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Investigations of the Roles of the Distal Heme Environment and the Proximal Heme Iron Ligand in Peroxide Activation by Heme Enzymes via Molecular Engineering of Myoglobin

SHIN-ICHI OZAKI,[†,‡,§] MARK P. ROACH,[†,⊥] TOSHITAKA MATSUI,[†,‡,∥] AND YOSHIHITO WATANABE*[,†,‡]

Institute for Molecular Science, and Department of Structural Molecular Science, The Graduate University for Advanced Studies, Myodaiji, Okazaki, 444-8585, Japan

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ABSTRACT

To pursue structure−function relationships of heme enzymes in the activation of peroxides, we have chosen to use myoglobin as the framework for our molecular engineering studies. Comparison of the crystal structures of myoglobin and peroxidases reveals differences in the arrangement of amino acid residues in heme active sites. On the basis of these structural differences and the reaction mechanisms of peroxidases, we have converted myoglobin into a peroxidase-like enzyme by alternation of the heme distal pocket via site-directed mutagenesis. The replacement of the proximal histidine with cysteine and the exogenous substituted imidazoles slightly accelerates the peroxide O−O bond cleavage due to the electron donor characteristics. However, we have not observed an enhancement in the activation of peroxide by the proximal mutant with tyrosine, the exogenous phenolate, and benzoate. A clear understanding of the absolute role of the proximal ligand remains elusive.

Introduction

The four general biological functions of heme proteins are the transport of electrons (e.g., cytochrome b_5),[1] the transport of oxygen (e.g., hemoglobin, myoglobin),[2−5] the sensing of oxygen or carbon monoxide (e.g., FixL, CooA),[6−8] and the catalysis of redox reactions (e.g., horseradish peroxidase, cytochrome P450, catalase, NO synthase, NO reductase).[9−18] Despite the differences in functions, all of the proteins have iron protoporphyrin IX (heme) as a common prosthetic group. The amino acid residues surrounding the prosthetic group appear to control the intrinsic functions of the heme.

The electron carrier hemoproteins have two strong axial ligands.[19] On the other hand, in the case of globins, the coordination site on the distal side of the heme pocket is either vacant or occupied with a water molecule, which is easily exchanged for other ligands such as an oxygen molecule.[20,21] A histidine residue on the proximal side serves as an axial ligand, and the distal histidine stabilizes binding of O_2 through a hydrogen-bonding interaction.[3,4,22] The heme in FixL, an oxygen-sensing hemoprotein, appears to have one accessible coordination site, as observed in globins.[7] CooA from *Rhodospirillum rubrum*, which regulates the expression of the *coo* operons with carbon monoxide as an effector, is another sensor hemoprotein to play a role in homeostasis.[8]

The heme iron usually has one coordination site for peroxides, molecular oxygen, or other ligands to bind and react with.[23−28] Generally speaking, peroxidases react with H_2O_2 to produce a species known as compound I, an oxoferryl ($O{=}Fe^{IV}$) species paired with a porphyrin radical cation (Figure 1).[7] In compound I formation, the distal histidine first serves as a general base to allow the binding of H_2O_2 to the heme iron. The protonated distal histidine then transfers the proton to the singly protonated oxygen atom of the bound HO_2^-. Heterolytic O−O bond cleavage results in the release of a water molecule. The positively charged distal arginine also contributes to this process by polarization of the O−O bond. The combination of catalytic functions of the distal histidine and arginine residues has been described as the "pull effect".[8−13]

On the other hand, the cytochrome P450 monooxygenases bind O_2 instead of H_2O_2 and require the input of

* To whom correspondence should be addressed. Phone: 81-564-55-7430. Fax: 81-564-54-2254. E-mail: yoshi@ims.ac.jp.

† Institute for Molecular Science.

‡ The Graduate University for Advanced Studies.

§ Current address: Faculty of Education, Human Life and Environmental Sciences, Yamagata University, Kojirakawa, Yamagata, 990-8560, Japan.

⊥ Current address: Isis Pharmaceuticals Inc., 2292 Faraday Ave. Carlsbad, CA, 92008.

∥ Current address: Institute for Chemical Reaction Science, Tohoku University, Sendai, 980-8577, Japan.

Shin-ichi Ozaki was born in Nagoya, Japan, in 1964, and received his undergraduate education at Keio University in Japan and the University of Maryland. He obtained his Ph.D. in chemistry at Texas A&M University in 1992 and did his postdoctoral work on hemoenzymes at the University of California at San Francisco and the Institute for Molecular Science. He is currently Associate Professor of Biological Chemistry at Yamagata University, and his interests are in the areas of metalloproteins and biosynthesis of natural products.

