

Accounts

Introduction of P450, Peroxidase, and Catalase Activities into Myoglobin by Site-Directed Mutagenesis: Diverse Reactivities of Compound I

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(Received January 6, 2003)

We have prepared several myoglobin (Mb) mutants which are protein models for peroxidase, P450, and catalase. In most cases, the distal site of Mb was modified by site-directed mutagenesis on the basis of mechanisms of peroxidase, P450, and catalase reactions. The most important feature of the Mb mutants is the direct observation of their active intermediates, so called compound I. Under catalytic oxidations of sulfides and styrene by H_2O_2, up to 97% of enantioselectivity was observed. Introduction of an aromatic substrate, tryptophan, near the heme by the site directed mutagenesis of Mb allowed us to proceed almost stoichiometric aromatic hydroxylation. In addition, compound I of Mb mutants exhibit the catalase activity. These results demonstrate that the compound I of Mb mutants are capable to proceed all of the peroxidase, P450, and catalase reactions.

By sharing the heme prosthetic group, heme proteins and enzymes play versatile roles including one-electron oxidation, monooxygenation, NO synthesis, dismutation of H_2O_2, four-electron reduction of molecular oxygen, NO reduction, electron transfer, carriage and storage of molecular oxygen, and sensing of small molecules such as CO. Typical examples of these proteins and enzymes are peroxidases, P450s, NOS, catalase, oxidases, P450nor, hemoglobin and myoglobin, and CooA (Table 1).[1–6] Among them, peroxidases, P450s and catalase are the enzymes responsible for a variety of substrate oxidations achieved by the use of a common active intermediate, O=Fe(Ⅳ) porphyrin π-cation radical or its equivalent, so called compound I.[1,2,7] Though these enzymes utilize compound I as the key intermediates of the oxidations, each enzyme catalyzes its own oxidation(s) but not others, i.e., each enzyme is responsible for its own biological functions.

What could be the reasons, for example, why cannot peroxidase be catalase? Both catalase and peroxidases, such as horseradish peroxidase (HRP) and cytochrome *c* peroxidase (C*c*P), react with H_2O_2 to afford compound I at a rate of $\sim 10^7\ M^{-1}\,s^{-1}$ ($1\ M = 1\ mol\,dm^{-3}$), indicating that it is easy for H_2O_2 to access to the heme site of these enzymes.[2] Once compound I of these enzymes is formed, the heme site could be readily accessible for second H_2O_2. Thus, catalase oxidizes a second H_2O_2 at a rate comparable to the compound I formation.[2,8] If this is also the case for HRP, for example, why cannot HRP compound I exhibit the dismutation of H_2O_2 at the rate comparable to catalase?[9] The same question is also applicable for HRP and catalase, i.e., why is it hard for HRP and catalase to catalyze epoxidation and hydroxylation? Efforts to apply HRP and cytochrome *c* peroxidase mutants for the peroxide-dependent oxidation (peroxygenation) of sulfides and aniline were focused on the improvement of the oxidation activities by the introduction of a space for the substrate access to or substrate accommodation nearby the heme, while the rates are still very much low.[10,11] The heme cavities of HRP[12] and beef liver catalase (BLcase)[13] are shown in Fig. 1. The distal site of both enzymes consists of histidine as a general acid-base catalyst and a polar residue, arginine for HRP and asparagine for catalase. Thus, these distal site structures are very similar. On the other hand, one apparent difference in these enzymes is the axial (proximal) ligand, histidine for HRP and tyrosine for catalase. Thus, the difference in the reactivity of compound I of HRP and catalase could be attributed to the different axial ligand. In the case of P450, ligation of cysteine to the heme might perturb the reactivity of compound I to allow its high activity for the hydroxylation (Fig. 1c).[14] Higuchi et al. reported that synthetic P450 models consisting

Table 1. Biological Function of Hemoproteins/Heme Enzymes

O_2 transportation/storage	hemoglobin, myoglobin
Respiratory chain	cytochrome oxidase
Monooxygenation	cytochrome P450
H_2O_2 activation	peroxidase
H_2O_2 dismutation	catalase
NO synthesis	NO synthase
NO reduction	P450nor
Heme metabolism	heme oxygenase
Sensing	FixL, CooA, etc

Published on the web July 15, 2003; DOI 10.1246/bcsj.76.1309

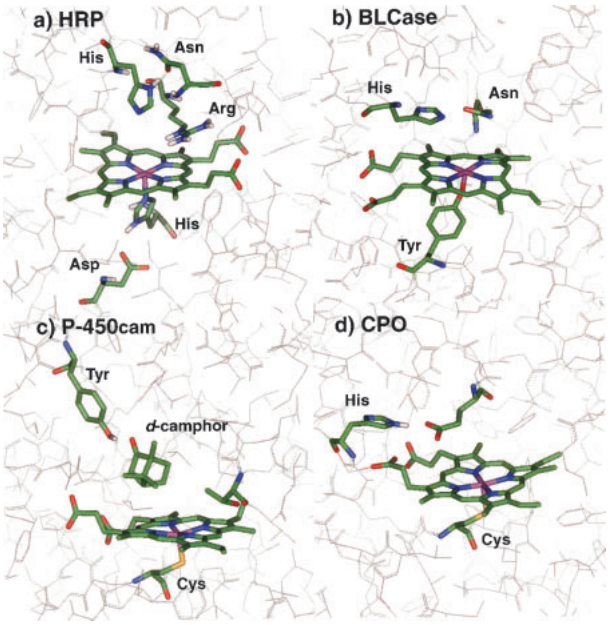


Fig. 1. Active site structures of horseradish peroxidase (HRP) (a), beef liver catalase (BLCase) (b), $P450_{cam}$ (c), and chloroperoxidase (CPO) (d).

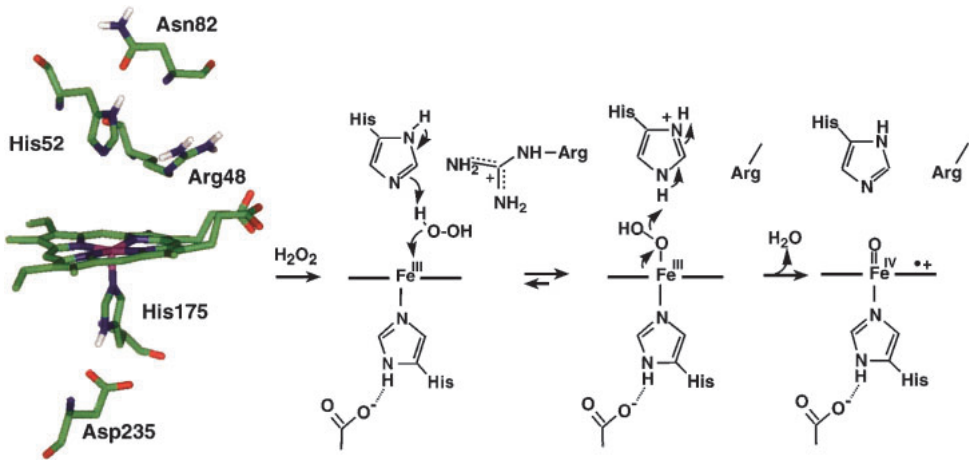
of iron porphyrins bearing thiolate ligand exhibit higher hydroxylation/epoxidation ratios compared with those having some other ligand such as chloride or pyridine.[15]

In our early works, we have prepared a myoglobin mutant in which proximal histidine ligand (His93) was replaced by cysteine to mimic the coordination structure of P450.[16,17] The His93Cys Mb mutant shows spectroscopic properties (including NMR, resonance Raman and UV-vis) that were similar to those of P450. More importantly, the cysteine ligation exclusively enhanced the heterolytic O–O bond cleavage of cumene hydroperoxide bound to the heme. Unfortunately, it was still hard for the cysteine mutant to oxidize foreign compounds such as sulfides and olefins. In this review article, we describe a new strategy for the construction of myoglobin mutants which are able to exhibit enzymatic activities of peroxidase, P450, and catalase.

