were made. At all pH values, plots of the initial rate versus the hydrogen peroxide concentration gave a Michaelis-Menten-type saturation curve. The kinetic parameters at each pH values are shown in Table 1 and Figure 2. Wild-type P450st showed the highest Michaelis constant value (~80 mM) for hydrogen peroxide (K_m^{H2O2}) at pH 7. This value was reduced to about one-third at pH 5 and 10, indicating that the affinity of P450st for hydrogen peroxide is increased in weak-acidic and weak-basic solutions. These changes in the K_m^{H2O2} value could not be due to changes in the protonation state of hydrogen peroxide due to its high pK_a value (p$K_a \approx 12$ in water). Thus, some changes in the protein structure and/or the electronic properties of heme could affect the affinity of P450st for hydrogen peroxide.

On the other hand, the turnover rate (k_{cat}) exhibited an S-shaped curve with increasing pH until pH 10 (Figure 2B). The apparent acid dissociation constant (pK_a^{app}) value was 7.0, which was estimated by fitting the curve to the Henderson-Hasselbalch equation. These findings indicate that the rate-limiting step is any deprotonation reaction with a pK_a value of 7.0.

Modulation of the Lewis acidity of the heme by mutating the residues around the proximal heme site. Previously, we prepared a series of P450st mutants in which the heme binding motif residues were mutated [21]. In one of these mutants, the conserved phenylalanine (Phe310) is substituted with alanine and Ala320 is substituted with a glutamine. This mutant (F310A/A320Q) exhibits a 30 mV positive-shift in redox potential for the Fe^{III}/Fe^{II} couple compared with that of wild-type P450st due to the weakening of the push effect of the proximal thiolate in the mutant. This result indicates that the electron density around the heme is decreased, and thus, the Lewis acidity of the heme is expected to be increased. We also revealed that the F310A/A320Q mutant maintains the higher thermal stability than typical P450s [21]. Hence, this mutant is suitable to confirm the effects of increasing of the Lewis acidity of the heme on the peroxygenase activity of P450st.

A previous spectroscopic study of wild-type P450st has noted that the Lewis acidity of the heme can be evaluated by the pK_a value of "the acid-alkaline transition", which is due to the protonation equilibrium between Fe^{III}-H_2O and Fe^{III}-OH^- (pK_a^{AA}) [23]. To investigate effects of the mutations on the Lewis acidity of the heme, the pH dependence of the spectroscopic properties of the F310A/A320Q mutant was investigated. The optical absorption spectra of the mutant at various pH values are shown in Figure 3A. The mutant exhibited a typical spectrum of a ferric low-spin heme at pH 3-7, as previously demonstrated by optical absorption spectroscopy and resonance Raman spectroscopy at pH 7.5 [21]. Below pH 2, the mutant lost the heme due to cleavage of the Fe-S bond; heme dissociation occurs below pH 1.5 for wild-type P450st [23]. This difference in tolerance for acid is likely due to a slight decrease in the structural stability of the mutant [21].

In weak-basic solutions, the mutant exhibited a type II-like spectral change with no aggregation and/or precipitation, as was previously observed for wild-type P450st [23]. This change was reversible and showed well-defined isosbestic points at 376, 423, and 516 nm, demonstrating that only two states are present during this transition. These findings demonstrate that the observed spectral change is the acid-alkaline transition. The pK_a^{AA} value was calculated as 8.2 ± 0.1 by fitting the curve to the Henderson-Hasselbalch equation. This value is lower than that of wild-type P450st (pK_a^{AA} = 8.7 ± 0.1) [23], demonstrating that the Lewis acidity of the heme is increased by the mutations as expected. The high Lewis acidity of the mutant reflects the low electron density around the heme.

pH dependence of the peroxygenase reactions catalyzed by the F310A/A320Q mutant. By mutating F310 and A320, the peroxygenase activities were significantly increased over a broad pH range. Figure 4 shows the specific activities of the peroxygenase reactions (ethylbenzene hydroxylation and styrene epoxidation) catalyzed by the F310A/A320Q mutant at various pH values. Similar to wild-type P450st, the mutant converted ethylbenzene and styrene to 1-phenylethanol (with a small amount of unknown byproduct) and styrene epoxide, respectively. The pH dependences of these reactions were changed to the bell-shaped pH dependences with maximum activities near pH 5. At pH 5, the mutant was over 15-fold more active than wild-type P450st. Notably, the pH dependence of styrene epoxidation was in good agreement with that of ethylbenzene hydroxylation even for the mutant. This result suggests that, similar to the case of wild-type P450st, the rate-limiting steps of both reactions are the same.

The kinetic parameters of hydrogen peroxide-driven ethylbenzene hydroxylation by the F310A/A320Q mutant are shown in Table 2. Similar to wild-type P450st, plots of the initial rate versus the hydrogen peroxide concentration gave a Michaelis-Menten-type saturation curve at all pH values investigated. The K_m^{H2O2} values were significantly decreased in the mutant at all of the examined pH values, while its pH

dependence was unchanged. The binding of hydrogen peroxide is most likely facilitated due to the increase in the Lewis acidity of the heme iron.

