Reactivities of Oxo and Peroxo Intermediates Studied by Hemoprotein Mutants

YOSHIHITO WATANABE,*,†
HIROSHI NAKAJIMA,‡ AND TAKAFUMI UENO†
Research Center for Materials Science and Department of Chemistry, Graduate School of Science, Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464-8602, Japan

Received December 20, 2006

ABSTRACT
A series of myoglobin mutants, in which distal sites are modified by site-directed mutagenesis, are able to catalyze peroxidase, catalase, and P450 reactions even though their proximal histidine ligands are intact. More importantly, reactions of P450, catalase, and peroxidase substrates and compound I of myoglobin mutants can be observed spectroscopically. Thus, detailed oxidation mechanisms were examined. On the basis of these results, we suggest that the different reactivities of P450, catalase, and peroxidase are mainly caused by their active site structures, but not the axial ligand. We have also prepared compound 0 under physiological conditions by employing a mutant of cytochrome c_{552}. Compound 0 is not able to oxidize ascorbic acid.

Introduction

Hemoproteins and heme enzymes play a variety of biological roles, including carriage and storage of O_2 (hemoglobin and myoglobin), electron transfer (cytochromes), one-electron oxidation (peroxidase) and monooxygenation (cytochrome P450) of foreign substrates, dismutation of H_2O_2 (catalase), NO synthesis (NOS), four-electron reduction of O_2 (oxidase), sensing of small molecules such as CO (CooA), etc. Among these enzymes, we have been studying the enzymes related to oxidation and oxygenations, i.e., peroxidases, catalases, and cytochromes P450. While the reaction center of these enzymes is the heme, each enzyme is responsible for its own function(s) but not for others. It is still not quite clear to us why only P450 exhibits monooxygenase activity among the heme enzymes except for chloroperoxidase, since these enzymes use either H_2O_2 or O_2, 2 e^-, and 2 H^+ for the preparation of a very reactive intermediate, O=Fe^{IV} porphyrin π-cation radical, so called compound I, as the common oxidant. One could attribute different activities of these enzymes to the difference in their axial ligands, i.e., histidine in most peroxidases, tyrosine in catalases except for catalase peroxidases, cysteine in P450s (Figure 1). We believe that the different reactivity is mainly caused by their active site structures, but not the axial ligand. For example, the active site of horseradish peroxidase (HRP) is surrounded by phenylalanine residues to prevent the direct access of foreign substrates (Figure 2a). In fact, elimination of one phenyl group in HRP by site-directed mutagenesis allows the HRP mutant to catalyze sulfoxidation.[1] In the case of catalase, the active site is buried deep inside of the proteins; thus, it is impossible for catalase to catalyze the P450-type reactions (Figure 2, MLC). To improve our understanding of the general reactivity of compound I, we have prepared a series of myoglobin mutants that afford compound I as an observable species. On the other hand, a heme iron–hydroperoxo complex, compound 0, has been considered as a potent oxidant in P450 reactions, while its reactivity is still controversial.[2,3] Through the mutation work of *thermophilus* cytochrome c_{552}, we have observed compound 0 with a lifetime sufficiently long for examination of its reactivity. In this Account, we discuss the reactivity of compound I as well as compound 0 as models for the reactive intermediates of peroxidase, catalase, and P450.

Preparation and Reactivity of Compound 0 and Compound I

Production of Durable Compound 0 in a Mutant of Cytochrome c_{552}. Detailed studies on the reactive intermediates (compounds 0, I, and II) are very important for our understanding of the catalytic reactions of P450, peroxidases, and catalase. It is also crucial for the design of hydrogen peroxide-dependent monooxygenases (peroxygenases) via utilization of structurally well-defined hemoproteins. Scheme 1 shows mechanisms of oxygen activation and reactions catalyzed by heme enzymes, while some of the intermediates are not well characterized yet. Although a number of experimental and theoretical characterizations of electronic structure and reactivities

Yoshihito Watanabe was born in Iwate, Japan, in 1953. He received his B.Sc. degree from Tohoku University and Ph.D. degree under the direction of Shigeru Oae of Tsukuba University in 1982. He joined John T. Groves' group at the University of Michigan as a postdoctoral fellow and moved to Princeton University as a research staff member in 1985. In 1987, he was appointed as an Assistant Professor in the Department of Biochemistry, Medical School of Keio University, in Japan. In 1989, he joined as a senior research scientist the National Chemical Research Laboratories in Tsukuba. Then, he was appointed as an Associate Professor at Kyoto University in 1990. He spent 8 years at the Institute for Molecular Science from 1994 as a professor, and he joined to the current faculty at Nagoya University as a full professor in 2002. His current research interests are design of hydrogen peroxide-dependent monooxygenase and construction of metalloenzymes having synthetic complexes in the catalytic centers.

Hiroshi Nakajima was born in Osaka, Japan, in 1967. He received his Ph.D. at The Graduate University for Advanced Studies supervised by Prof. Koji Tanaka in 1996. He was a research associate in Prof. Shigetoshi Aono's research group at the Japan Advanced Institute of Science and Technology from 1996 to 2002 and at the Institute of Molecular Science for 1 year. He joined the current faculty at Nagoya University as an associate professor in 2003. His current research efforts are focused on chemical and biological transformation of thermally tolerant proteins and design of artificial sensor proteins based on elucidation of the signal transducing mechanism of natural sensor proteins.

Takafumi Ueno was born in Hokkaido, Japan, in 1971. After receiving his Ph. D. at Osaka University in Japan under the supervision of Prof. Akira Nakamura (1998), he spent two years in Mitsubishi Chemical Corp. He started his scientific research as a research associate with Prof. Yoshihito Watanabe at the Institute of Molecular Science (2000) and moved to Nagoya University as an assistant professor (2002). In 2004–2005, he joined the research group of Prof. John E. Johnson at The Scripps Research Institute. His current research interests lie in the design of artificial metalloenzymes and bio-nanomaterials.

* Corresponding author.
† Research Center for Materials Science.
‡ Department of Chemistry, Graduate School of Science.

10.1021/ar600046a CCC: $37.00
Published on Web 06/14/2007

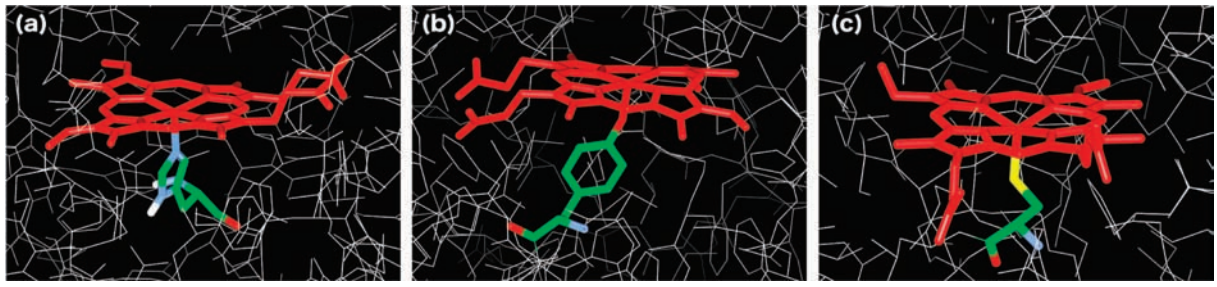


FIGURE 1. Heme ligands: histidine in cytochrome *c* peroxidase (C*c*P) (a, PDB entry 1CCA), tyrosine in catalase (MLC) (b, PDB entry 4BLC), and cysteine in P450cam (c, PDB entry 2CPP).