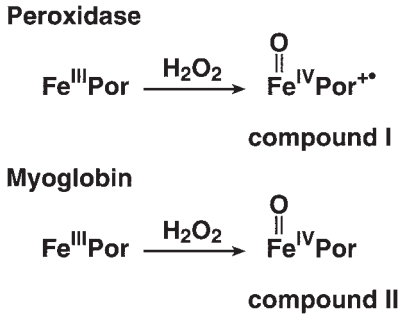
1. Background

In 1980, Poulos and Kraut successfully solved the crystal structure of cytochrome *c* peroxidase and proposed roles of both proximal- and distal-histidine in the formation of its compound I.[18] The peroxidase reaction cascade begins with the incorporation of H_2O_2 near the heme (Scheme 1). The ligation of H_2O_2 is a reversible process, however, deprotonation of H_2O_2 assisted by distal histidine as a general base accelerates the peroxide ligation. Once a peroxide-heme intermediate is formed, protonated distal histidine serves as a general acid to help the heterolytic O–O bond cleavage (pull effect). Under usual experimental conditions, the peroxide bound heme is not an observable species,[19] indicating that the relative rate of the O–O bond cleavage over the peroxide binding rate is very much greater. The major driving force for the formation of compound I can be the concomitant formation of a very stable water molecule. At the same time, an anionic proximal histidine ligand is expected to encourage the heterolysis of the peroxide bound heme by introducing an electron into the σ^*-orbital of the peroxide through the heme (push effect).[18,20] Apparently, each step for the formation of compound I is an ionic process; thus, the polar atmosphere of the distal heme site by a protonated arginine residue (Arg48) is also an important factor for efficient compound I formation. In fact, replacement of His52 or Arg48 by a nonpolar amino acid residue such as leucine reduced the rate of compound I formation by 5 or 2 orders of magnitude, respectively.[21,22]

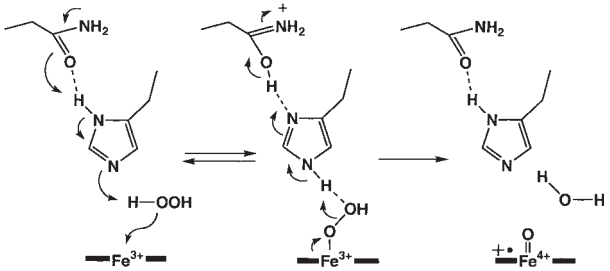
Myoglobin is a hemoprotein responsible for the storage of molecular oxygen. Comparison of the crystal structures of myoglobin[23] and cytochrome *c* peroxidase (Fig. 2) reveals that the heme binding site structures are very similar, while the reaction of myoglobin with H_2O_2 does not afford compound I, instead, compound II (O=Fe(IV)) is observed (Scheme 2) at the rate of 10^3 M^{-1} s^{-1}, 4 orders of magnitude slower than peroxidases.[24] Inspection of Fig. 2 leads us to recognize three differences in these protein structures. 1) Proximal histidine (His175) in cytochrome *c* peroxidase interacts with aspartic acid (Asp235), while that in myoglobin interacts with serine 92. 2) Distal histidine (His52) in cytochrome *c* peroxidase interacts with asparagine (Asn82) through a hydrogen bond. 3) The distance between the heme iron and distal histidine



Scheme 1. Role of distal histidine of cytochrome *c* peroxidase on the formation of compound I.



Scheme 2. Compound I and II formations by the reaction of H_2O_2 with peroxidase and myoglobin, respectively.



Scheme 3. Roles of the hydrogen bond between distal histidine and asparagine for the formation of compound I of peroxidases.

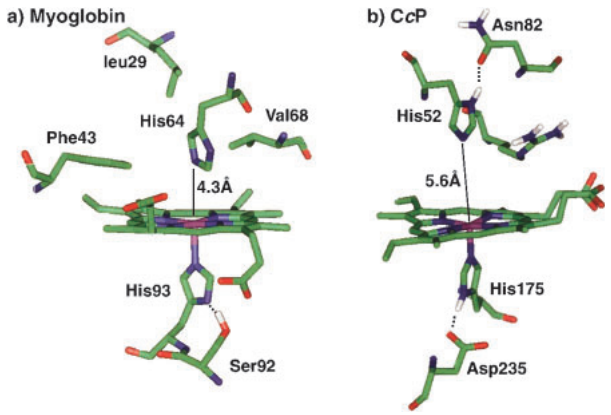


Fig. 2. Comparison of the heme sites in myoglobin (a) and cytochrome *c* peroxidase (C*c*P) (b).

(N_ε) in myoglobin is about 1 Å shorter than that of cytochrome *c* peroxidase. In order to understand the role of hydrogen bonding between His175 and Asp235, Kraut et al. replaced Asp235 with asparagine and observed almost no effect on the rate of compound I formation.[25] Therefore, the push effect described in Scheme 1 is not as great as expected before. However, one should recall that the formation of the hydroperoxide-heme adduct as a precursor for the compound I is also affected by the hydrogen bond. Anionic nature of proximal histidine by the hydrogen bond disfavors adduct formation in equilibrium between the resting state and ligation of H_2O_2 (trans effect), while the O–O bond cleavage of the peroxide bound to the heme is expected to be accelerated by anionic histidine ligation. Thus, these two opposite effects result in the overall effect of the hydrogen bond being very small. On the other hand, Goodin et al. prepared a cytochrome *c* peroxidase mutant in which His175 was replaced to glycine (His175Gly C*c*P).[26] The reaction of His175Gly C*c*P and H_2O_2 caused heme destruction instead of the compound I formation, suggesting an additional role of distal histidine to fix the heme at a proper position in the heme cavity.

The deletion of the hydrogen bonding between distal histidine and asparagine was also examined by using a horseradish peroxidase (HRP) mutant, in which asparagine 70 was replaced with valine.[27] The reaction of Asn70Val HRP with H_2O_2 gave compound I at a rate of 1.2×10^6 M^{-1} s^{-1}, 10 fold slower than that by native HRP. The result could be attributed to the character of distal histidine in Asn70Val HRP mutant being less basic than that in native HRP. Scheme 3 summarizes the role of the distal histidine-asparagine hydrogen bond in the reaction of peroxidase and H_2O_2.

In the studies described above, amino acid residues that are expected to be very important for their enzymatic functions were deleted by site-directed mutagenesis. Therefore, decreased activities are the measure of the effects. On the other hand, the approach we have been taking in the last several years is very different from these methods, i.e., we have been trying to introduce the targeted enzymatic functions into myoglobin by constructing the heme vicinity on the basis of the reaction mechanisms.[28] The first example of this approach was the introduction of a general acid-base histidine at a proper position for the formation of compound I.

2. Introduction of Peroxidase Activity into Myoglobin by Mechanism Based Molecular Design

For the formation of compound I, the peroxide bound to a heme iron must cleave heterolytically with concomitant production of a water molecule. Thus, charge separation between two oxygens of the heme bound peroxide is expected at the transition state as shown in Fig. 3 (inset). On the basis of the crystal structure of C*c*P[18] (Fig. 2), the N_ε-proton of distal histidine in cytochrome *c* peroxidase is able to interact exclusively with the negatively charged leaving (distal) oxygen to stabilize the charge separation. On the other hand, the fact that distal histidine of myoglobin is located 1 Å closer to the heme[23] than that of cytochrome *c* peroxidase allows N_ε-proton of distal histidine in myoglobin to interact with both oxygens as shown in Fig. 3a. Therefore, the hydrogen bond in myoglobin is not able to stabilize the transition state and it is hard for myoglobin to facilitate the heterolytic O–O bond cleavage. If this is the case, one may assume that the relocation of distal histidine in myoglobin to a position similar to that of peroxidases and catalase could allow myoglobin to yield compound I efficiently. Even though no crystal structures of peroxide bound to heme are available, we will be able to estimate its structure from oxy forms of heme proteins formed by the coordination of molecular oxygen to the ferrous heme (Eq. 1). The crystal structures of the oxy-forms of cytochrome *c* peroxidase[29] and myoglobin[30] show that the distances between N_ε and both proximal- and distal-oxygens are 3.9, 3.0 and 2.95, 2.72 Å respectively (Fig. 3b and c). These structures support the proposed structures for the peroxide bound to myoglobin and cytochrome *c* peroxidase that are shown in Fig. 3a.