Earlier, we revealed that wild-type P450st exhibited an S-shaped pH-k_{cat} profile with a pK_a^{app} value of 7.0 (Figure 2B). For the mutant, the pH-k_{cat} profile was changed to a bell-shaped curve with a maximum value at neutral pH (pH 5 ≈ pH 10 < pH 7). At pH 7, the k_{cat} value was increased by 2.7-fold for the mutant. This increase was likely due to the acceleration of the rate-limiting deprotonation of the Fe^{III}-H_2O_2 complex by the increase in the Lewis acidity of the heme. On the other hand, the k_{cat} value was decreased by 0.6-fold for the mutant at pH 10. This change indicates a change in the rate-limiting step.

DISCUSSION

We have investigated the peroxygenase property of wild-type P450st and a mutant at various pH values. For wild-type P450st, hydrogen peroxide-driven ethylbenzene hydroxylation and styrene epoxidation were activated in weak-acidic and weak-basic solutions, and the pH dependences of these reactions were in good agreement with each other. The k_{cat} value exhibited an S-shaped curve with increasing pH and the pK_a^{app} value of 7.0. These results indicate that the rate-limiting step is any deprotonation reaction before the formation of the active oxidant with a pK_a value of 7.0.

As shown in Scheme 1, the reaction mechanism of peroxygenase reactions by P450s involves many protonation/deprotonation steps [1, 2, 24]. The resting state of P450s has a heme with a water ligand (or a hydroxide ligand at high pH) [23] at the sixth coordination site. When a substrate and a hydrogen peroxide access the active site, the ligand at the sixth coordination site is substituted by the hydrogen peroxide and the ferric-hydroperoxide (Fe^{III}-H_2O_2) complex is generated. Then, this complex is deprotonated and converted to a ferric-hydroperoxo species (compound 0). Because compound 0 is a good Lewis base, it undergoes protonation to the outer oxygen atom. This protonation induces heterolytic O-O bond cleavage and forms an oxoferryl porphyrin π-cation radical species (compound I), which is expected to be the major active oxidant of hydroxylation by P450s. During these steps, there is only one deprotonation step, the formation of compound 0 by the deprotonation of the Fe^{III}-H_2O_2 complex (Scheme 1, a). Therefore, this step should be the rate-limiting step of the peroxygenase reactions catalyzed by wild-type P450st.

The pK_a value of the Fe^{III}-H_2O_2 complex of P450st and other P450s has not yet been experimentally confirmed. Thus, we decided to estimate this value from the pK_a^{AA} value. Egawa et al. revealed that the formation of compound 0 by several myoglobins via reaction with hydrogen peroxide is activated in weak-basic solutions with a pK_a^{app} value of 7.00-7.27 [25]. They also elucidated that the pK_a^{app} values are ~1.7 units lower than the pK_a^{AA} values. The relationship between these two pK_a values should be applicable to P450st. The pK_a^{AA} value of wild-type P450st has been previously estimated as 8.7 [20], suggesting that the pK_a value of the formation of compound 0 by the deprotonation of the Fe^{III}-H_2O_2 complex is 7.0. This value is completely in agreement with the pK_a^{app} value that was calculated from the pH dependence of the k_{cat} value. This agreement confirms that the rate-limiting step of peroxygenase reactions by P450st is the deprotonation of the Fe^{III}-H_2O_2 complex.

This rate-limiting step is quite different from the case of typical monooxygenation reactions by P450s: the rate-limiting steps are considered to be any step after formation of the active oxidant (e.g. C-H bond breaking or product dissociation from P450s) as well as the electron transfer steps [26-30]. If the active oxidant is formed rapidly enough, the rate-limiting step of peroxygenase reaction should be the substrate

oxidation or the conformational change in the substrate-bound complex as described in recent studies of CYP119 compound I [31-33].

Then, we attempted to improve the peroxygenase activity of P450st on the basis of the reaction mechanism by a site-directed mutagenesis study. P450s have highly conserved residues around the proximal cysteine ligand, which are collectively called the "heme-binding" motif. Previous studies have revealed that several residues within this region control the redox potential of the heme. For example, several site-directed mutagenesis studies of cytochrome P450BM3 have reported that a highly conserved phenylalanine residue within the heme binding loop (Phe393 in P450BM3) controls electron donation from the thiolate ligand [34-37]. In the present study, the F310A/A320Q mutant of P450st, which is also mutated around the heme-binding motif, was utilized. We previously revealed that this mutant keeps the high thermostability and exhibits a positive-shift in redox potential for the Fe^{III}/Fe^{II} couple due to decrease of the electron donation from the thiolate ligand [21].