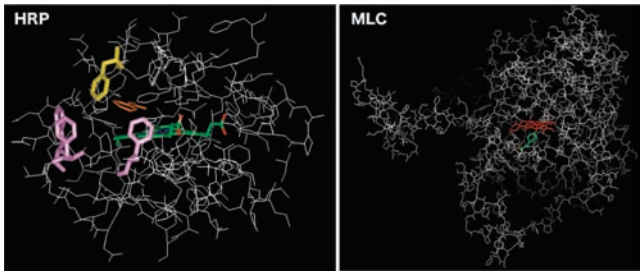
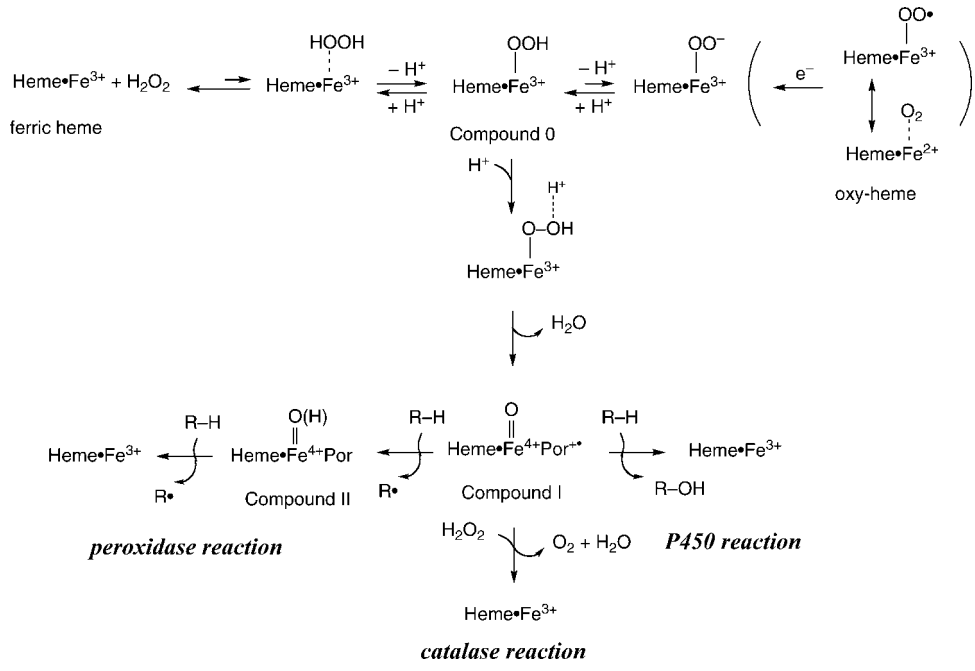


FIGURE 2. Active site structures of horseradish peroxidase (HRP, PDB entry 1ATJ) and catalase (MLC, PDB entry 4BLC).

of compounds I and II of P450 and peroxidase have been carried out, the reactivity of compound 0, which is expected to be a precursor of compound I by the reaction of ferric heme with H_2O_2, is still controversial. For example, possibly due to the transient character of a low steady state concentration of compound 0 under physiological conditions, clear spectroscopic characterization of compound 0 has been unsuccessful in the reaction cycle. In an earlier work, we reported that polyethylene glycolated HRP could stabilize a peroxide-bound heme species in chlorobenzene at low temperatures.[4] This implies that the heterolytic O–O bond cleavage of hydroperoxide bound to the heme could be repressed by the exclusion of a proton in the active site since the peroxidases utilize protonation on the distal oxygen of compound 0 for the heterolytic O–O bond cleavage (Scheme 1).[5–7] Accordingly, a heme cavity that is hydrophobic but still accessible by H_2O_2 might provide an appropriate environment for producing compound 0 having a lifetime sufficiently long for observation in aqueous media. On the basis of these considerations, we have employed cytochrome c_{552} (cyt c_{552}) from thermophilic bacterium *Thermus thermophirus*. In fact, we have successfully observed compound 0 by modifying a hydrophobic heme cavity of cytochrome c_{552} under physiological conditions.[8] An analogous result has been reported in the reaction of myoglobin mutants with H_2O_2.[9] Recently, another study of detection of compound 0 has been reported with the reaction of *Aplysia limacine* Mb with H_2O_2 by Svistunenko et al.[10] As described in the study, *A. limacine* Mb is not furnished with a histidine residue in the heme cavity to form a hydrogen bond with O_2 bound to heme, nor are any other amino acid residues that render the cavity hydrophilic placed. Consistent with our results, *A. limacine* Mb gives detectable compound 0 via a normal EPR technique on the reaction with H_2O_2 at 4 °C.

Scheme 1



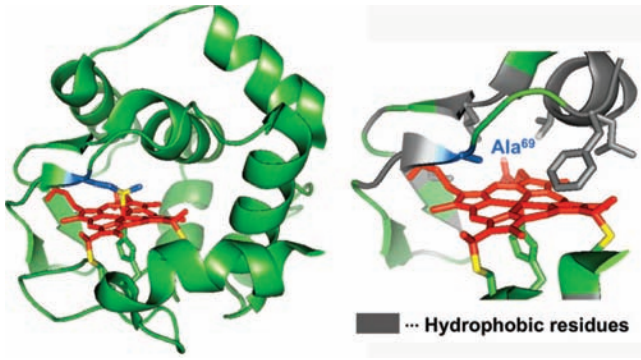


FIGURE 3. Crystal structure of cyt c_{552} (PDB entry 1C52) and an expected structure of its M69A mutant.

We have prepared an M69A mutant in which the sixth axial ligand, Met^{69}, is replaced with alanine to render the heme iron reactive to H_2O_2 with minimal change in the other properties of the heme cavity. As shown in Figure 3, the heme cavity of M69A consists of hydrophobic amino acid residues.

Figure 4 shows time-dependent EPR spectral changes of 100 μM M69A upon addition of 10 mM H_2O_2. The spectra evidence participation of two sets of rhombic low-spin signals associated with peroxide-bound heme species, together with the organic radical ($g = 2.007$) formed by internal oxidation of an amino acid residue by compound I. According to the previous studies of compound 0 by cryoradiolysis of hemoproteins[11,12] and with model compounds,[13,14] the sets of signals in Figure 4 are tentatively assigned to compound 0 ($g = 2.290$, 2.150, and 1.950; [heme·Fe^{3+}–OOH]) and peroxo heme ($g = 2.241$, 2.150, and 1.967; [heme·Fe^{3+}–OO^-]$^-$) species. According to the radiolytic reduction of oxy forms of hemoproteins, [heme·Fe^{3+}–$O_2^{\bullet}$] or [heme·Fe^{2+}–O_2], at cryo temperatures, a [heme·Fe^{3+}–OO^-]$^-$ species is formed as the primary intermediate, followed by the protonation to yield a [heme·Fe^{3+}–OOH] species. The tentative assignments of the EPR signals in Figure 4 are based on these studies. In the reaction of M69A with H_2O_2, either one or two protons of H_2O_2 must be eliminated for the formation of [heme·Fe^{3+}–OOH] or [heme·Fe^{3+}–OO^-]$^-$, respectively. The first deprotonation from a [heme·Fe^{3+}–HOOH]$^+$ species is likely. Equilibrium between water-bound ferric porphyrin and its hydroxy form is a rather common event. If the pK_a values of water (~15) and hydrogen peroxide (~12) are taken into account, equilibrium between [Fe^{3+}–HOOH]$^+$ and [Fe^{3+}–OOH] could, therefore, be available even without any acid–base catalysts such as the distal histidine of peroxidases and catalases. In addition, the deprotonation gives a neutral heme species to shift the equilibrium from the charged [Fe^{3+}–HOOH]$^+$ to neutral [Fe^{3+}–OOH] species. By contrast, the formation of the [Fe^{3+}–OO^-]$^-$ species is a less favorable process, since the pK_a value of HOO^- is more than 15 and abstraction of a proton from [Fe^{3+}–OOH] induces a negative charge in the hydrophobic space. Although we cannot rule out the formation of the [Fe^{3+}–OO^-]$^-$ species, this process seems less unlikely; thus, we suggest a conformational isomer of the [Fe^{3+}–OOH] species gives two sets of EPR signals. The reaction of the M69A mutant with D_2O_2 in place of H_2O_2 also supports this explanation (Figure 4b); i.e., no deuterium effect on the intensity ratio between the signal sets suggests that the migration process of a proton is not involved between the $g = 2.290$ ([Fe^{3+}–OOH]) and $g = 2.241$ species. Additional mutagenesis of Val^{49} to aspartic acid results in the loss of detectable compound 0, which strongly supports the crucial role of hydrophobicity of the heme cavity in retarding the subsequent reaction of compound 0.