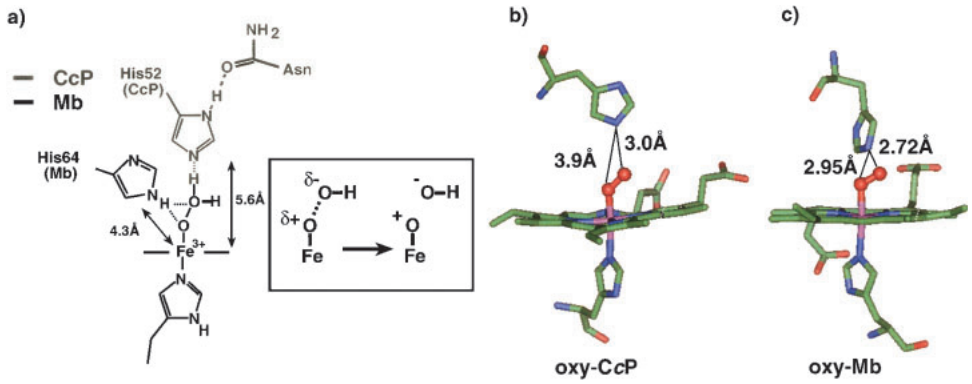
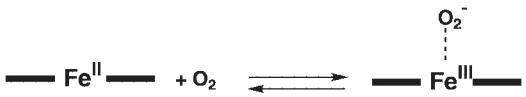


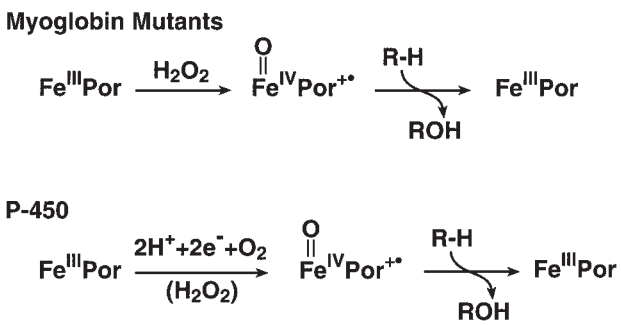
Fig. 3. Schematic drawing of distal histidine in myoglobin (Mb) and cytochrome *c* peroxidase (C*c*P) (a) and oxy forms of C*c*P (b) and Mb (c).



(1)

On the basis of these considerations, we have prepared two sets of myoglobin mutants, in which new distal histidine was introduced at the position of Leu29 or Phe43.[31,32] At the same time, distal histidine (His64) was replaced with leucine for both mutants. Thus, the mutants we have prepared are Leu29-His/His64Leu and Phe43His/His64Leu Mb (L29H/H64L and F43H/H64L). Fortunately, we have solved their crystal structures (Fig. 4).[32] The distances between N_ε of distal histidine and the heme iron are 6.5 and 5.7 Å for L29H/H64L and F43H/H64L Mb, respectively. The reaction of these two myoglobin mutants and *m*-chloroperbenzoic acid (*m*CPBA) was monitored by a stopped-flow spectrophotometer. On a 60 ms time scale, we have observed compound I formation, for the first time, followed by slow decay of compound I to compound II (O=Fe^{IV} Por) (Fig. 5).[31] Under the same conditions, native myoglobin directly affords compound II in 2 s. Peroxidase substrates such as 2,2′-azinobis(3-ethyl-2,3-dihydrobenzothiazole-6-sulfonic acid) (ABTS) and guaiacol were readily oxidized by these mutants.

Due to the replacement of distal histidine (His64) in myoglobin to leucine, a substrate binding space immediately above the heme was introduced (Fig. 4). Thus, the myoglobin mutants were expected to exhibit monooxygenase activities (oxo-transfer into substrates) as observed for P450. However, there is a major difference between myoglobin catalyzed monooxygenation and that catalyzed by P450, i.e., the myoglobin mutants use hydrogen peroxide as an oxidant while P450 utilizes molecular oxygen and two electrons as shown in Scheme 4.



Scheme 4. Peroxide-depenent oxidation (myoglobin) and reductive oxygen activation by P450.

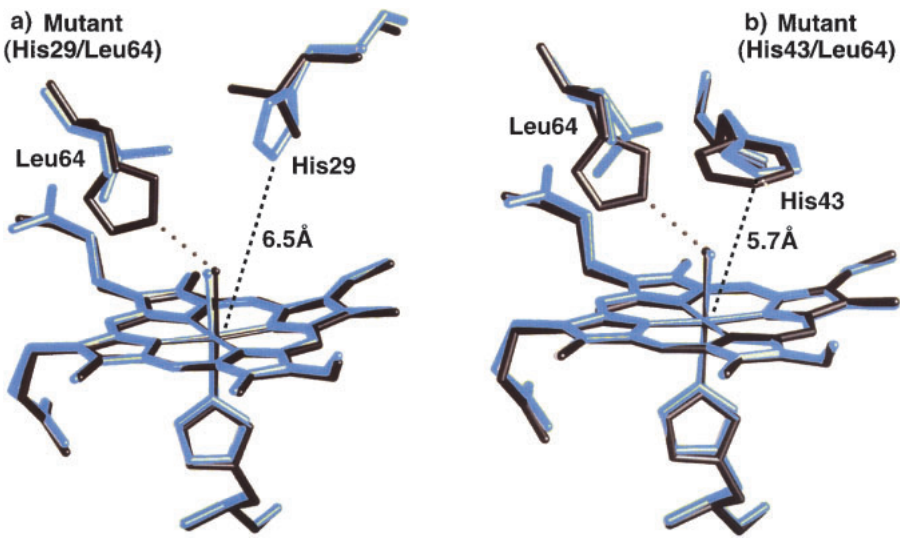


Fig. 4. Crystal structures of L29H/H64L (a) and F43H/H64L Mb (b) superimposed on native Mb (black).

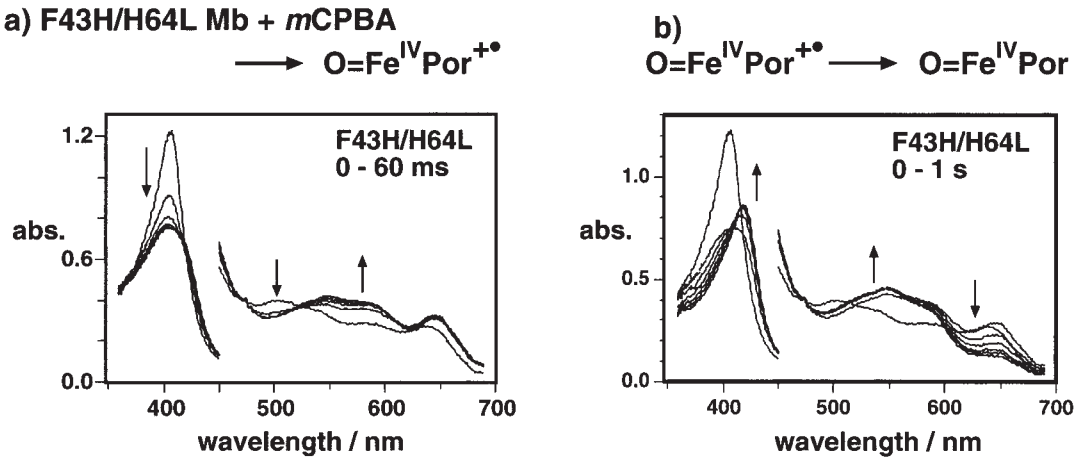
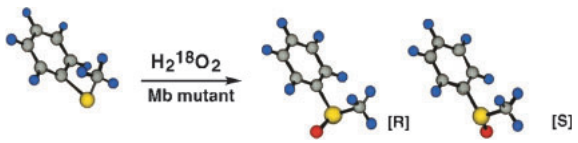
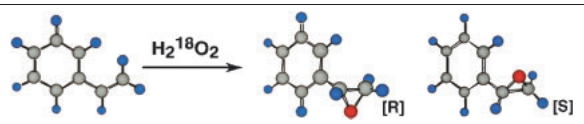


Fig. 5. Formation of myoglobin compound I (a) and its decay to compound II (b).

Table 2. Oxidation of Thioanisole and Styrene by Myoglobin and Its Mutants



Protein	Turnover /m	^{18}O content	e.e. (*R*)
Sperm Whale Myoglobin	0.13	92	25
His64Leu	0.045	ND	27
Leu29His	3.9	ND	91
L29H/H64L	5.5	97	97
F43H/H64L	47	96	85



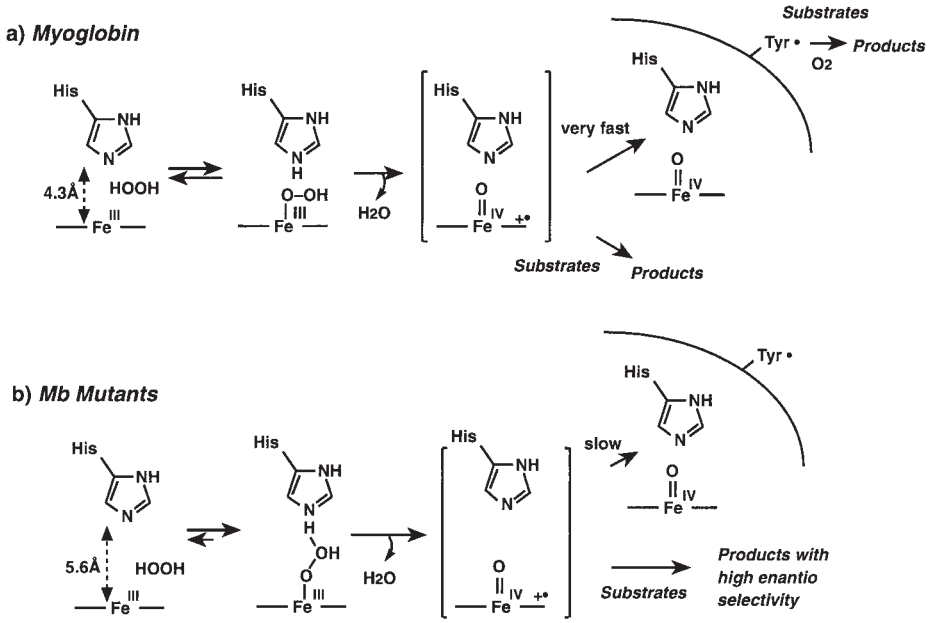
Protein	Turnover /m	$H_2{}^{18}O_2$	e.e. (*R*)
Sperm Whale Myoglobin	0.015	20	9*R*
L29H/H64L	0.14	94	80*R*
F43H/H64L	4.5	94	64*R* (70*R*[a])

a) *m*CPBA was used as the oxidant.