The decreased pK_a^{AA} value of the F310A/A320Q indicated that the Lewis acidity of the heme is increased by the mutations. The peroxygenase activity of this mutant was higher than that of wild-type P450st over a broad pH range, and the pH-k_{cat} profile was changed to a bell-shaped curve with a maximum value at neutral pH. This change of the pH-k_{cat} profile indicates a change in the rate-limiting step in weak-basic solutions. The increase in the Lewis acidity of the heme not only accelerates the deprotonation of the heme ligand but also inhibits its protonation. Especially in basic solutions, the protonation of the heme ligand should be strongly inhibited. Thus, it is likely that a change in the rate-limiting step of the peroxygenase reaction from the deprotonation of the Fe^{III}-H_2O_2 complex to the formation of compound I by the protonation of compound 0 results in the decrease in the k_{cat} value at pH 10. This hypothesis is reasonable because the heterolytic cleavage of the O-O bond of compound 0 to form compound I must be disturbed in the mutant due to the weak push effect of the proximal thiolate.

We previously reported that the F310A/A320Q mutant catalyzes styrene epoxidation via the peroxide shunt pathway more efficiently than wild-type P450st in neutral solutions [21]. This report suggested that the activation of the peroxygenase reaction in spite of the weakening push effect of the proximal heme ligand indicates the participation of compound 0 in this reaction. However, this assumption is contradicted by the present study because ethylbenzene hydroxylation, which is not catalyzed by compound 0, is also activated with the mutant. Kinetic studies over a wide pH range revealed that ethylbenzene hydroxylation and styrene epoxidation progress with the same rate-limiting step, the deprotonation of the Fe^{III}-H_2O_2 complex. As revealed in the present study, the previously reported acceleration of styrene epoxidation via the peroxide shunt pathway for the mutant is thus attributed to the acceleration of this step. These studies confirm that rates of the peroxygenase reactions catalyzed by P450st are adjustable by changing the Lewis acidity of the heme.

Peroxygenase reactions catalyzed by P450s were discovered in the 1970's, and enormous efforts to understand this type of reaction have been undertaken. The reaction mechanism has been gradually elucidated, and recently, highly reactive P450 mutants have been developed by protein engineering. In addition, studies of some P450 derivatives (fungal peroxygenases) have revealed the property of peroxygenase reaction. However, further investigation (e.g. kinetics, pH dependence, thermodynamic analysis, and structural insight) is required to better understand this reaction. In this study, we revealed the pH dependence of the peroxygenase reactions catalyzed by wild-type P450st and identified the rate-limiting step of this reaction. We also succeeded to increase the peroxygenase activity of P450st on

the basis of this mechanistic insight. These findings provide detailed insights into peroxygenase reactions catalyzed by P450s.

The kinetic parameters determined in this study show that the low affinity for hydrogen peroxide and the interruption of the deprotonation of the Fe^{III}-H_2O_2 complex are primary problems for the efficient progression of peroxygenase reactions. We demonstrated that an increase in the Lewis acidity of the heme is an effective solution for these problems. This method should be applicable to other P450s because the reaction mechanism of peroxygenase reactions is common for P450s. Our observations also revealed that the reaction rate of the mutant is reduced in weak-alkaline solutions due to a change in the rate-limiting step. This result indicates that the Lewis acidity of the heme must be optimized according to the reaction conditions, taking into account the efficiency of the protonation/deprotonation steps in the reaction cycle. To address this issue, arrangement of proton donor/accepters around the distal heme site seems to be effective as well as modulation of the Lewis acidity of the heme only if substrate binding of substrates is not disturbed.

EXPERIMENTAL PROCEDURE

Expression, purification, and spectroscopic observation of P450st. Wild-type P450st and the F310A/A320Q mutant were expressed in *Escherichia coli* BL21 (DE3) (Merck) and purified as reported previously [16, 21]. Optical absorption spectroscopy of the F310A/A320Q mutant was carried out with a Shimadzu UV-2450 spectrometer at 25°C in several buffers (40 mM): glycine-HCl (pH 2-3), acetate (pH 4-5), potassium phosphate (pH 6-8), and glycine-NaOH (pH 9-12). The pK_a value of the acid-alkaline transition ($Fe^{III}H_2O \leftrightarrow Fe^{III}OH^- + H^+$) of the mutant was determined by the shift of the Soret region with fitting to the Henderson-Hasselbalch equation (eq. 1) as reported previously [23].

$$pH = pK_a + \frac{[Fe^{III}OH^-]}{[Fe^{III}H_2O]} \qquad \text{(eq. 1)}$$

Specific activities of the peroxygenase reactions. The specific activities of the peroxygenase reactions catalyzed by wild-type P450st and the F310A/A320Q mutant were examined at 25°C in the various 40 mM buffers (pH 2-12) described above. The reaction mixtures (total volume of 200 μl) contained 40 μM enzyme, 2 mM substrate (ethylbenzene or styrene), and 3 mM hydrogen peroxide. The substrates were dissolved in acetonitrile, and 2 μl substrate was added to the reaction mixture. The reaction was initiated by the addition of enzyme. Control incubations were performed without enzyme or hydrogen peroxide. The samples were extracted with dichloromethane containing an internal standard (styrene for ethylbenzene hydroxylation, and 2-phenylethanol for styrene epoxidation). The combined dichloromethane layers were concentrated by evaporation and then analyzed by isothermal gas chromatography (GC-4000, GL Science) at 80°C on an InterCap 1 column (30 m × 0.25 mm inner diameter, GL Science). Under these conditions, the retention times for ethylbenzene, 1-phenylethanol, styrene, and styrene oxide were 3.0, 6.9, 3.3, and 7.0 min, respectively. The products formations were quantified using relative peak areas with respect to the internal standards.