As mentioned above, the involvement of the compound 0 species in P450-catalyzed substrate oxygenations such as hydroxylation and epoxidation is still controversial[2,3] due to the difficulty in detecting compound 0 under physiological conditions.[15–17] Therefore, we have examined the reactivity of compound 0 obtained under the conditions shown in Figure 4. Upon addition of ascorbic acid to the solution used for Figure 4a, the signal corresponding to a tyrosine radical of the protein disappeared immediately to give the signal that can be assigned to the resting state heme (ferric high-spin) as shown in Figure 4c, while the signals assigned to compound 0 remained unchanged. The results imply the exclusive reduction of compound II (EPR silent) and a protein radical by ascorbic acid. This is the first experimental evidence indicating that the peroxide-bound heme species is an inactive intermediate as an oxidation agent even for oxidatively fragile ascorbic acid.

Our study clearly shows that the heme cavity of thermally tolerant Cyt c_{552} variants provides the suitable environment for the successful capture of the compound 0 intermediate that has been considered to be a labile intermediate and difficult to detect in the reaction with H_2O_2 under ambient conditions. This study is in good agreement with our early study using the modified HRP in an organic solvent.[1] A highly hydrophobic heme cavity accounts for the suitable environment that affords durable compound 0. The reaction of the compound 0 species with ascorbic acid reveals the incompetence of the species for oxidations even such as an oxidatively fragile substrate, ascorbic acid.

Reactivity of Compound I for the Peroxidase Reactions. A series of myoglobin mutants, in which distal histidine was replaced with other amino acid residues, gave compound I in their reactions with *m*-chloroperoxybenzoic acid (*m*CPBA) as observable species.[18,19] These compound I species readily oxidize peroxidase substrates such as guaiacol and ABTS.[20] Horseradish peroxidase (HRP) was reported to catalyze oxidation of thioanisole to the corresponding sulfoxide,[21] while peroxidases typically catalyze two sequential one-electron oxidations.[22] The sulfoxidation involves an oxygen-transfer process from an oxoferryl species to sulfides, since ^{18}O in $H_2{}^{18}O_2$ was shown to be incorporated into the sulfoxide.[21,23] The oxygen transfer was suggested to proceed via electron transfer and/or oxygen coupling (overall two-elelctron oxo transfer) in competition with typical sequential electron transfer based on the kinetic study of the reaction of HRP compound I with *p*-methoxythioanisole.[24,25] Such an

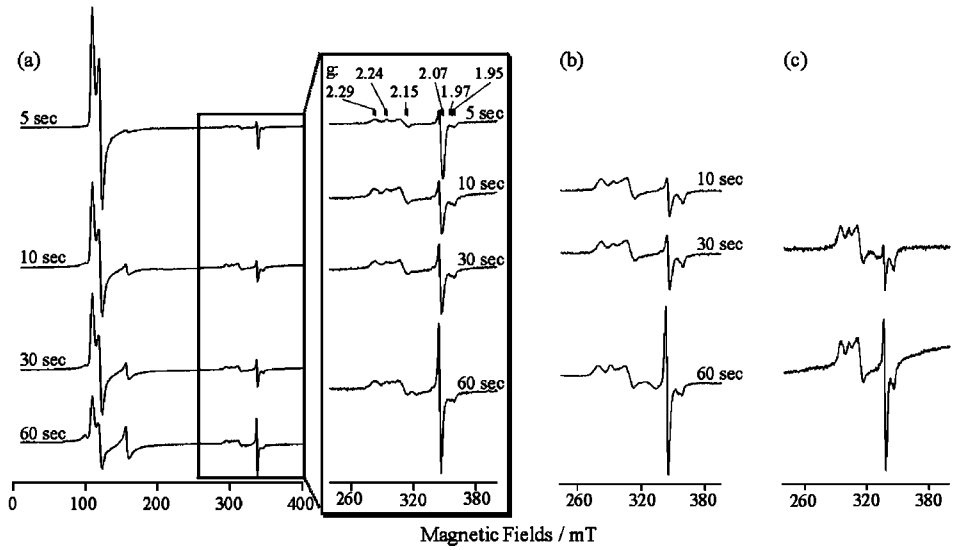


FIGURE 4. EPR spectra of M69A (100 μM) (a) after being mixed with H_2O_2 (10 mM) in 20 mM MES-NaOH buffer (pH 5.0), (b) after being mixed with D_2O_2 (10 mM) in 20 mM MES-NaOD buffer (pD 5.0) under conditions otherwise the same as those for spectrum a, and (c) 10 s after being mixed with H_2O_2 (10 mM) in the presence (top) and absence (bottom) of ascorbic acid (400 μM). All the reactions were performed at 25 °C, and the spectra were recorded at 4 K using a microwave power of 2.5 mW at 9.546 GHz and a field modulation of 0.8 mT at 100 kHz.

electron trasfer–oxygen coupling mechanism was also proposed for sulfoxidations catalyzed by P450.[26–29] We have studied the mechanisms of sulfoxidation catalyzed by compound I of heme enzymes, including HRP, the His64Ser Mb mutant, and O=$Fe^{IV}TMP^{+\bullet}$ (TMP = 5,10,15,20-tetramesitylporphyrin dianion).[30] The reaction of thioanisole and compound I of HRP was directly monitored by UV–vis spectroscopy. Upon addition of thioanisole, compound I was reduced to the resting state of HRP with partial accumulation of compound II as an intermediate. The yield of sulfoxide by a stoichiometric reaction of HRP compound I with thioanisole was only 25%. On the other hand, the same sulfoxidation by both His64Ser Mb and the Fe(TMP) complex exclusively exhibited a two-electron process, resulting in quantitative formation of sulfoxide. When 1,5-dithiacyclooctane (DTCO) was employed as a substrate, the reaction of His64Ser Mb compound I with DTCO exhibits rapid formation of compound II, which decayed to the ferric state due to the low oxidation potential of DTCO. The observed rate constants (log k_{obs}) of the reactions of compound I of the Fe(TMP) complex, HRP, and His64Ser Mb with a series of para-substituted thoanisoles correlated with the one-electron oxidation potentials ($E°_{ox}$) of the sufides. A comparison of these correlations with the established correlation between log k_{obs} and $E°_{ox}$ for the corresponding electron-transfer reactions of substituted *N,N*-dimethylanilines[31] has revealed that the initial step of the sulfoxidation reactions by compound I is the one-electron transfer process. The major factor discriminating the HRP from Mb mutants and the Fe(TMP) complex is a closed active site structure of HRP that prevents the substrate from accessing the oxygen bound to the heme iron but not the nature of compound I of HRP.