The hydrogen peroxide-dependent monooxygenation (peroxygenation) activity of the myoglobin mutants was examined by employing thioanisole and styrene as substrates.[31,33]

Myoglobin exhibits sulfoxidation activity at a turnover frequency of 0.13 /m with moderate enantioselectivity (Table 2). In addition, ^{18}O in $H_2{}^{18}O_2$ was mostly incorporated into the sulfoxide. As expected, both myoglobin mutants are capable of catalyzing the sulfoxidation with up to 360-fold higher activity. More importantly, the L29H/H64L mutant gave *R*-isomer almost exclusively. Likewise the sulfoxidation, wild type myoglobin also catalyzes styrene epoxidation, however, only 20% of ^{18}O in $H_2{}^{18}O_2$ was introduced into the epoxide. According to Ortiz de Montellano, active species of myoglobin for the epoxidation was a peroxide radical formed by the reaction of molecular oxygen and a phenoxyl radical of a tyrosine residue on the myoglobin surface.[34] On the other hand, the incorporation of ^{18}O in the epoxide catalyzed by the mutants is evident from the results shown in Table 2. Thus, the myoglobin mutants are still able to proceed the P450 type oxo-transfer reaction with high enantioselectivity (70–80% e.e. for *R*-isomer) and high reactivity compared to wild type myoglobin. In addition, replacement of the oxidant from H_2O_2 to *m*CPBA in the epoxidation by F43H/H64L Mb gave the same enantioselectivity, indicating that the two oxidants afford the same active species, compound I as shown in Scheme 4. These results imply that compound I could be formed even in the case of wild type myoglobin, however, two reactions of compound I, i.e.; the oxidation of substrates and substrate independent reduction of compound I compete with each other. If the substrate is less reactive, the latter reaction becomes the major process to afford compound II, coupled with one electron oxidation of amino acid residues such as tyrosine and tryptophan through distal histidine (His64), since His64 in wild-type myoglobin is located closer to the heme compared to the mutants. In fact, radical species of those residues in wild type myoglobin were detected by spin-trapping experiments.[35,36] As already discussed, these radicals are expected to react with molecular oxygen to yield peroxyl radicals, which could be the species responsible for the epoxidation (Scheme 5).[34] In order to test for possible involvement of distal histidine in the compound I reduction, we have replaced distal histidine with non-oxidizable amino acid residues such as alanine and leucine (His64Ala and His64Leu Mb, respectively). These histidine deletion mutants reacted with *m*CPBA and afforded their compound I as an observable intermediate by stopped flow spectroscopy.[37]

Very high enantiomeric selectivity in the sulfoxidation and epoxidation by Mb mutants could be caused by chiral environment of the substrate binding site in the Mb mutants. Further attempts to apply the peroxygenase activity for the hydroxylation of ethylbenzene and indane by F43H/H64L Mb were not successful (0.2–7 turnover/h) under conditions similar to sulfides and olefin oxidations.[38]



Scheme 5. Compound I formation and its reduction by a surface tyrosine for myoglobin (a) and slow reduction of compound I in the case of the mutants due to the relocation of distal histidine (b).

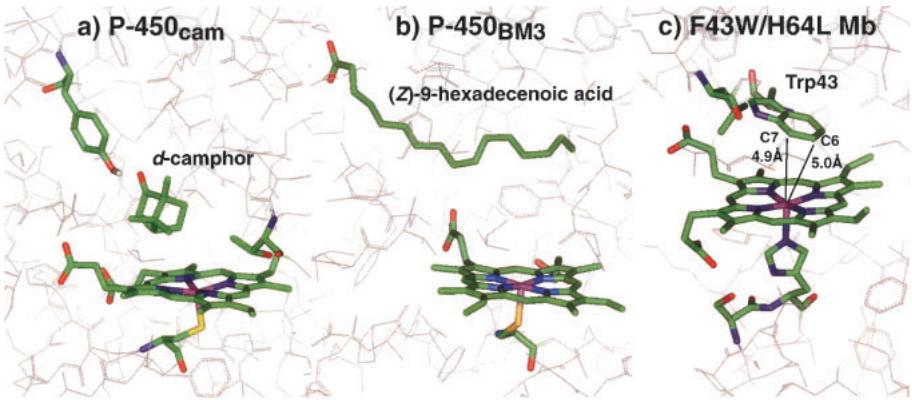


Fig. 6. Crystal structures of the resting state of $P450_{cam}$ (a) and $P450_{BM3}$ (b), and expected structure of F43W/H64L Mb mutant (c).

3. Facile Oxidation of Aromatic Ring by Myoglobin; Introduction of P450 Activity into Myoglobin

While the enantioselective sulfoxidation and epoxidation are successful examples of P450 type functions introduced into myoglobin, it was still very hard for myoglobin mutants to catalyze the hydroxylation, which is the most important biological function of P450. Thus, L29H/H64L and F43H/H64L Mb are still far away from being suitable protein models for P450. However, it doesn't seem to be fair to myoglobin mutants to compare their hydroxylation activity with that of P450. For example, Fig. 6A and B show crystal structures of $P450_{cam}$[14] and $P450_{BM3}$[39] at the resting state. Apparently, the substrates (*d*-camphor and (*Z*)-9-hexadecenoic acid for $P450_{cam}$ and $P450_{BM3}$, respectively) are captured near the heme and are ready to be oxidized. While we have provided a substrate binding space 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(s) such as hydrogen bond or charge interaction. Therefore, substrates may not steadily remain near the heme. In order to make the fair competition of hydroxylation activity between P450 and myoglobin mutants, we have introduced a substrate, tryptophan, nearby the heme of myoglobin by site-directed mutagenesis.[40] At the same time, distal histidine was replaced by leucine (F43W/H64L Mb) to avoid possible reduction of compound I through distal histidine. Though we do not have its crystal structure yet, molecular dynamics simulation suggests that the C6 and C7 positions of tryptophan 43 are closest to the heme among the tryptophan side chain.[41] Thus, we assumed the oxidation site of the tryptophan residue to be either the C6 or the C7 position. The reaction of F43W/H64L Mb and *m*CPBA was monitored by an electron spray ionization mass spectrophotometer (ESI-Mass), since the oxidative modification of amino acid residue causes the molecular weight change.