Steady-state kinetic analysis. All steady-state kinetic measurements of ethylbenzene hydroxylation were performed at 30°C in several 40 mM buffers (pH 4-10). The reaction mixtures (total volume of 200 μl)

contained 10 μM enzyme, 10 mM ethylbenzene, and 1-300 mM hydrogen peroxide. The reaction, extraction, and product detection were performed as described above (in the "*Specific activities of the peroxygenase reactions*" section). The kinetic constants were calculated with Hanes-Woolf plots.

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REFERENCES

1. Ortiz de Montellano PR (2005) *Cytochrome P450: Structure, Mechanism, and Biochemistry*, 3rd ed. Kluwer Academic/Plenum Publishers, NY.
2. Kadlubar FF, Morton KC & Ziegler DM (1973) Microsomal-catalyzed hydroperoxide-dependent c-oxidation of amines. *Biochem Biophys Res Commun* **54**, 1255-1261.
3. Hrycay EG, Gustafsson JÅ, Ingelman-Sundberg M & Ernster L (1975) Sodium periodate, sodium chlorite, organic hydroperoxides, and H_2O_2 as hydroxylating agents in steroid hydroxylation reactions catalyzed by partially purified cytochrome P-450. *Biochem Biophys Res Commun* **66**, 209-216.
4. Matsunaga I, Yamada M, Kusunose E, Nishiuchi Y, Yano I, & Ichihara K (1996) Direct involvement of hydrogen peroxide in bacterial α-hydroxylation of fatty acid. *FEBS Lett* **386**, 252-254.
5. Matsunaga I, Ueda A, Fujiwara N, Sumimoto T, & Ichihara K (1999) Characterization of the *ybdT* gene product of *Bacillus subtilis*: Novel fatty acid β-hydroxylating cytochrome P450. *Lipids* **34**, 841-846.
6. Joo H, Lin Z, & Arnold FH (1999) Laboratory evolution of peroxide-mediated cytochrome P450 hydroxylation. *Nature* **399**, 670-673.
7. Girhard M, Schuster S, Dietrich M, Dürre P, & Urlacher VB (2007) Cytochrome P450 monooxygenase from *Clostridium acetobutylicum*: A new α-fatty acid hydroxylase. *Biochem Biophys Res Commun* **362**, 114-119.
8. Hrycay EG, & Bandiera SM (2012) The monooxygenase, peroxidase, and peroxygenase properties of cytochrome P450. *Arch Biochem Biophys* **522**, 71-89.
9. Li QS, Ogawa J, & Shimizu S (2001) Critical role of the residue size at position 87 in H_2O_2-dependent substrate hydroxylation activity and H_2O_2 inactivation of cytochrome P450BM-3. *Biochem Biophys Res Commun* **280**, 1258-1261.
10. Cirino PC, & Arnold FH (2003) A self-sufficient peroxide-driven hydroxylation biocatalyst. *Angew Chem Int Ed* **42**, 3299-3301.
11. Ullrich R, & Hofrichter M (2005) The haloperoxidase of the agaric fungus *Agrocybe aegerita* hydroxylates toluene and naphthalene. *FEBS Lett* **579**, 6247-6250.
12. Kluge MG, Ullrich R, Scheibner K, & Hofrichter M (2007) Spectrophotometric assay for detection of aromatic hydroxylation catalyzed by fungal haloperoxidase-peroxygenase. *Appl Microbiol Biotechnol* **75**, 1473-1478.
13. Praj-Kobielska M, Kinne M, Ullrich R, Scheibner K, Kayser G, Hammel KE, & Hofrichter M (2011) Preparation of human drug metabolites using fungal peroxygenases. *Biochem Pharmacol* **82**, 789-796.
14. Wright RL, Harris K, Solow B, White RH, & Kennelly PJ (1996) Cloning of a potential cytochrome P450 from the Archaeon *Sulfolobus solfataricus*. *FEBS Lett* **384**, 235-239.
15. Yano JK, Blasco F, Li H, Schmid RD, Henne A, & Poulos TL (2003) Preliminary characterization