Mark Roach was born in Montreal, Canada, in 1970. He received his B.Sc. degree from Mount Allison University, Sackville, NB, Canada, in 1992 and Ph.D. degree under the direction of John H. Dawson at the University of South Carolina in 1997. He was a Japan Society for the Promotion of Science postdoctoral fellow in Yoshihito Watanabe's research group at the Institute for Molecular Science from 1997 to 1999 and a postdoctoral associate in David B. Goodin's group at the Scripps Research Institute, La Jolla, CA, from 1999 to 2000. He is now a technical writer at Isis Pharmaceuticals, Inc., Carlsbad, CA.

Toshitaka Matsui was born in Osaka, Japan, in 1971 and received his undergraduate and graduate education at Kyoto University and the Okazaki National Research Institute, Graduate University for Advanced Studies, respectively. He is currently a research associate at the Institute for Chemical Reaction Science of Tohoku University in Sendai. His research interests are in the areas of molecular mechanism and engineering of metalloenzymes.

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's 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 at the National Chemical Research Laboratories in Tsukuba. He was then appointed as an Associate Professor at the Kyoto University in 1990. He has been a Professor at the Institute for Molecular Science since 1994.

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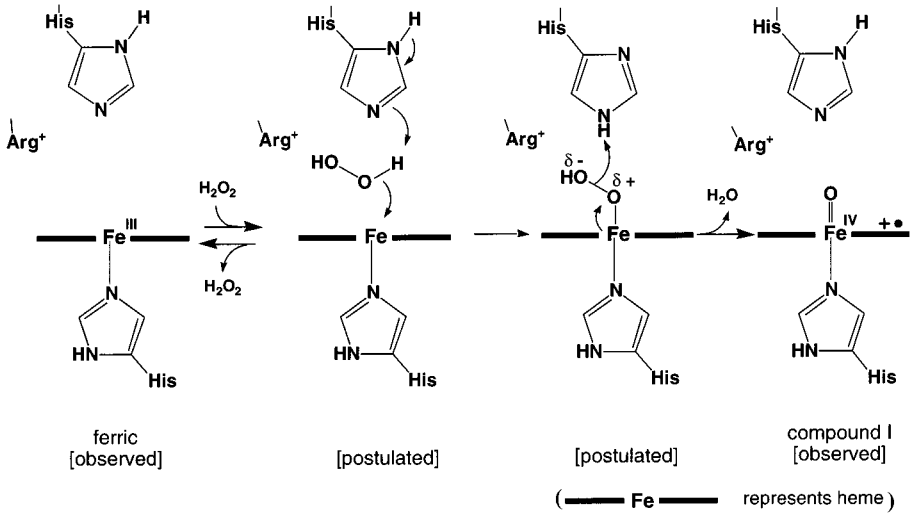


FIGURE 1. The proposed mechanism of compound I formation. The distal histidine first functions as a general base, and then the protonated imidazole serves as a general acid to cleave the O−O bond.