The ESI-Mass spectrum of intact myoglobin shows its apoform as the major peak, with a weak holo-myoglobin peak due

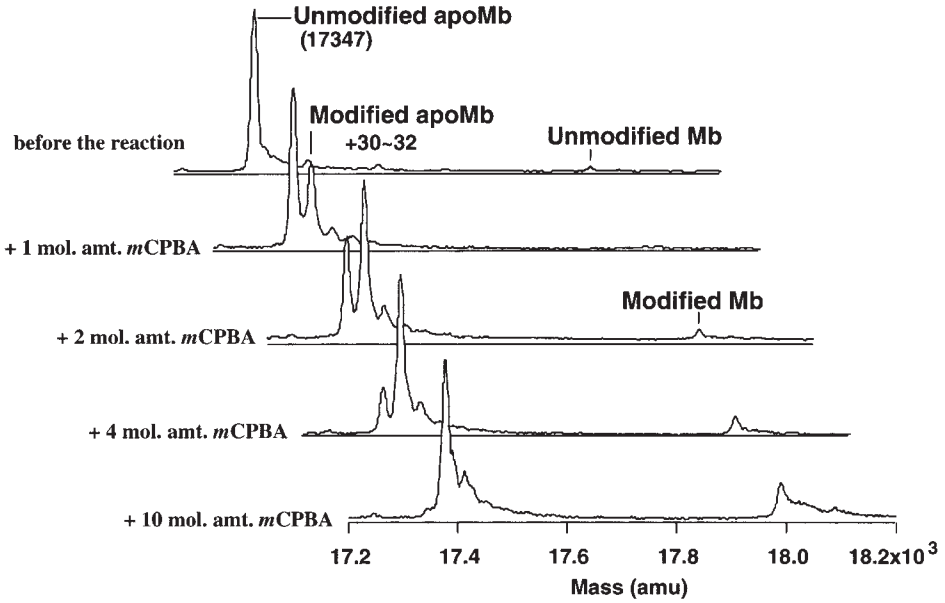
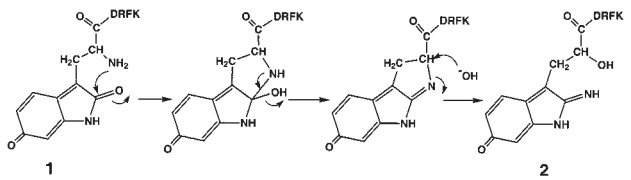


Fig. 7. ESI-Mass spectral changes of Phe43Trp/His64Leu Mb upon the aliquot addition of *m*CPBA.



Scheme 6. The structures of oxidatively modified tryptophan (**1** and **2**) and transformation mechansim from **1** to **2**.

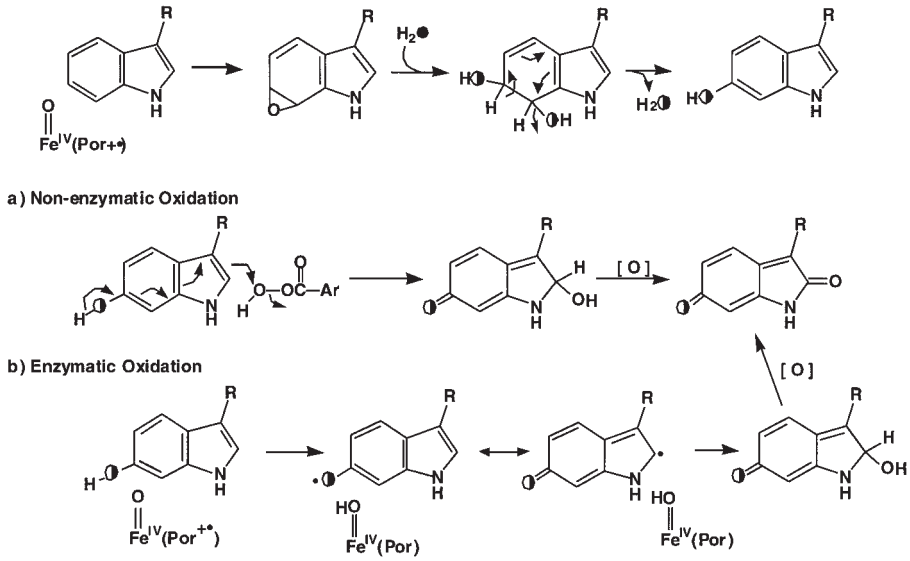
to the release of heme under the ionization process. The addition of 1 equimolar amount of *m*CPBA to the myoglobin mutant causes a decrease of the intact myoglobin peak with a concomitant appearance of a new peak in the ESI-Mass spectrum. Further titration of the mutant by *m*CPBA indicated that 4 molar amounts of *m*CPBA is enough for completion of the spectral change with an increased mass of 30–32 Da (Fig. 7).[42] In order to identify the modified amino acid residue(s), the oxidized mutant was digested by Lys-C achromobacter, followed by the isolation of the modified fragments by FPLC. Two fragments were finally obtained as the oxidation products. Amino acid sequence analysis, mass-mass measurement, and ^{1}H- and ^{13}C-NMR measurements of the fragments allowed us to identify their structures (**1** and **2**) as shown in Scheme 6. The primary product is **1**, which is readily transformed to **2** in the presence of Lys-C achromobacter. Thus, the N-O exchange could proceed via a Schiff base intermediate during the digestion of the whole enzyme, as shown in Scheme 6.[42]

Modified tryptophan **1** and **2** are products 6 electron oxidized from its parent indole structure; thus, the oxidative modification of tryptophan by *m*CPBA proceeded almost stoichiometrically since 4 molar amounts of *m*CPBA (8 electron oxidizing equivalent) allowed the complete oxidation (Fig. 7). The oxidation at the position of C6 in the indole ring suggests that the initial oxidation was the epoxidation of the C6–C7 double bond. Hydrolysis of the epoxide followed by aromatization could yield 6-hydroxyindole. Either enzymatic or non-enzymatic oxidation of 6-hydroxyindole finally gave indole-2,6-dione, **1** (Scheme 7). If the mechanistic consideration is correct, we expect that 50% of the oxygen in the 6-carbonyl group comes from water molecules, while the oxygen of 2-carbonyl is derived exclusively from the oxidant. Thus, we have examined the reaction either with Ar–CO–^{18}O–^{18}OH, in $H_2{}^{18}O$, or under $^{18}O_2$ atmosphere, where ^{18}O contents are 72, 91, and 98%, respectively. Table 3 shows the results of ^{18}O-incorporation in **1**, consistent with the mechanism depicted in Scheme 7.[43]

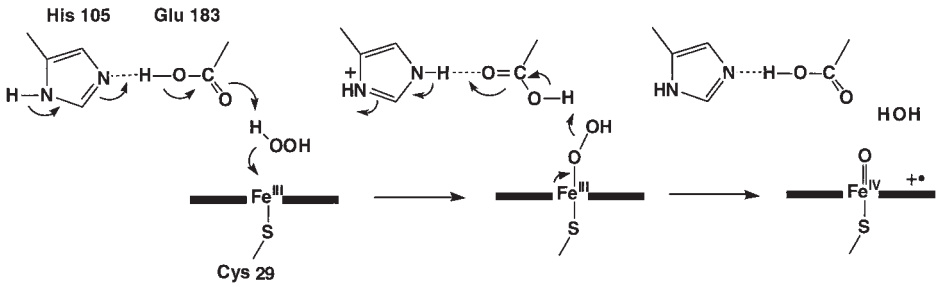
Almost stoichiometric oxidation of the aromatic ring of tryptophan at the position near the heme demonstrates that *compound I of myoglobin can oxidize an aromatic ring if the substrate is placed near the heme and this is the exact feature of P450 mimicked by the myoglobin mutant.*

4. Myoglobin Compound I Formation by the Reaction with H_2O_2

4.1 Modeling of Chloroperoxidase. The distal histidine had been relocated for the myoglobin mutants, F43H/H64L and L29H/H64L Mb. While they gave compound I in the reaction by *m*CPBA as observable species, *m*CPBA is a stronger oxidant than H_2O_2 and is not a natural oxidant.[31] Thus, we have to prepare myoglobin mutants which give compound I by their reactions with H_2O_2 to really introduce the peroxidase feature into myoglobin. For this purpose, we have mimicked the distal structure of chloroperoxidase from the marine fungus *Caldariomyces fumago*.[44] Chloroperoxidase is a chimera type heme enzyme since the distal site consists of a histidine-glutamate couple whereas the proximal ligand is a cysteine thiolate as observed for P450. Scheme 8 shows the role of Glu183 in chloroperoxidase, which works together with His105 as a general acid base catalyst. By mimicking chloroperoxidase, we have replaced distal histidine in myoglobin with aspartic acid (H64D Mb).[45] The reaction of H64D Mb with H_2O_2 is shown in Fig. 8. The compound I formation is apparent from

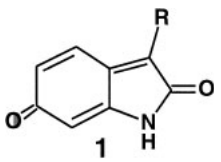


Scheme 7. A proposed oxidation mechanism of tryptophan 43 by *m*CPBA in $H_2{}^{18}O$.



Scheme 8. Active site structure and acid-base function of glutamic acid (Glu183) in chloroperoxidase.

Table 3. ^{18}O Incorporation in the Product **1** under Various ^{18}O Labeled Conditions



1

	Contents of ^{18}O in **1**		
	^{18}O–^{18}O	^{16}O–^{18}O	^{16}O–^{16}O
	obs. (calc.)		
^{18}O-*m*CPBA (72%)[a)]	25 (26)	50 (56)	25 (18)
$H_2{}^{18}O$ (91%)[a)]	0 (0)	39 (45)	61 (55)
$^{18}O_2$ (98%)[a)]	0 (0)	0 (0)	100 (100)

a) Content of ^{18}O-labelling.