and crystal structure of a thermostable cytochrome P450 from *Thermus thermophilus*. *J Biol Chem* **278**, 608-616.
16. Oku Y, Ohtaki A, Kamitori S, Nakamura N, Yohda M, Ohno H, & Kawarabayashi Y (2004) Structure and direct electrochemistry of cytochrome P450 from the thermoacidophilic crenarchaeon, *Sulfolobus tokodaii* strain 7. *J Inorg Biochem* **98**, 1194-1199.
17. Nishida CR, & Ortiz de Montellano PR (2005) Thermophilic cytochrome P450 enzymes. *Biochem Biophys Res Commun* **338**, 437-445.
18. Ho WW, Li H, Nishida CR, Ortiz de Montellano PR, & Poulos TL (2008) Crystal structure and properties of CYP231A2 from the thermoacidophilic archaeon *Picrophilus torridus*. *Biochemistry* **47**, 2071-2079.
19. McLean MA, Maves SA, Weiss KE, Krepich S, & Sligar, SG (1998) Characterization of a cytochrome P450 from the acidothermophilic archaea *Sulfolobus solfataricus*. *Biochem Biophys Res Commun* **252**, 166-172.
20. Koo LS, Tschirret-Guth RA, Straub WE, Moënne-Loccoz P, Loehr TM, & Ortiz de Montellano PR (2000) The active site of the thermophilic CYP119 from *Sulfolobus solfataricus*. *J Biol Chem* **275**, 14112-14123.
21. Matsumura H, Wakatabi M, Omi S, Ohtaki A, Nakamura N, Yohda M, & Ohno H (2008) Modulation of redox potential and alteration in reactivity via the peroxide shunt pathway by mutation of cytochrome P450 around the proximal heme ligand. *Biochemistry* **47**, 4834-4842.
22. Matsumura H, Matsuda K, Nakamura N, Ohtaki A, Yoshida H, Kamitori S, Yohda M, & Ohno H (2011) Monooxygenation by a thermophilic cytochrome P450 via direct electron donation from NADH. *Metallomics* **3**, 389-395.
23. Hayakawa S, Matsumura H, Nakamura N, Yohda M, & Ohno H (2013) Spectroscopic characterization of the acid-alkaline transition of a thermophilic cytochrome P450. *FEBS Lett* **587**, 94-97.
24. Hollenberg PF (1992) Mechanisms of cytochrome P450 and peroxidase-catalyzed xenobiotic metabolism. *FASEB J* **6**, 686-694.
25. Egawa T, Yoshioka S, Takahashi S, Hori H, Nagano S, Shimada H, Ishimori K, Morishima I, Suematsu M, & Ishimura Y (2003) Kinetic and spectroscopic characterization of a hydroperoxy compound in the reaction of native myoglobin with hydrogen peroxide. *J Biol Chem* **278**, 41597-41606.
26. Bell LC, & Guengerich FP (1997) Oxidation kinetics of ethanol by human cytochrome P450 2E1. Rate-limiting product release accounts for effects of isotopic hydrogen substitution and cytochrome b_5 on steady-state kinetics. *J Biol Chem* **272**, 29643-29651.
27. Guengerich FP (1999) Cytochrome P-450 3A4: Regulation and role in drug metabolism. *Annu Rev Pharmacol Toxicol* **39**, 1-17.
28. Guengerich FP (2002) Rate-limiting steps in cytochrome P450 catalysis. *Biol Chem* **383**, 1553-1564.
29. Schenkman JB, & Jansson I (2003) The many roles of cytochrome b_5. *Pharmacol Ther* **97**, 139-152.
30. Shinkyo R, & Guengerich FP (2011) Cytochrome P450 7A1 cholesterol 7α-hydroxylation. Individual reaction steps in the catalytic cycle and rate-limiting ferric iron reduction. *J Biol Chem* **286**, 4632-4643.
31. Rittle J, & Green MT (2010) Cytochrome P450 compound I: Capture, characterization, and C-H bond activation kinetics. *Science* **330**, 933-937.
32. Su Z, Chen X, Horner JH, & Newcomb M (2012) Rate-controlling isomerizations in fatty acid oxidations by a cytochrome P450 compound I. *Chem Eur J* **18**, 2472-2476.
33. Su Z, Horner JH, & Newcomb M (2012) Rates of fatty acid oxidations by P450 compound I are pH

dependent. *ChemBioChem* **13**, 2061-2064.
34. Ost TWB, Miles CS, Munro AW, Murdoch J, Reid GA, & Chapman SK (2001) Phenylalanine 393 exerts thermodynamic control over the heme of flavocytochrome P450 BM3. *Biochemistry* **40**, 13421-13429.
35. Ost TWB, Munro AW, Mowat CG, Taylor PR, Pesseguiero A, Fulco AJ, Cho AK, Gheesman MA, Walkinshaw MD, & Chapman SK (2001) Structural and spectroscopic analysis of the F393H mutant of flavocytochrome P450 BM3. *Biochemistry* **40**, 13430-13438.
36. Ost TWB, Clark J, Mowat CG, Miles CS, Walkinshaw MD, Reid GA, Chapman SK, & Daff S (2003) Oxygen activation and electron transfer in flavocytochrome P450 BM3. *J Am Chem Soc* **125**, 15010-15020.
37. Chen Z, Ost TWB, & Schelvis JPM (2004) Phe393 mutants of cytochrome P450 BM3 with modified heme redox potentials have altered heme vinyl and propionate conformations. *Biochemistry* **43**, 1798-1808.