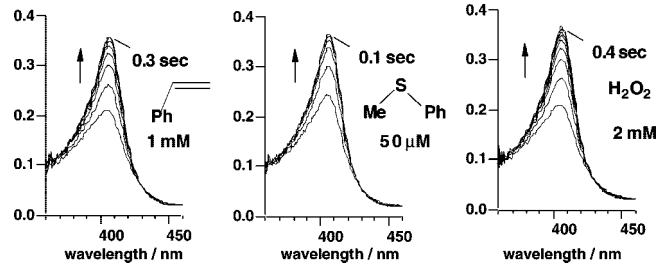


FIGURE 5. Spectral changes of Mb compound I showing the oxidation of styrene, thioanisole, and hydrogen peroxide.

Reactivity of Compound I for the Catalatic Reactions. Catalase catalyzes dismutation of 2 mol of H_2O_2 to 1 mol of O_2 and 2 mol of H_2O by its reaction with first H_2O_2 to form Compound I, which oxidizes second H_2O_2 to O_2 (Scheme 1). The catalase reaction has been known since 1940s, and it is suggested that the distal histidine is important for the deprotonation of hydrogen peroxide,[32,33] although the detailed mechanism of the H_2O_2 oxidation of compound I (catalatic reaction) is not clear. One of the major reasons is that the rate of formation of compound I by H_2O_2 and its rate of reaction with H_2O_2 are comparable;[32,34,35] thus, the reaction of catalse and H_2O_2 immediately affords a steady state mixture of compound I and the resting state. On the other hand, we have reported that compound I of myoglobin mutants prepared by their reactions with *m*CPBA is able to oxidize H_2O_2 through a mechanism similar to that of catalase.[36] More importantly, this process can be observed as an UV–vis spectral change (Figure 5).[37] To elucidate the detailed catalatic reaction mechanism, we have determined the kinetic isotope effect (KIE) on catalatic reactions (k_2) of catalase and Mb mutants in H_2O and D_2O (Table 1).[36]

The deuterium isotope effect on the reaction of *Micrococcus lysodeikticus* catalase (MLC) was calculated to be 4.0 on the basis of a complicated kinetic treatment.[36]

Table 1. Rate Constants (k_2) of the Catalatic Reactions

Mb	k_{2H}[a] (M^{-1} s^{-1})	k_{2D}[b] (M^{-1} s^{-1})	k_{2H}/k_{2D}
H64A Mb[c]	$(5.26 \pm 0.06) \times 10^3$	$(2.32 \pm 0.02) \times 10^2$	23
H64S Mb[c]	$(6.91 \pm 0.01) \times 10^3$	$(2.38 \pm 0.01) \times 10^2$	29
H64D Mb[c]	$(15.8 \pm 0.3) \times 10^3$	$(8.18 \pm 0.02) \times 10^2$	18
L29H/H64L Mb[c]	$(33.6 \pm 0.1) \times 10^3$	$(3.39 \pm 0.03) \times 10^3$	10
F43H/H64L Mb[c]	$(21.0 \pm 0.5) \times 10^3$	$(10.3 \pm 0.1) \times 10^3$	2.1
MLC[d]	$(1.20 \pm 1.98) \times 10^7$	$(3.08 \pm 0.32) \times 10^6$	4.0

[a] In H_2O buffer. [b] In D_2O buffer. [c] Mb, 50 mM sodium acetate buffer (pL 5.0) at 5.0 °C. [d] MLC, 50 mM sodium phosphate buffer (pL 7.0) at 5.0 °C.

F43H/H64L Mb also gave a small KIE (2.1) determined by direct observation of the Mb-I reaction with H_2O_2. These results indicate that hydrogen peroxide is easily deprotonated by the distal histidine and then reacts with compound I of MLC and F43H/H64L Mb. On the other hand, in the reactions of H_2O_2 and Mb-I of L29H/H64L and H64X (X = D, A, and S), the KIEs are greatly increased (10–29) compared with those of F43H/H64L Mb and MLC (Table 1). Large KIEs are caused due to the tunneling effect, which is confirmed by the Arrhenius parameter ratio.[38] Evolution of O_2 catalyzed by H64D or F43H/H64L Mbs from a solution containing a 50:50 mixture of $H_2{}^{18}O_2$ and $H_2{}^{16}O_2$ shows formation of $^{18}O_2$ and $^{16}O_2$ with no indication of $^{16}O^{18}O$. This demonstrates that the catalase reactions by the Mb mutants proceed without breakage of the O–O bond that has been found in the deprotonation process with catalase and chloroperoxidase.[39,40] Our previous studies of myoglobin mutants showed that the designed histidine (His43) in F43H/H64L Mb serves as a general acid–base catalyst to form compound I in the reaction with H_2O_2.[20] On the other hand, the distal aspartic acid (Asp64) in H64D and the distal histidine (His29) in L29H/H64L Mb hardly participate as the catalyst.[20,41] The crystal structure of F43H/H64L Mb shows the distance between the N^{ϵ} of His43 and the ferric heme iron to be 5.7 Å, being similar to structurally known peroxidases and MLC (Figure 6).[20,33,42] On the other hand, the distance in L29H/H64L Mb from the heme iron is too great (6.6 Å) for it to serve as a general acid–base catalyst.[20] Thus, the catalatic reaction of MLC and F43H/H64L Mb, in which the general acid–base catalyst is located at a proper position in the active site, could proceed via an ionic mechanism with a small KIE (<4) (Scheme 2A), while the other Mb mutants oxidize H_2O_2 via a mechanism involving hydrogen abstraction with a large KIE (10–29) due to absence of the general acid–base catalyst (Scheme 2B). The hydrogen abstraction by compound I has been proposed for the alkane hydroxylation by cytochrome P450, its model complexes, and even non-heme enzymes.[43,44] In these reactions, large KIEs in a range of 9–29 are commonly observed due to a tunneling effect.[38]

While the histidine residue at position 43 helps the ionic oxidation of hydrogen peroxide, the absolute k_{2H} value of F43H/H64L Mb is virtually the same as that for H64D Mb and L29H/H64L Mb (Table 1). The crystal structure of H64D/V68A Mb suggests that an Asp residue at position 64 could help to incorporate hydrogen peroxide in the active site because of the enlargement of the active site as well as hydrogen bonding.[45] Although His at position 29 of L29H/H64L Mb is located too far from the heme iron to play the role of the acid–base catalyst, L29H/H64L Mb also provides a larger space for the accommodation of hydrogen peroxide than the F43H/H64L mutant, and His29 might help to stabilize Mb-I by a polar effect.[20] Our results suggest that important factors