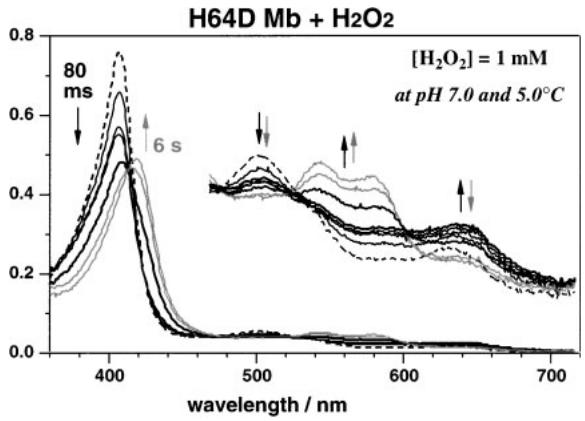


Fig. 8. Spectral changes upon the addition of H_2O_2 to His64Asp Mb at pH 7.0.

the spectral changes. In this particular case, compound I formation is much faster than its decay to compound II; this allowed us to observe compound I. More importantly, the rate for the formation of compound I by H_2O_2 is very much faster than that estimated for other myoglobin mutants we have prepared before. Thus, H64D mutant is expected to oxidize sulfides much faster than F43H/H64L and L29H/H64L Mb mutants. Table 4 shows the comparison of relative oxidation activities of H64D Mb to those of wild-type Mb.[45] These results as well as the results shown in Table 2 provide a clear demonstration for the introduction of peroxidase and P450 features into myoglobin by simple mutation.

4.2 Fine Tuning of H64D Mb as a Highly Enantioselective Oxidation Catalyst. While these H_2O_2-dependent oxidation activities of H64D Mb are higher than those of any rationally designed Mb mutants, H64L/F43H and H64L/L29H Mbs which show up to 97% e.e. for sulfoxidation, the H64D Mb gave mostly racemic sulfoxide. It has yet to be clarified how the enantioselectivity of these Mb mutants in

Table 4. Comparison of Relative Reactivities of Mb and His64Asp Mb in Different Types of Oxidations

Oxidation	Relative reactivity (H64D Mb/wild type Mb)
Peroxidase type	
Guaiacol oxidation	51
ABTS oxidation	69
P450 type	
Thioanisole oxidation	580
Styrene oxidation	818

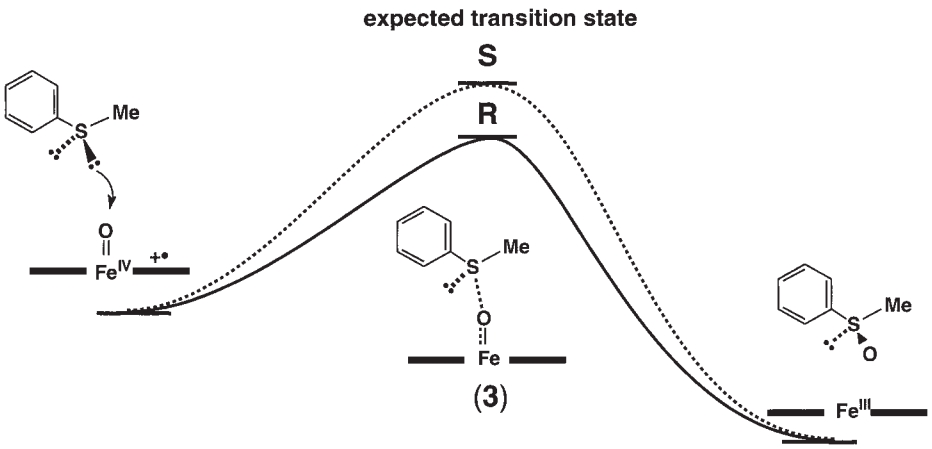
sulfoxidation is determined. Structural studies of wild-type myoglobin indicated that the access of small substrates such as O_2 and CO to the active site is limited by His-64 and Val-68 residues located immediately above the heme (Fig. 2).[46] Thus, Val-68 in H64D Mb was replaced by a smaller residue, Ala or Ser, to prepare H64D/V68A and H64D/V68S, respectively. These two mutants showed dramatic improvement in the H_2O_2-dependent enantioselective sulfoxidation (84 and 88% ee) with catalytic activity comparable to that for H64D Mb (Table 5).[47] The results clearly indicate that the residue at the position of 68 is important for the enantioselective sulfoxidation.

Table 5. Enantioselective Sulfoxidation of Thioanisole by Mbs with H_2O_2

Mutant	Rate (turnover/m)	e.e./% (*R*)
H64D/V68A	121	84
H64D/V68S	64	88
H64D	145	6

4.3 Use of Transition State Analogue for the Sulfoxidation. The sulfoxidation by myoglobin compound I is expected to proceed, in the simplest case, according to the reaction coordinate shown in Scheme 9. Preferable formation of *R*-sulfoxide is suggestive of lower activation enthalpy for the transition state of R-sulfoxidation. An expected structure of the transition state, **3** is shown in Scheme 9. While it is impossible to observe **3**, we will be able to estimate its structure and stability by employing a transition state analogue, 1-phenylethylamine, for the thioanisole oxidation (Fig. 9). Thus, we have studied enantioselective ligation of (*R*)- and (*S*)-1-phenylethylamine to H64D/V68A and H64D/V68S Mbs in comparison with the sulfoxidation of thioanisole.[47]

Ligation of the (*R*)- or (*S*)-amine to myoglobin mutants was



Scheme 9. The simplest sulfoxidation mechanism by myoglobin compound I and a proposed transition state structure (**3**) for the preferable formation of *R*-sulfoxide.

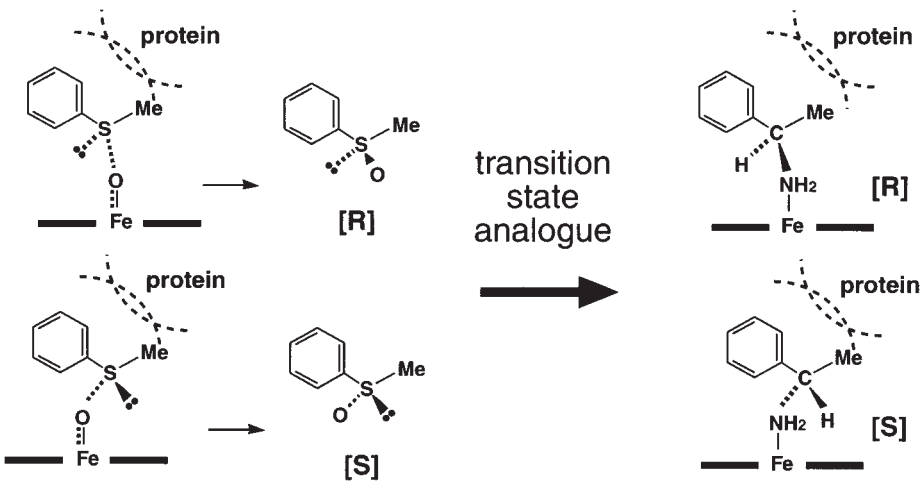


Fig. 9. Transition state analogues, (*R*)- and (*S*)-1-phenylethylamine, for the sulfoxidation.

Table 6. Kinetic Parameters for 1-Phenylethylamine Binding to Mb Mutants

	$k_1/M^{-1}\,s^{-1}$		k_{-1}/s^{-1}		K/M^{-1}	
	R	*S*	*R*	*S*	*R*	*S*
H64D/V68A	$1.3 \pm 0.1 \times 10^4$	$1.3 \pm 0.1 \times 10^4$	16	0.59	8.1×10^2	2.2×10^4
H64D/V68S	$2.2 \pm 0.3 \times 10^3$	$2.7 \pm 0.1 \times 10^3$	22	0.24	1.0×10^2	1.1×10^4
H64D	30 ± 10	4 ± 2	0.096	0.057	3.1×10^2	70

confirmed by UV-vis and EPR spectral changes; their binding constants (K) are listed in Table 6. If the structure of the amine bound to the heme is similar to the transition state shown in Fig. 9, we expect greater (*R*)-amine binding affinity over the (*S*)-isomer, since the activation enthalpy of the transition state for the (*R*)-sulfoxide formation is smaller than that for the (*S*)-sulfoxide formation. In contrast to the expectation, the K values of (*S*)-1-phenylethylamine with H64D/V68A and H64D/V68S are 27-fold and 112-fold larger than those of the corresponding (*R*)-amine, respectively. In the case of H64D Mb, which affords mostly racemic sulfoxide, differences in the enantioselective binding for (*R*)- and (*S*)-amine are very small (Table 4). In order to determine the chiral discrimination step in the amine binding, we have measured both on rate (k_1) and off rate (k_{-1}) of amine binding to the Mb mutants. The on rates (k_1) of (*R*)- and (*S*)-α-methylbenzylamine to H64D/V68A and H64D/V68S Mb are almost identical: 1.3×10^4 $M^{-1}\,s^{-1}$ and $2.2–2.7 \times 10^4$ $M^{-1}\,s^{-1}$, respectively. In contrast, a large difference is observed for the off rate. This indicates that the chiral discrimination of the (*S*)-amine ligation over the (*R*)-amine by H64D/V68A and H64D/V68S Mb is exclusively caused by a very small off rate of the (*S*)-amine relative to the (*R*)-amine: 1:27 for H64D/V68A and 1:92 for H64D/V68S. These selectivities would correspond to 93 and 98% e.e. for the amine binding, respectively. If we apply these results for the sulfoxidation, the off rate for the (*S*)-sulfoxide formation may also be much smaller than that for the (*R*)-sulfoxide due to the higher activation energy of the former. Thus, enantioselectivity in the sulfoxidation of thioanisole by H64D/V68A and H64D/V68S Mb may be determined by the off rate of sulfoxide with the energy profile of the reaction coordinate shown in Fig. 10.