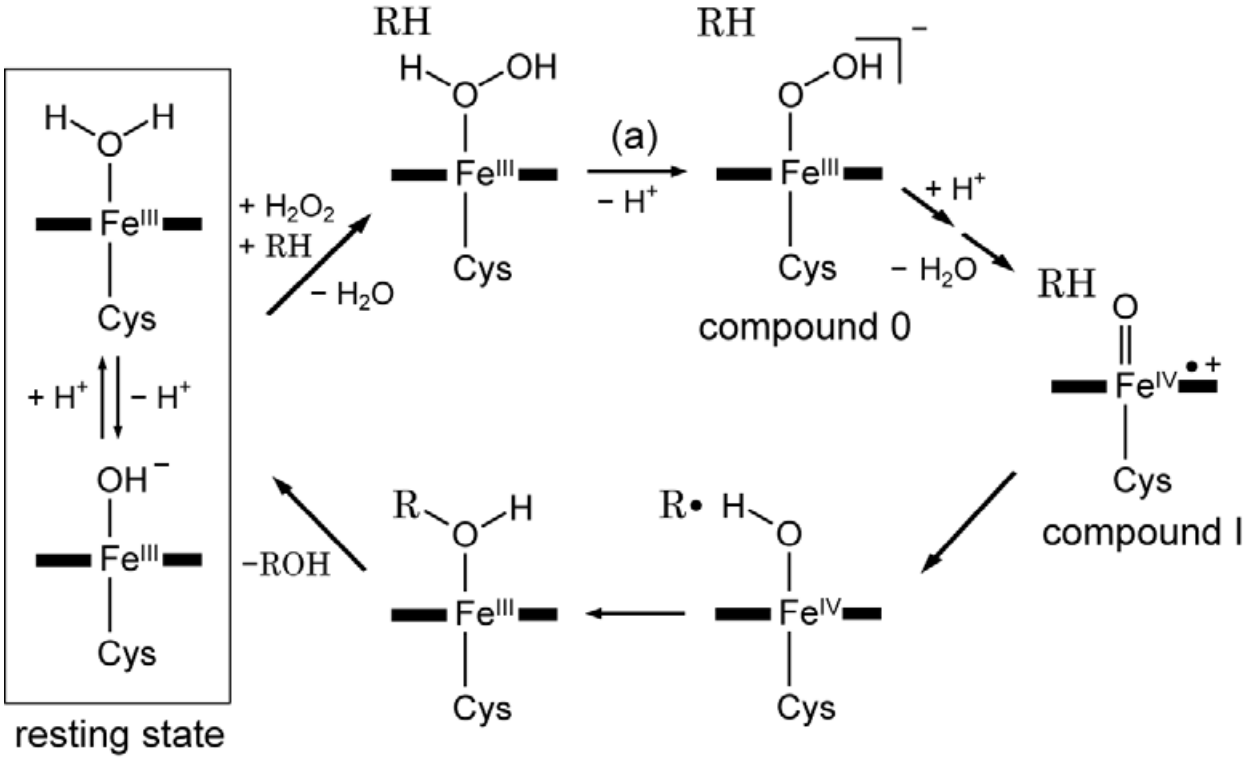
Table 1. Kinetic parameters of hydrogen peroxide-driven ethylbenzene hydroxylation by wild-type P450st at various pH values.

	K_m^{H2O2} (mM)	k_{cat} (min^{-1})	k_{cat}/K_m^{H2O2} ($\times 10^{-2}$ min^{-1} mM^{-1})
pH 5	23.2 ± 2.0	5.6 ± 1.4	24.0 ± 4.9
pH 7	81.4 ± 3.6	7.5 ± 0.5	9.3 ± 0.7
pH 10	26.2 ± 13.9	9.5 ± 3.1	41.5 ± 13.6

Table 2. Kinetic parameters of hydrogen peroxide-driven ethylbenzene hydroxylation by the F310A/A320Q mutant at various pH values.

	K_m^{H2O2} (mM)	k_{cat} (min^{-1})	k_{cat}/K_m^{H2O2} ($\times 10^{-2}$ min^{-1} mM^{-1})
pH 5	7.2 ± 2.4	4.3 ± 0.6	62.2 ± 14.5
pH 7	30.6 ± 1.6	20.4 ± 5.2	66.7 ± 17.4
pH 10	9.3 ± 1.7	5.3 ± 0.5	57.8 ± 5.4

SCHEME 1. The reaction mechanism of hydroxylation by P450s via the peroxide shunt pathway.