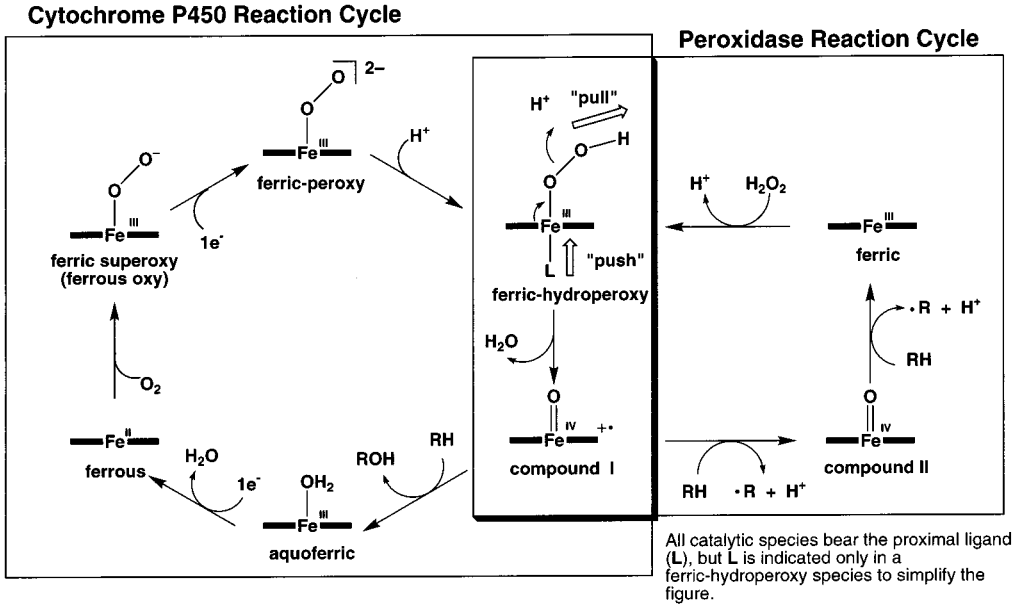


FIGURE 2. Comparison of reaction cycles of peroxidase and cytochrome P450. Compound I, a ferryl porphyrin radical cation, is a critical catalytic species for both hemoenzymes.

two electrons for the formation of a putative hydroperoxide bound to the iron. Heterolytic O−O bond cleavage generates a highly reactive catalytic species equivalent to compound I (Figure 2).[10] The cytochrome P450 monooxygenase reaction cycle differs from that of the peroxidases in that the oxo-ferryl oxygen atom is transferred to the substrates. Figure 2 indicates the overlapping nature of the types of reactive intermediates involved in peroxidase and monooxygenase chemistry. Structural features of the cytochrome P450s include a cysteine thiolate ligand, the absence of obvious catalytic functional residues in the distal heme pocket, and the presence of a water shuttle pathway from the exterior of the protein to the distal heme pocket.[26] The catalytic efficiency of the cytochrome P450s has long been attributed to O−O bond cleavage induced by strong electron donation of the thiolate ligand, the so-called "push effect".[29]

Myoglobin (Mb) normally functions as an oxygen carrier[2] but could be converted into a hemoenzyme if the active site is designed on the basis of our current knowledge of how protein active site structure influences its functional reactivity. The extensively accumulated biochemical and biophysical data on Mb, including the X-ray crystal structures of native, wild-type, and various myoglobin mutants, provide us with a good starting point.[3,4,7,8,21,22,30−35] In our pursuit of an understanding of how hemoprotein structure influences its function, we have chosen myoglobin as a template for the addition of various structural features (Figure 3). Our results, together with studies on the formation of compound I for heme

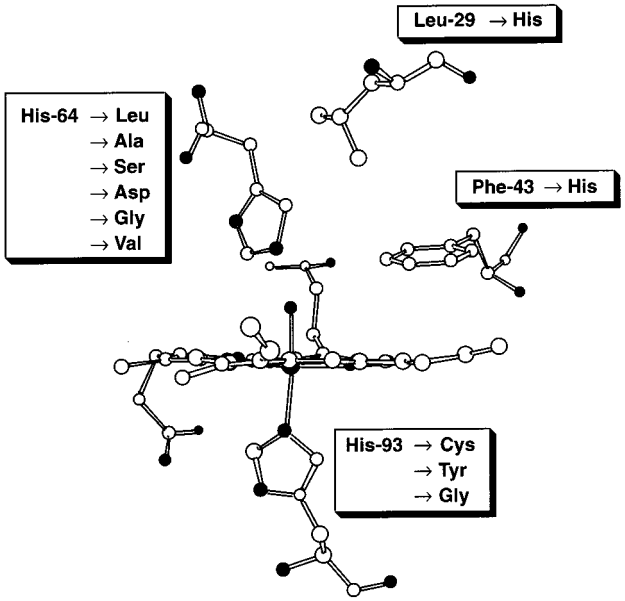


FIGURE 3. A listing of active site myoglobin (Mb) mutants discussed in this Account.

model porphyrins,[36,37] have provided insights into the mechanistic details.

I. The Role of the Distal Heme Environment in the Activation of Peroxide

A. The Distal Histidine Relocation Myoglobin Mutants F43H/H64L and L29H/H64L. Comparison of the crystal structures of the oxy forms of W191F cytochrome *c* peroxidase (C*c*P) mutant and sperm whale Mb allows us to examine the details of compound I formation (Figure 4).[22,38] The distances between Nϵ of the distal histidine and the oxygen atoms of oxy-Mb are 2.7 Å for the terminal oxygen atom (O1) and 3.0 Å for the oxygen atom bound to the iron (O2), while the values for oxy-W191F C*c*P are 3.0 and 3.9 Å, respectively. It is expected that when C*c*P reacts with H_2O_2, the protonated imidazole forms a hydrogen bond only with the terminal oxygen atom (O1) during initiation of heterolysis of the O−O bond. On the other hand, in the reaction of Mb with H_2O_2, both oxygen atoms would be positioned to accept a proton from Nϵ of the distal histidine. We expect that this arrangement is responsible for the poor reactivity of Mb with H_2O_2. To investigate if the alignment of the distal histidine is critical for the activation of H_2O_2, we have constructed the F43H/H64L and L29H/H64L Mb mutants. The two double mutants are termed "distal histidine relocation mutants".