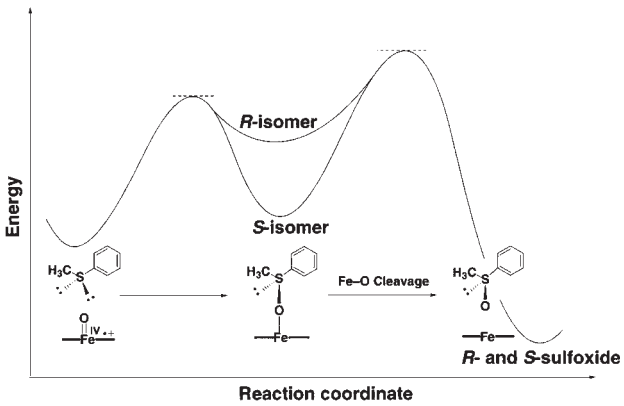
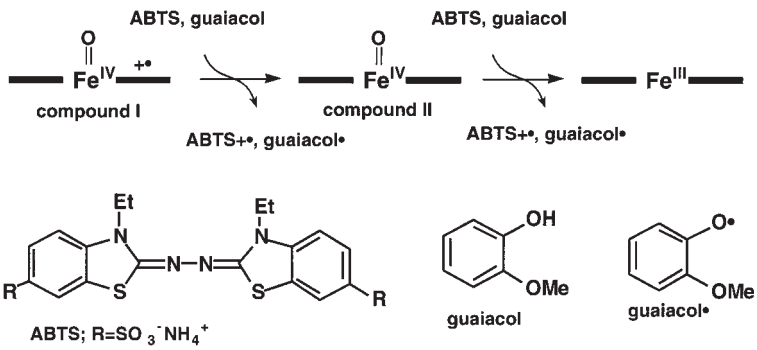


Fig. 10. Reaction coordinate and energy profile of sulfoxidation by H64D Mb mutants.

5. Advantage in Use of Myoglobin Mutants for the Mechanistic Studies of the Oxo-Transfer as Protein Models for P450

While we have explained that myoglobin mutants are able to exhibit peroxidase and P450 activities, the catalytic activity is less than that of those enzymes. What could be the advantage in use of myoglobin mutants as models for P450? In the case of P450 such as $P450_{cam}$, oxygenation activity is quite high; however, it is impossible for us to directly observe the oxo-transfer (oxygenation) process, since compound I is, in most cases, not observable.[7,48] *How about compound I of peroxidases such as horseradish peroxidase (HRP)?* So far, no one has ever claimed the use of peroxidase compound I as a protein model for P450. The peroxidase-catalyzed oxidation is, in general, a one-electron oxidation of substrates but not the oxygenation (two electron oxidation of substrates). For example, ABTS and guaiacol are the most common peroxidase substrates and the $ABTS^{+\bullet}$ formation can be monitored as an absorbance change to measure the peroxidase activity (Scheme 10).[27,49] In *N*,*N*-dimethylaniline and thioanisole oxidations by HRP compound I, we were able to observe two stepwise one electron oxidations spectroscopically.[50] When a variety of substituents were introduces on the phenyl groups of *N*,*N*-dimethylaniline and thioanisole, the oxidation potentials (E^0_{ox}) are perturbed. Fig. 11a shows plots of the oxidation potential of these *p*-substituted substrates vs oxidation rates by



Scheme 10. Typical peroxidase reactions.

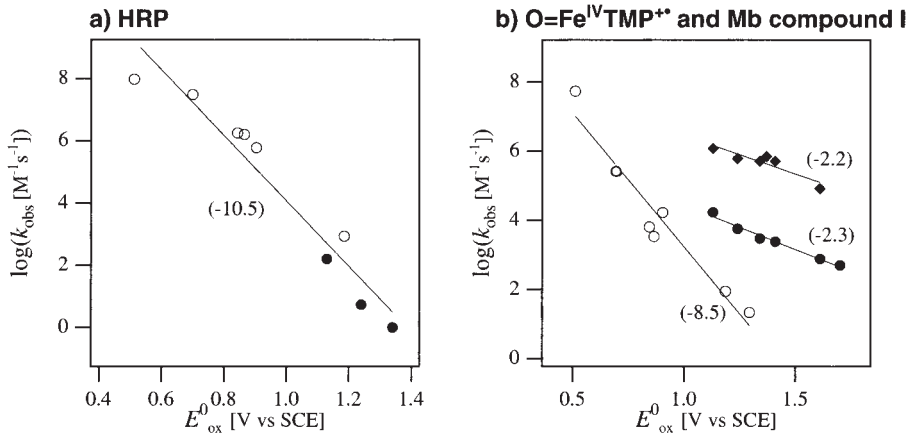


Fig. 11. Bimolecular rate constants of dimethylanilines (○) and thioanisoles (●) oxidation by HRP compound I (a), and of dimethylanilines (○) and thioanisoles (●) oxidation by **4** (b), along with thioanisole (◆) oxidation by compound I of H64S Mb (b).

compound I of HRP ($\log k_{obs}$). Apparently, there is a single linear correlation between $\log k_{obs}$ and E^0_{ox} even though *p*-substituted dimethylanilines and thioanisoles are plotted together. Thus, the rate constants for the oxidation of these substrates by HRP compound I are expressed by a common relationship (Eq. 2):[50]

$$\log k_{obs} = -10.5E^0_{ox} + 14.6 \quad (2)$$

Such a unified correlation clearly indicates that the oxidation proceeds via one electron transfer rather than via direct oxygen transfer. We have also examined the same oxidations by employing a synthetic model of compound I, O=Fe^{IV}TMP$^{+\bullet}$ (**4**) (TMP = 5,10,15,20-tetramesitylporphyrin dianion).[51] We have already demonstrated that the oxidation of dimethylanilines by **4** proceeds via a one-electron transfer mechanism. Thus, the parallel relationship between two plots of HRP compound I and **4** was obtained.[50] The same treatment was applied to thioanisole oxidation by **4**. Fig. 11b summarizes correlations between $\log k_{obs}$ for the sulfoxidation and E^0_{ox} of thioanisoles, in comparison with a linear correlation between $\log k_{obs}$ for the electron transfer from dimethylanilines to **4** and E^0_{ox} of dimethylanilines.[52] In contrast with the case of HRP compound I in Fig. 11a, the k_{obs} values of thioanisoles in Fig. 11b are at least two-orders of magnitude larger than the k_{obs} values of electron transfer from dimethyanilines to **4** when they are compared with the same E^0_{ox} values. In addition, the k_{obs} values of thioanisoles are much less sensitive to the E^0_{ox} values of thioanisoles, as compared to the large slope observed in the linear correlation between $\log k_{obs}$ for the electron transfer from dimethylaniline to **4** and E^0_{ox}. This indicates that the reduction of **4** by thioanisoles proceeds via direct oxygen transfer as shown in Scheme 9 rather than via a process including the electron transfer.