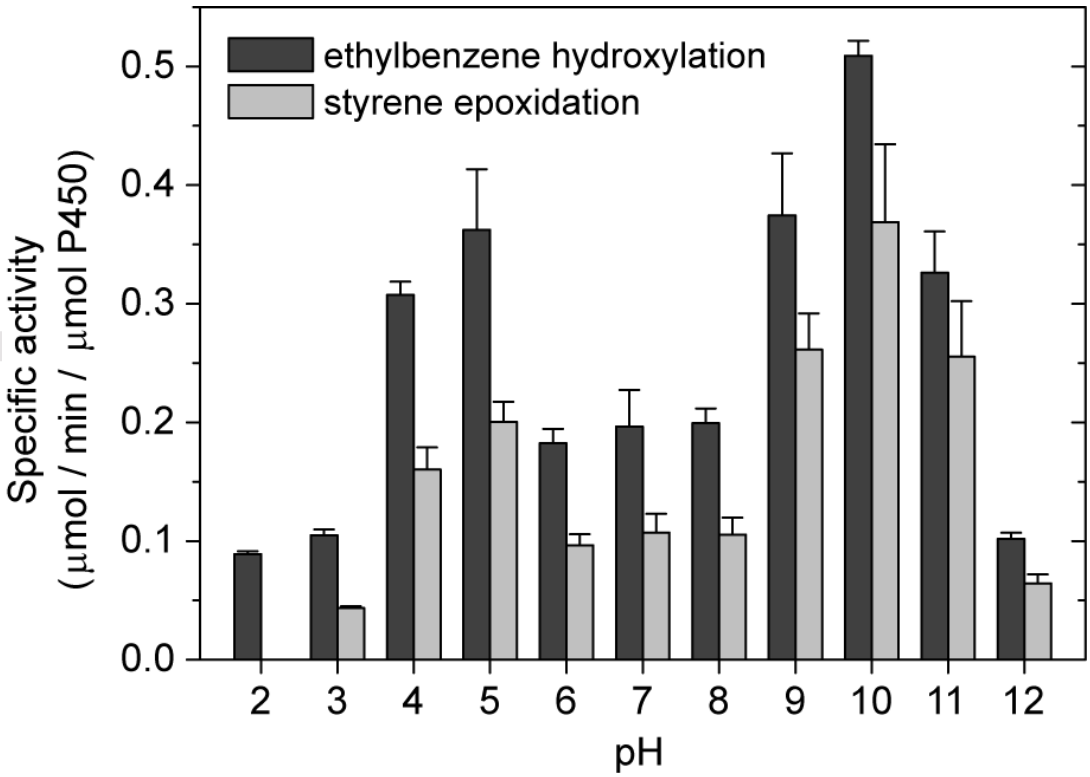


FIGURE 1. Specific activities of the peroxygenase reactions (ethylbenzene hydroxylation (*black*) and styrene epoxidation (*gray*)) catalyzed by wild-type P450st at pH 2-12.

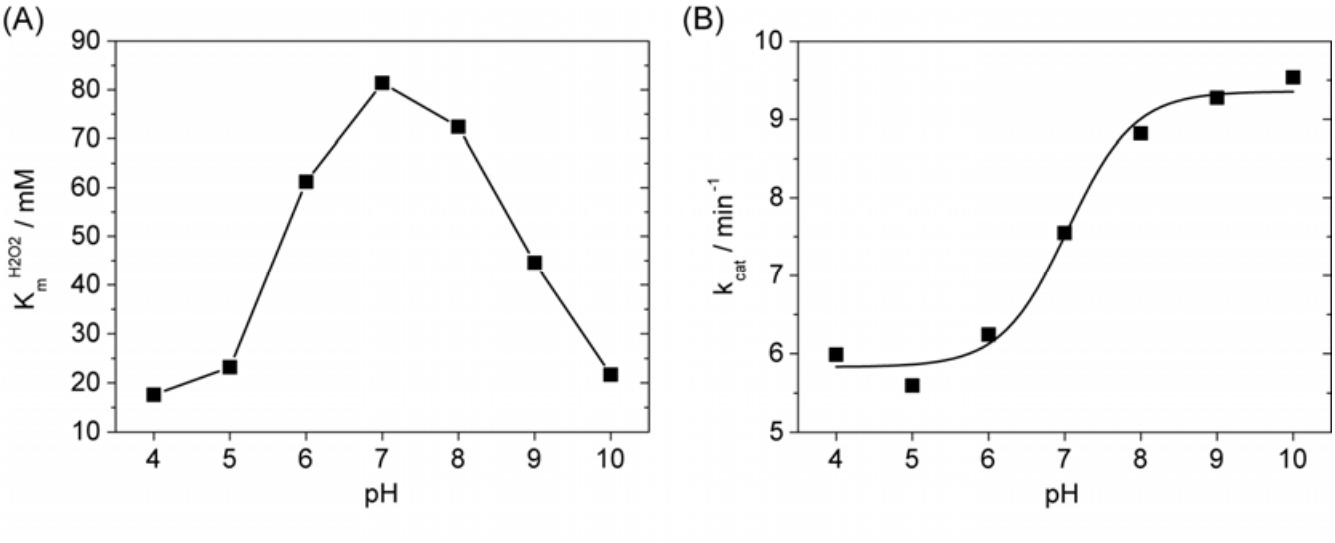


FIGURE 2. The pH dependences of (A) the K_m value for hydrogen peroxide (K_m^{H2O2}) and (B) the k_{cat} value of hydrogen peroxide-driven ethylbenzene hydroxylation by wild-type P450st. Plots of the k_{cat} value as a function of pH were fitted to the Henderson-Hasselbalch equation for calculating the apparent acid dissociation constant (pK_a^{app}).