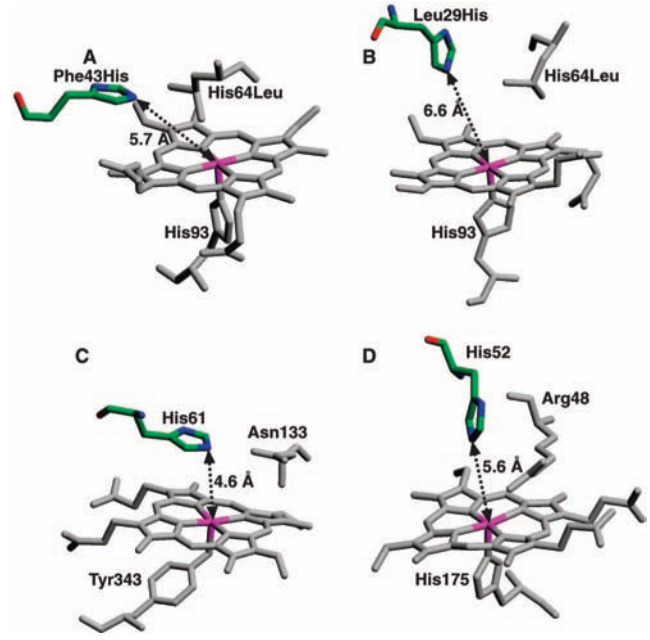
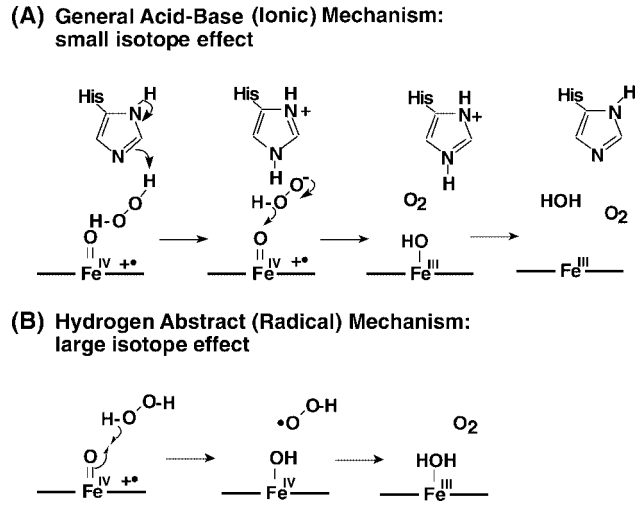


FIGURE 6. Location of the distal histidine in H64L/F43H Mb (A, PDB entry 1OFK), H64L/L29H Mb (B, PDB entry 1OFJ), MLC (C, PDB entry 4BLC), and C*c*P (D, PDB entry 1CCA).

Scheme 2. Ionic (A) and Radical (B) Mechanisms of Oxidation of Hydrogen Peroxide by Compound I



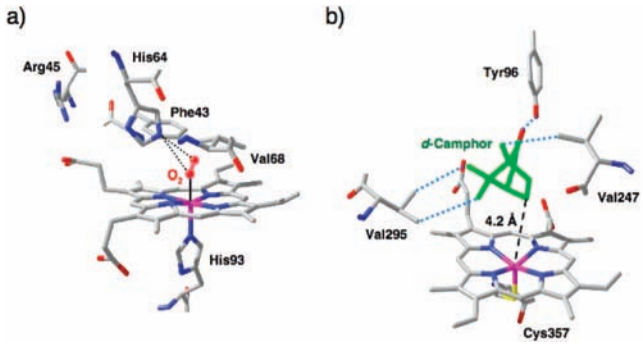


FIGURE 7. Crystal structures of Mb and $P450_{cam}$ taken from PDB entries 1A6M and 2CPP, respectively.

for the catalatic reaction are not only the deprotonation but also enough space and polarity for the accommodation of hydrogen peroxide at a suitable position in the active site. In fact, distal cavity mutation of a Trp residue in catalase-peroxidase completely depressed its catalase activity but preserved peroxidase activity. It has been concluded that the indole ring is involved in the binding of a H_2O_2 molecule.[46,47]

Effect of the Substrate Binding Environment for the Oxidation of the Aromatic Ring by Compound I

We have reported the hydroxylation of the aromatic ring by heme protein mutants other than P450s and chloroperoxidase.[37,48,49] A primary factor regulating the hydroxylation is likely the presence of an appropriate binding site that positions the substrate the correct distance from and the correct orientation with respect to the reactive compound I intermediate. For example, Figure 7 shows crystal structures of an oxy form of Mb and the resting state of $P450_{cam}$, including their substrates (O_2 for Mb and *d*-camphor for $P450_{cam}$) at the active sites. Apparently, *d*-camphor is captured near the heme and is ready to be oxidized. On the other hand, the heme vicinity of Mb is suitable for accommodation of O_2 but not suitable for accepting organic substrates. While we have provided space, large enough for substrates, immediately above the heme of the myoglobin mutants by replacing distal histidine with leucine, there are no amino acid residues able to capture substrates by specific interaction such as hydrogen bonding or charge interaction. Therefore, substrates are not expected to remain in the space. To compare the hydroxylation activity between P450 and myoglobin mutants under similar conditions, we have introduced a substrate, tryptophan, near the heme of myoglobin by site-directed mutagenesis. Substitution of the Val68 residue in H64D variants of Mb has been shown to accelerate the peroxygenase reaction rates and increase the stereoselectivity of the products.[45,50] For example, H64D/V68I Mb exhibits a peroxygenase activity 1600-fold greater than that of wild-type Mb.[45] Thus, we have prepared Phe43Try/His64Asp/Val68Ile (WDI) Mb for the comparison of its oxidation activity to that of P450.[51]

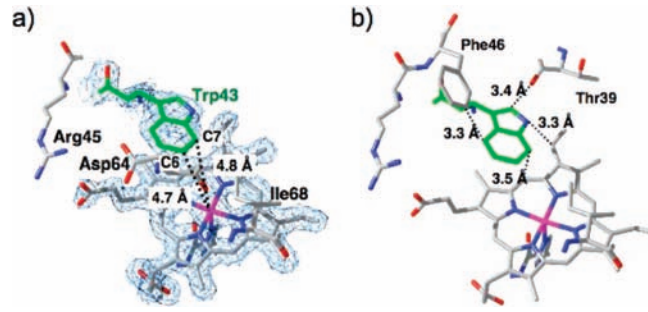


FIGURE 8. Crystal structure of WDI Mb (PDB entry 2E2Y).

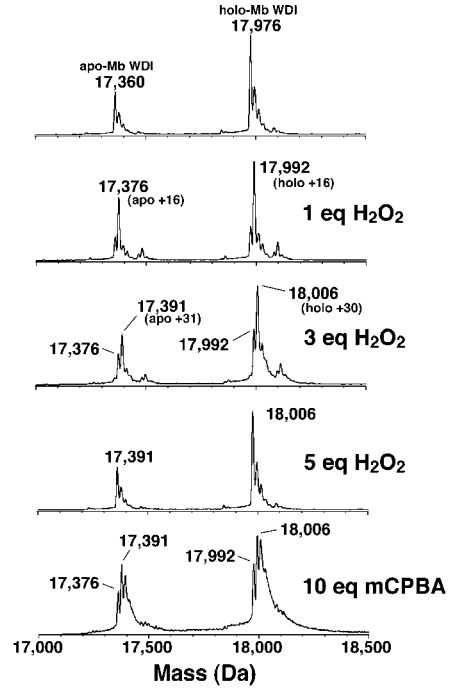
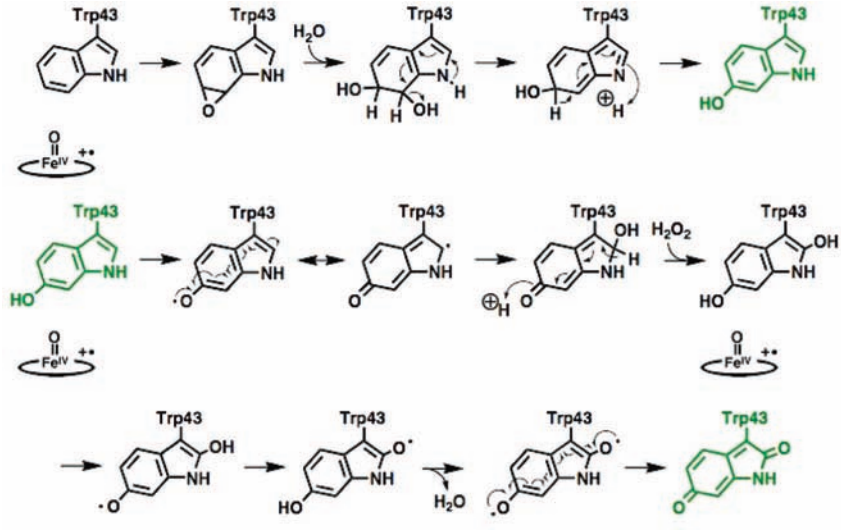


FIGURE 9. ESI-TOF mass spectral changes upon addition of H_2O_2 to WDI Mb.