We have also employed the His64Ser Mb mutant (H64S Mb) as a P450 model to compare the reactivity of H64S Mb compound I toward thioanisoles with that of the synthetic model, **4**.[50] The reactions of H64S Mb compound I and thioanisoles were examined by observing the spectral changes as shown in Fig. 12.[50] In a separate experiment, quantitative sulfoxide formation was confirmed. The results are compared with those of **4** in Fig. 11b, where the $\log k_{obs}$ values are plotted against the E^0_{ox} values of thioanisoles. There is a parallel relationship between the plots of **4** and H64S Mb compound I, both of which are far above the electron transfer correlation for the reduction of HRP compound I by dimethylanilines and thioanisoles. This indicates that the sulfoxidation of thioanisoles by H64S Mb compound I also proceeds via direct oxygen transfer as shown in Scheme 9 rather than the electron transfer/oxygen rebound pathway. *The most important feature of Mb compound I is that the oxygenation process is observable, thus, we are able to examine detailed mechanisms of P450 type reactions.*

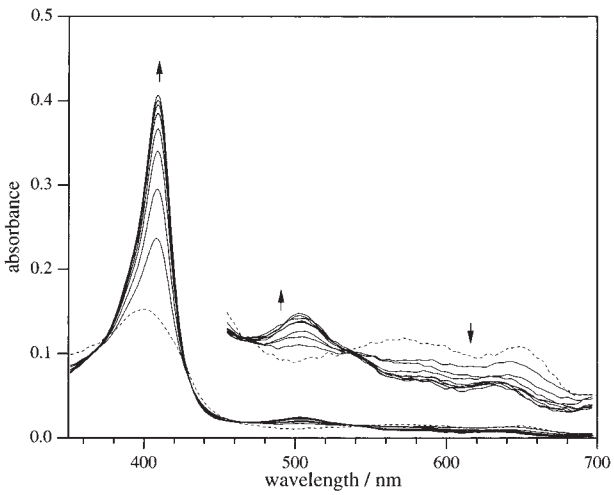


Fig. 12. Spectral changes upon the addition of thioanisole to H64S Mb compound I in sodium acetate buffer (pH 5.0) at 277 K.

6. Catalase Activity of Myoglobin Compound I

We have already discussed the use of H_2O_2 as an oxidant for the formation of compound I of H64D Mb (Fig. 8).[45] The same reaction with Mb mutants such as L29H/H64L Mb gave compound II, even though the replacement of the oxidant from H_2O_2 to *m*CPBA allowed us to observe compound I. On the other hand, F43H/H64L Mb was found to show a slight accumulation of compound I in its reaction with H_2O_2,[37] since F43H/H64L Mb is more reactive than L29H/H64L Mb

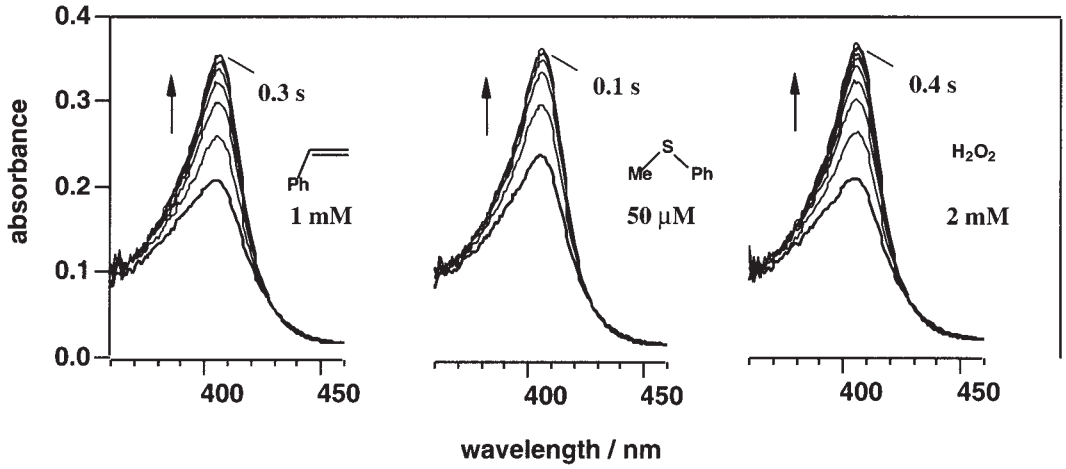


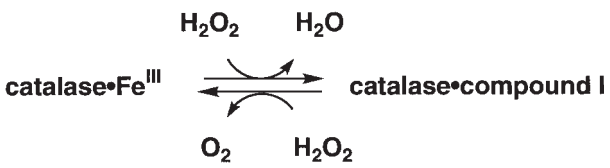
Fig. 13. Spectral changes of H64A myoglobin compound I upon the addition of styrene, thioanisole, and H_2O_2 (from left to right).

Table 7. Reaction of Mb with Hydrogen Peroxide

Mb	O_2 evolution (turnover/min)
Wild-type	1.7
L29H/H64L	8.8
F43H/H64L	79

(Table 2). There are two possible reasons for not observing compound I of these mutants in the reaction with H_2O_2: 1) the formation rate of compound I is much slower than its decay to compound II under the stopped flow conditions; Alternatively, 2) compound I could react with H_2O_2 to afford O_2, known as the catalase reaction. To examine the latter possibility, we have measured the O_2 evolution in the reaction of Mb mutants with H_2O_2 and we have observed the O_2 formation (Table 7).[45] Further confirmation of catalase activity was spectroscopically examined as follows. By using double mixing technique, we have initially prepared H64A Mb compound I by a stoichiometric amount of *m*CPBA. After the confirmation of the compound I formation, H_2O_2 was introduced to the resulting solution to observe the oxidation of H_2O_2 by Mb compound I (Fig. 13). Fig. 13 is the first direct observation of H_2O_2 oxidation by compound I. In order to compare the relative reactivity of H_2O_2, the oxidations of styrene and thioanisole by compound I of the same Mb mutant under the same conditions are also shown in Fig. 13.

It is impossible for catalase to observe the H_2O_2 oxidation by its compound I, since the ferric state of catalase formed by the reaction of compound I and H_2O_2 immediate reproduces compound I; i.e., comparable rates for the formation of compound I and its reaction with H_2O_2 gives a mixture of compound I and the resting state of catalase in the steady state condition (Eq. 3).



(3)

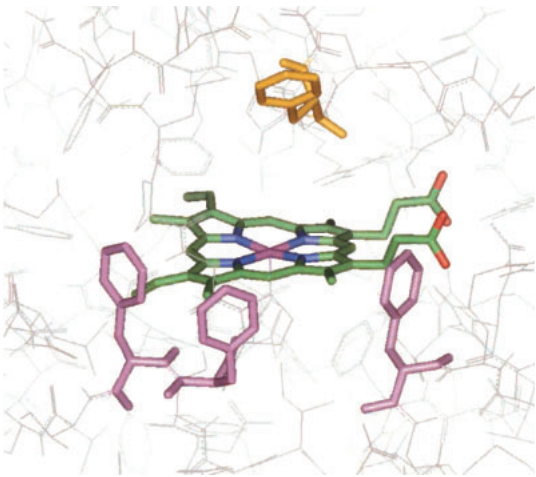


Fig. 14. Crystal structure of HRP and four phenylalanine sidechains are highlighted by a stick expression.

7. Conclusion

In this account, we have described our recent efforts toward the construction of heme enzymes by utilizing myoglobin as a framework of heme enzymes. Through the studies, we have successfully prepared several myoglobin mutants which are protein models for peroxidase, P450, and catalase. In most cases, the distal site of myoglobin was modified by the site-directed mutagenesis on the basis of mechanisms of peroxidase, P450, and catalase. These results clearly indicate that the different proximal ligand in peroxidase, P450, and catalase is not the crucial factor for discriminating the catalytic functions of these enzymes. Apparently, the compound I formation is the key for their catalytic activity. At the same time, we have to address the fact that the different proximal ligand may regulate their reactivity under the physiological conditions as a secondary factor. In addition, the tertiary structure of enzymes is also very important for discriminating catalytic activities of heme enzymes. For example, horseradish peroxidase (HRP) does not have a proper substrate binding site nor a pathway for the introduction of substrate to the heme site, as is commonly provided in cytochrome P450. Fig. 14 shows a crystal struc-

ture of HRP.[12] Four phenyl groups tightly cover the heme to prevent the substrate access to the heme. This structural feature may allow the one-electron oxidation of substrates by compound I instead of the P450 type oxidation.

The molecular design of enzymes for the use of synthetic processes is a very important field of bioscience; mechanistic studies provide very important aspects for the rational design of enzymes. We are very pleased if our account stimulates chemists to utilize some current methodologies commonly used in molecular biology.

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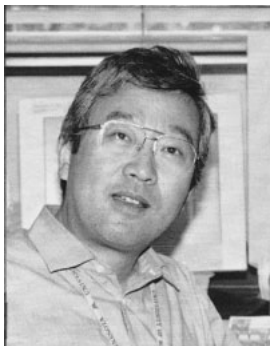

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