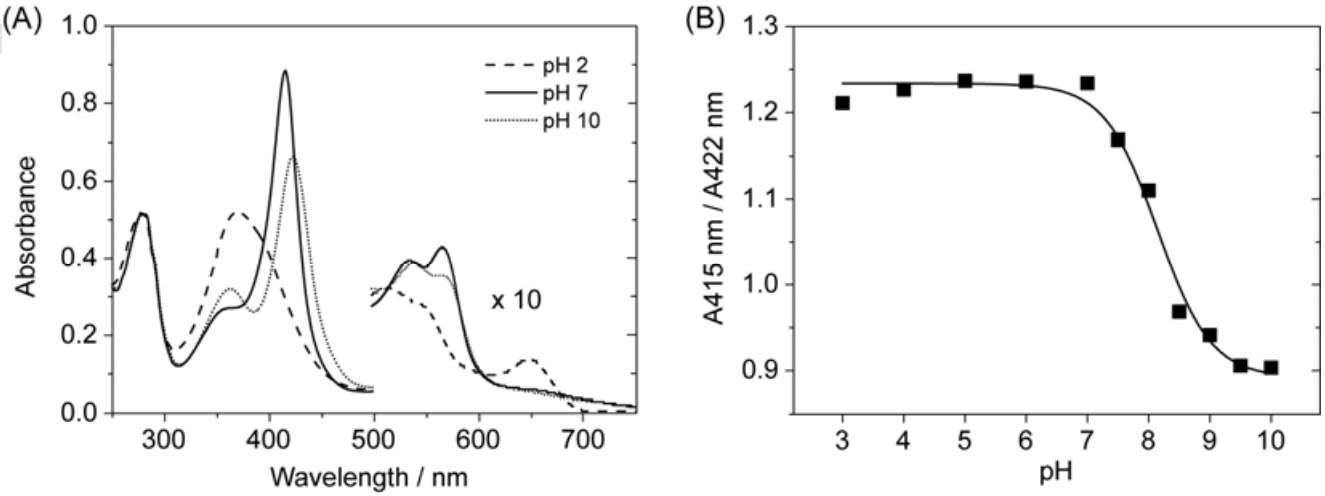


FIGURE 3. (A) Optical absorption spectra of the ferric state of the F310A/A320Q mutant at pH 2 (*dashed line*), pH 7 (*solid line*), and pH 10 (*dotted line*). (B) Shift of the Soret region as a function of pH. The plots were fitted to the Henderson-Hasselbalch equation to calculate the pK_a value.

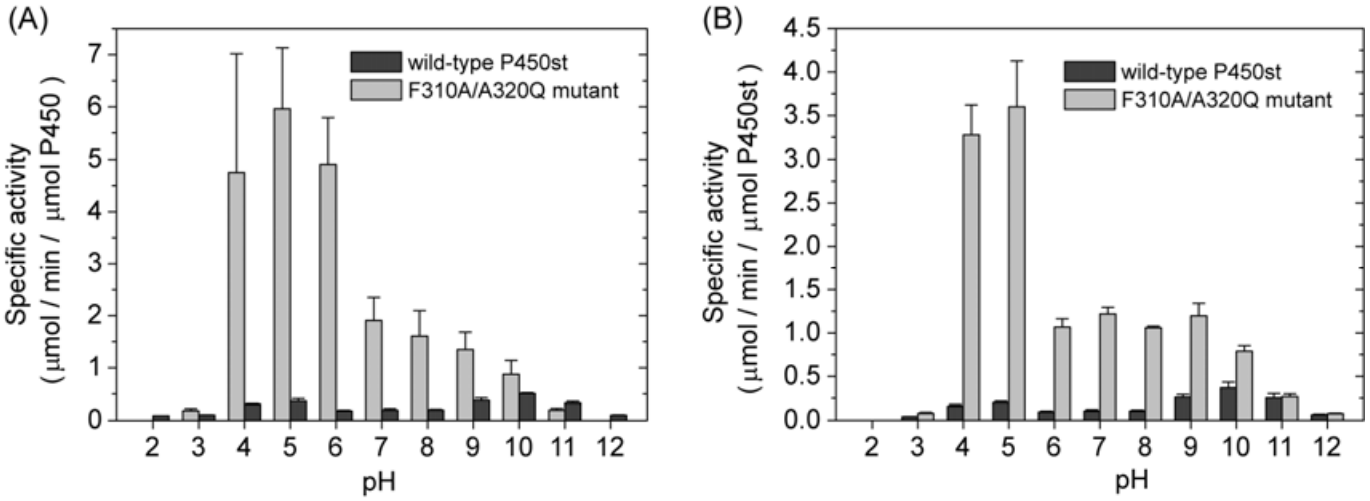


FIGURE 4. Specific activities of the peroxygenase reactions (ethylbenzene hydroxylation (A) and styrene epoxidation (B)) catalyzed by wild-type P450st (*black*) and the F310A/A320Q mutant (*gray*) at pH 2-12.