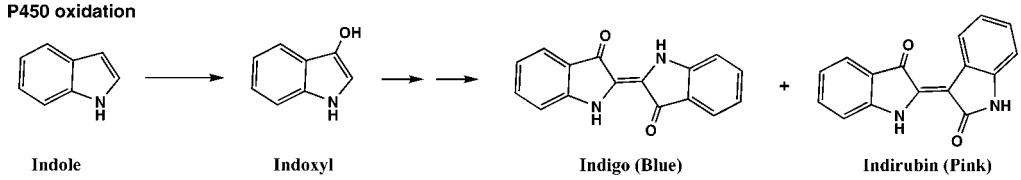
The crystal structure of WDI Mb shows that the C_6 and C_7 atoms of the indole ring in tryptophane are located close to the heme, 4.7 and 4.8 Å, respectively, from the heme iron (Figure 8). The orientation of the indole ring is restricted by noncovalent interactions such as π–π, CH–π, and hydrogen bonding with the adjacent residues.

The reaction of WDI Mb and H_2O_2 was monitored via electrospray ionization time-of-flight mass spectrophotometry (ESI-TOF MS). One equivalent of H_2O_2 was enough to complete the reaction to give an oxidatively modified myoglobin mutant having a mass increased by 16 Da, from 17360 to 17376 Da (Figure 9). The addition of 3 equiv of H_2O_2 resulted in the almost complete conversion to a species with an increase in mass of 30 Da. The ESI-TOF MS spectrum after the addition of 5 equiv of H_2O_2 showed a clear conversion to the +30 Da species (Figure 9). There are several reports describing the similar mass increase for heme proteins upon reaction with H_2O_2, in which modification of the hemes such as covalent bond formation with the protein[52–54] and formation of d-type chlorin[55] accounts for the mass increase. On the other hand, the mass increase was assigned to the hydroxylation of Trp43 by NMR and MS analysis for the

Scheme 3. A Plausible Oxidation Mechanism of Trp43 Oxidation



Scheme 4. Oxidation of Indole by P450



digested mutant in our study. Digestion of the H_2O_2-treated mutant by Lys-C acromobacter followed by isolation of the modified fragments showed the oxidative modification in the tryptophan 43 residue. The structures of the +16 and +30 Da products were determined to be 6-hydroxy-Trp43 and 2,6-dioxoindole-Trp43, respectively, by NMR and MS analyses of the digested fragments of the oxidized WDI Mb (Scheme 3). The hydroxylation at C_6 in the indole ring is suggestive of the initial epoxidation of the C_6═C_7 bond, since 46% of the hydroxy oxygen in 6-hydroxy-Trp43 came from water; i.e., hydrolysis of the C_6–C_7 epoxide followed by aromatization could yield C_6-hydroxyindole with 50% incorporation of water oxygen into the product (Scheme 3).

To elucidate the mechanistic details of the hydroxylation, the reaction of WDI Mb with H_2O_2 was monitored by stopped-flow experiments. The time-dependent absorbance change at 408 nm of WDI Mb shows the completion of the oxidation within a few seconds without showing preformation of compound I (Figure 10B, inset). Under similar conditions, H64D Mb reacts with H_2O_2 to form compound I in 80 ms (Figure 10A), indicative of the initial formation of compound I followed by very fast oxidation of the aromatic ring. A slower formation of compound I in WDI could be due to the less hydrophilic environment of the heme vicinity in WDI Mb caused by the introduction of Trp43. To our knowledge, WDI Mb is the first enzyme to preferentially oxidize the aromatic ring of indole (Trp side chain) to produce a 6-OH derivative. P450 oxidation of indole has been shown to yield the pigments indigo and indirubin via 3-OH-indole (indoxyl) as shown in Scheme 4.[56]

A rapid oxidation of the aromatic ring by a stoichiometric amount of H_2O_2 is exactly the function of P450, while the P450 reactions utilize O_2, 2 e^-, and 2 H^+ instead of H_2O_2. Apparently, even myoglobin mutants are able to catalyze the oxidation of aromatic rings like P450 if we provide substrate binding sites in the heme cavity of mutants.

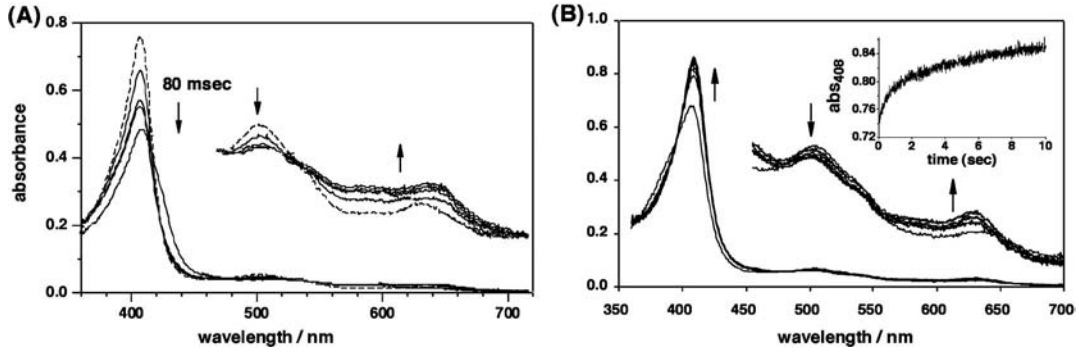


FIGURE 10. Time-dependent spectral changes of reactions of H64D Mb (A) and WDI Mb (B) with H_2O_2.

Conclusions

In this Account, we have described peroxidase, catalase, and P450 types of oxidations catalyzed by Mb mutants without modification of their proximal histidine ligands. These results support our proposals about the origin of different reactivities of P450, peroxidases, and catalase being primarily controlled by their active site structures. While the rates of compound I formation in Mb mutants are slower than the rates of peroxidases and catalases in their reactions with H_2O_2, it could be explained by the overall structural differences of the heme enzymes and our mutants. While we have not discussed roles of proximal ligands in the heme enzymes, they could modulate the rate of formation and reactivity of compound I. In addition, the direct observation of substrate oxidation processes by Mb mutants may allow us to examine detailed oxidation mechanisms. In the case of cytochrome c_{552}, we have successfully prepared a mutant which gives compound 0 as a durable intermediate for the study of its reactivity.

References

(1) Ozaki, S.; Ortiz de Montellano, P. R. Molecular Engineering of Horseradish-Peroxidase Thioether Sulfoxidation and Styrene Epoxidation by Phe-41 Leucine and Threonine Mutants. *J. Am. Chem. Soc.* **1995**, *117*, 7056–7064.

(2) Jin, S.; Makris, T. M.; Bryson, T. A.; Sligar, S. G.; Dawson, J. H. Epoxidation of Olefins by Hydroperoxo-Ferric Cytochrome P450. *J. Am. Chem. Soc.* **2003**, *125*, 3406–3407.

(3) Derat, E.; Kumar, D.; Hirao, H.; Shaik, S. Gauging the Relative Oxidative Powers of Compound I, Ferric-Hydroperoxide, and the Ferric-Hydrogen Peroxide Species of Cytochrome P450 Toward C-H Hydroxylation of a Radical clock Substrate. *J. Am. Chem. Soc.* **2006**, *128*, 473–484.

(4) Ozaki, S.; Inada, Y.; Watanabe, Y. Characterization of Polyethylene Glycolated Horseradish Peroxidase in Organic Solvents: Generation and Stabilization of Transient Catalytic Intermediates at Low Temperature. *J. Am. Chem. Soc.* **1998**, *120*, 8020–8025.

(5) Harrison, J. E.; Schultz, J. Studies on Chlorinating Activity of Myeloperoxidase. *J. Biol. Chem.* **1971**, *251*, 1371–1374.

(6) Rodriguez-Lopez, J. N.; Lowe, D. J.; Hernandez-Ruiz, J.; Hiner, A. N. P.; Garcia-Canovas, F.; Thorneley, R. N. F. Mechanism of Reaction of Hydrogen Peroxide with Horseradish Peroxidase: Identification of Intermediates in the Catalytic Cycle. *J. Am. Chem. Soc.* **2001**, *123*, 11838–11847.

(7) Poulos, T. L.; Kraut, J. The Stereochemistry of Peroxidase Catalysis. *J. Biol. Chem.* **1980**, *255*, 8199–8205.

(8) Ichikawa, Y.; Nakajima, H.; Watanabe, Y. Characterization of Peroxide Bound Heme Species Generated in Reaction of Thermally Tolerant Cytochrome c_{552} with Hydrogen Peroxide. *ChemBioChem* **2006**, *7*, 1582–1589.

(9) Brittain, T.; Baker, A. R.; Butler, C. S.; Little, R. H.; Lowe, D. J.; Greenwood, C.; Watmough, N. J. Reaction of variant sperm-whale myoglobins with hydrogen peroxide: The effects of mutating a histidine residue in the haem distal pocket. *Biochem. J.* **1997**, *326*, 109–115.

(10) Svistunenko, D. A.; Reeder, B. J.; Wankasi, M. M.; SilaghiDumitrescu, R.; Cooper, C. E.; Rinaldo, S.; Cutruzzola, F.; Wilson, M. T. Reaction of *Aplysia limacina* metmyoglobin with hydrogen peroxide. *Dalton Trans.* **2007**, *8*, 840–850.

(11) Denisov, I. G.; Makris, T. M.; Sligar, S. G. Formation and Decay of Hydroperoxo-Ferric Heme Complex in Horseradish Peroxidase Studied by Cryoradiolysis. *J. Biol. Chem.* **2002**, *277*, 42706–42710.

(12) Denisov, I. G.; Ikeda-Saito, M.; Yoshida, T.; Sligar, S. G. Cryogenic Absorption Spectra of Hydroperoxo-Ferric Heme Oxygenase, the Active Intermediate of Enzymatic Heme Oxygenation. *FBES Lett.* **2002**, *532*, 203–206.

(13) Loew, G.; Dupuis, M. Structure of a Model Transient Peroxide Intermediate of Peroxidases by Ab Initio Methods. *J. Am. Chem. Soc.* **1996**, *118*, 10584–10587.

(14) Harris, D. L.; Loew, G. H. Identification of Putative Peroxide Intermediates of Peroxidases by Electronic Structure and Spectra Calculations. *J. Am. Chem. Soc.* **1996**, *118*, 10588–10594.

(15) Chandrasena, R. E. P.; Vatsis, K. P.; Coon, M. J.; Hollenberg, P. F.; Newcomb, M. Hydroxylation by the Hydroperoxy-iron Species in Cytochrome P450 Enzymes. *J. Am. Chem. Soc.* **2004**, *126*, 115–126.

(16) Tajima, K.; Shigematsu, M.; Jinno, J.; Ishizu, K.; Ohya-Nishiguchi, H. Generation of Fe(III)oep-Hydrogen Peroxide Complex (Oep = Octaethylporphyrinato) by Reduction of Fe(II)oep-O_2 with Ascorbic-Acid Sodium-salt. *J. Chem. Soc., Chem. Commun.* **1990**, 144–145.

(17) Tajima, K.; Jinno, J.; Ishizu, K.; Sakurai, H.; Ohya-Nishiguchi, H. Direct Evidence of Heme-*tert*-butyl Peroxide Adduct Formation Demonstrated by Simultaneous esr and Optical Measurements. *Inorg. Chem.* **1989**, *28*, 709–715.

(18) Ozaki, S.; Matsui, T.; Watanabe, Y. Conversion of Myoglobin into a Peroxygenase: A Catalytic Intermediate of Sulfoxidation and Epoxidation by the F43H/H64L Mutant. *J. Am. Chem. Soc.* **1997**, *119*, 6666–6667.

(19) Matsui, T.; Ozaki, S.; Watanabe, Y. On the Formation and Reactivity of Compound I of the His-64 Myoglobin Mutants. *J. Biol. Chem.* **1997**, *272*, 32735–32738.

(20) Matsui, T.; Ozaki, S.; Liong, E.; Phillips, G. N., Jr.; Watanabe, Y. Effects of the Location of Distal Histidine in the Reaction of Myoglobin with Hydrogen Peroxide. *J. Biol. Chem.* **1999**, *274*, 2838–2844.

(21) Kobayashi, S.; Nakano, M.; Goto, T.; Kimura, T.; Schaap, A. P. An Evidence of the Peroxidase-dependent Oxygen-transfer from Hydrogen-peroxide to Sulfides. *Biochem. Biophys. Res. Commun.* **1986**, *135*, 166–171.

(22) Candeias, L. P.; Folkes, L. K.; Wardman, P. Factors Controlling the Substrate Specificity of Peroxidases: Kinetics and Thermodynamics of the Reaction of Horseradish Peroxidase Compound I with Phenols and Indole-3-Acetic Acids. *Biochemistry* **1997**, *36*, 7081–7085.

(23) Doerge, D. R.; Cooray, N. M.; Brewster, M. E. Peroxidase-catalyzed S-oxygenation: Mechanism of oxygen transfer for lactoperoxidase. *Biochemistry* **1991**, *30*, 8960–8964.

(24) Pérez, U.; Dunford, H. B. Transient-state Kinetics of the Reactions of 1-Methoxy-4-(Methylthio)Benzene with Horseradish-peroxidase Compound I and Compound II. *Biochemistry* **1990**, *29*, 2757–2763.

(25) Pérez, U.; Dunford, H. B. Spectral Studies on the Oxidation of Organic Sulfides (Thioanisoles) by Horseradish-peroxidase Compound-I and Compound-II. *Biochim. Biophys. Acta* **1990**, *1038*, 98–104.

(26) Watanabe, Y.; Iyanagi, T.; Oae, S. Kinetic-study on Enzymatic S-oxygenation Promoted by a Reconstituted System with Purified Cytochrome-P-450. *Tetrahedron Lett.* **1980**, *21*, 3685–3688.

(27) Watanabe, Y.; Numata, T.; Iyanagi, T.; Oae, S. Enzymatic Oxidation of Alkyl Sulfides by Cytochrome-P-450 and Hydroxyl Radical. *Bull. Chem. Soc. Jpn.* **1981**, *54*, 1163–1170.

(28) Watanabe, Y.; Iyanagi, T.; Oae, S. One Electron-transfer Mechanism in the Enzymatic Oxygenation of Sulfoxide to Sulfone Promoted by a Reconstituted System with Purified Cytochrome-P-450. *Tetrahedron Lett.* **1982**, *23*, 533–536.

(29) Modena, G.; Mariola, L. *Gazz. Chim. Ital.* **1957**, *87*, 1306.

(30) Goto, Y.; Matsui, T.; Ozaki, S.; Watanabe, Y.; Fukuzumi, S. Mechanisms of Sulfoxidation Catalyzed by High-valent Intermediates of Heme Enzymes: Electron-transfer vs. Oxygen-transfer Mechanism. *J. Am. Chem. Soc.* **1999**, *121*, 9497–9502.

(31) Goto, Y.; Watanabe, Y.; Fukuzumi, S.; Jones, J. P.; Dinnocenzo, J. P. Mechanisms of N-Demethylations Catalyzed by High-valent Species of Heme Enzymes: Novel Use of Isotope Effects and Direct Observation of Intermediates. *J. Am. Chem. Soc.* **1998**, *120*, 10762–10763.

(32) Dunford, H. B., Ed.; *Heme Peroxidases*; Wiley-VCH: New York, 1999.

(33) Fita, I.; Rossmann, M. G. The Active Center of Catalase. *J. Mol. Biol.* **1985**, *185*, 21–37.

(34) Chance, B.; Greenstein, D. S.; Roughton, F. J. W. The Machanism of Catalase Action. *Arch. Biochem. Biophys.* **1952**, *37*, 301–321.

(35) Kremer, M. L. Peroxidatic Activity of Catalase. *Biochim. Biophys. Acta* **1970**, *198*, 199–209.

(36) Kato, S.; Ueno, T.; Fukuzumi, S.; Watanabe, Y. Catalase Reaction by Myoglobin Mutants and Native Catalase. *J. Biol. Chem.* **2004**, *279*, 52376–52381.

(37) Watanabe, Y.; Ueno, T. Introduction of P450, Peroxidase, and Catalase Activities into Myoglobin by Site-directed Mutagenesis: Diverse Reactivities of Compound I. *Bull. Chem. Soc. Jpn.* **2003**, *76*, 1309–1322.

(38) Kwart, H. Temperature Dependence of the Primary Kinetic Hydrogen Isotope Effect as a Mechanistic Criterion. *Acc. Chem. Res.* **1982**, *15*, 401–408.

(39) Hager, L. P.; Doubek, D. L.; Silverstein, R. M.; Hargis, J. H.; Martin, J. C. Chloroperoxidase. IX. Structure of compound I. *J. Am. Chem. Soc.* **1972**, *94*, 4364–4366.

(40) Jarnagin, R. C.; Wang, J. H. Kinetics of the Exchange Reaction between Carbon-14-labeled Carbonate and Carbonato-bis-(trimethylenediamine)-Cobalt(III) Complex in Aqueous Solution. Effect of Steric Hindrance in a Ligand Substitution Process. *J. Am. Chem. Soc.* **1958**, *80*, 786–787.
(41) Matsui, T.; Ozaki, S.; Watanabe, Y. Formation and Catalytic Roles of Compound I in the Hydrogen Peroxide-Dependent Oxidations by His64 Myoglobin Mutants. *J. Am. Chem. Soc.* **1999**, *121*, 9952–9957.
(42) Poulos, T. L.; Freer, S. T.; Alden, R. A.; Edwards, S. L.; Skogland, U.; Takio, K.; Eriksson, B.; Xuong, N.; Yonetani, T.; Kraut, J. The Crystal Structure of Ccp. *J. Biol. Chem.* **1980**, *255*, 575–580.
(43) Neshein, J. C.; Lipscomb, J. D. Large Kinetic Isotope Effects in Methane Oxidation Catalyzed by MMO: Evidence for C-H Bond Cleavage in a Reaction Cycle Intermediate. *Biochemistry* **1996**, *35*, 10240–10247.
(44) Sorokin, A.; Robert, A.; Meunier, B. Intramolecular Kinetic Isotope Effects in Alkane Hydroxylations Catalyzed by Manganese and Iron Porhyrin Complexes. *J. Am. Chem. Soc.* **1993**, *115*, 7293–7299.
(45) Yang, H.; Matsui, T.; Ozaki, S.; Kato, S.; Ueno, T.; Phillips, G. N.; Fukuzumi, S.; Watanabe, Y. Molecular Engineering of Myoglobin: Influence of Residue 68 on the Rate and the Enantioselectivity of Oxidation Reactions Catalyzed by H64D/V68X Mb. *Biochemistry* **2003**, *42*, 10174–10181.
(46) Hillar, A.; Peters, B.; Pauls, R.; Loboda, A.; Zhang, H.; Mauk, A. G.; Loewen, P. C. Modulation of the Activities of Catalase-peroxidase HPI of *Escherichia coli* by Site-directed Mutagenesis. *Biochemistry* **2000**, *39*, 5868–5875.
(47) Regelsberger, G.; Jakopitsch, C.; Ruker, F.; Krois, D.; Peschek, G. A.; Obinger, C. Effect of Distal Cavity Mutation on the Formation of Compound I in Catalase-peroxidases. *J. Biol. Chem.* **2000**, *275*, 22854–22861.
(48) Hara, I.; Ueno, T.; Ozaki, S.; Itoh, S.; Lee, K.; Ueyama, N.; Watanabe, Y. Oxidative Modification of Tryptophan 43 in the Heme Vicinity of the F43W/H64L Myoglobin Mutant. *J. Biol. Chem.* **2001**, *276*, 36067–36070.
(49) Ozaki, S.; Hara, I.; Matsui, T.; Watanabe, Y. Molecular Engineering of Myoglobin: The Improvement of Oxidation Activity by Replacing Phe-43 with Tryptophan. *Biochemistry* **2001**, *40*, 1044–1052.
(50) Kato, S.; Yang, H. J.; Ueno, T.; Ozaki, S.; Phillips, G. N.; Fukuzumi, S.; Watanabe, Y. Asymmetric Sulfoxidation and Amine Binding by H64D/V68A and H64D/V68S Mb: Mechanistic Insight into the Chiral Discrimination Step. *J. Am. Chem. Soc.* **2002**, *124*, 8506–8507.
(51) Pfister, T. D.; Ohki, T.; Ueno, T.; Hara, I.; Adachi, S.; Makino, Y.; Ueyama, N.; Lu, Y.; Watanabe, Y. Monooxygenation of an Aromatic Ring by F43W/H64D/V68I Myoglobin Mutant and Hydrogen Peroxide: Myoglobin Mutants as a Model for P450 Hydroxylaation Chemistry. *J. Biol. Chem.* **2005**, *280*, 12858–12866.
(52) Catalano, C. E.; Choe, Y. S.; Ortiz de Montellano, P. R. Reactions of the protein radical in peroxidetreated myoglobin. Formation of a hemeprotein crosslink. *J. Biol. Chem.* **1989**, *264*, 10534–10541.
(53) Reeder, B. J.; Svistunenko, D. A.; Sharpe, M. A.; Wilson, M. T. Characteristics and Mechanism of Formation of Peroxide-Induced Heme to Protein Cross-Linking in Myoglobin. *Biochemistry* **2002**, *41*, 367–375.
(54) Osawa, Y.; Korzekwa, K. Oxidative modification by low levels of HOOH can transform myoglobin to an oxidase. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 7081–7085.
(55) Sugiyama, K.; Highet, R. J.; Woods, A.; Cotter, R. J.; Osawa, Y. Hydrogen peroxide-mediated alteration of the heme prosthetic group of metmyoglobin to an iron chlorin product: Evidence for a novel oxidative pathway. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 796–801.
(56) Gillam, E. M. J.; Notley, L. M.; Cai, H. L.; De Voss, J. J.; Guengerich, F. P. Oxidation of Indole by Cytochrome P450 Enzymes. *Biochemistry* **2000**, *39*, 13817–13824.

AR600046A