The crystal structure of F43H/H64L Mb was solved at 1.8 Å resolution to confirm the exact location of the distal histidine.[39] The result indicates that the distance between Nϵ of the distal histidine and the ferric heme iron is 5.7 Å (Figure 5), which is similar to those in peroxidases (5.5−6.0 Å).[24,28] As expected, the double mutation increases the reactivity by 11-fold relative to that of wild-type Mb. Consequently, the F43H/H64L mutant oxidizes 2,2′-aminobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and guaiacol approximately 6-fold faster than the wild type.[39] Moreover, the oxygenation of thioanisole and styrene is also improved by 200- and 300-fold, respectively (Figure 6).[40,41] In contrast, Nϵ of His-29 in L29H/H64L Mb is located 6.6 Å away from the iron.[39] The distal histidine in the L29H/H64L mutant lies too far from the heme center to participate in efficient activation of H_2O_2; therefore, the mutant exhibits the peroxidase activity, which is less than one-third of that for wild-type Mb.[39]

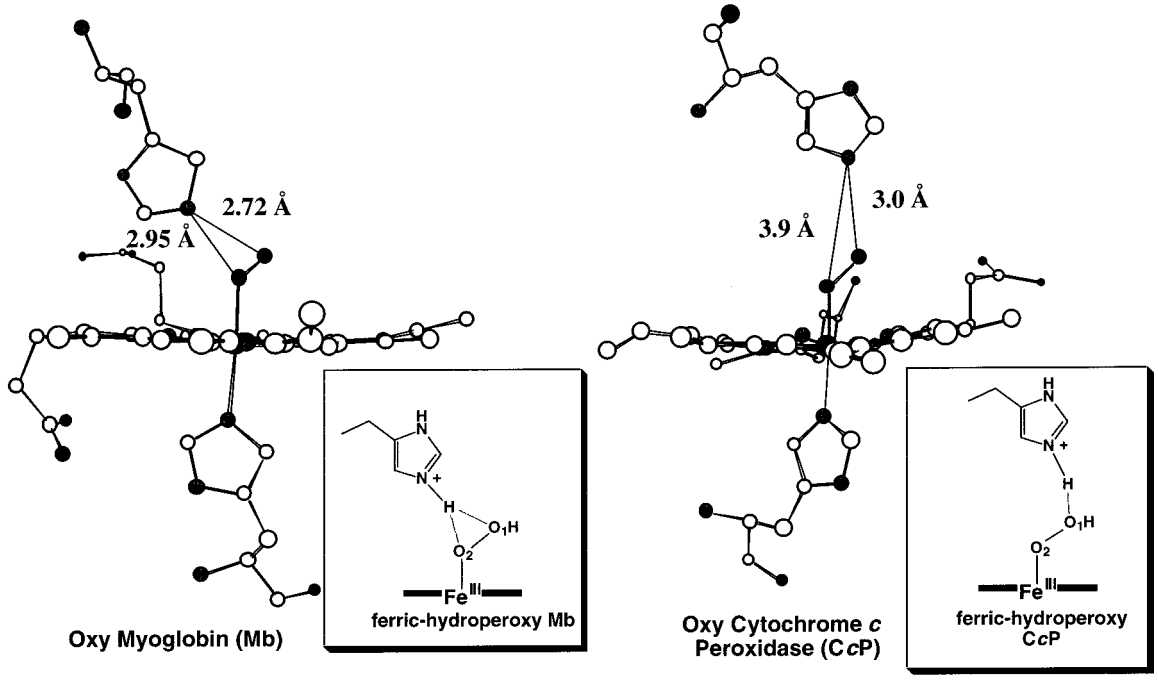


FIGURE 4. X-ray crystal structures of oxy forms of cytochrome *c* peroxidase mutant (W191F C*c*P) and myoglobin (Mb), used to postulate structures of ferric-hydroperoxy species, a precursor of compound I, as depicted in insets.

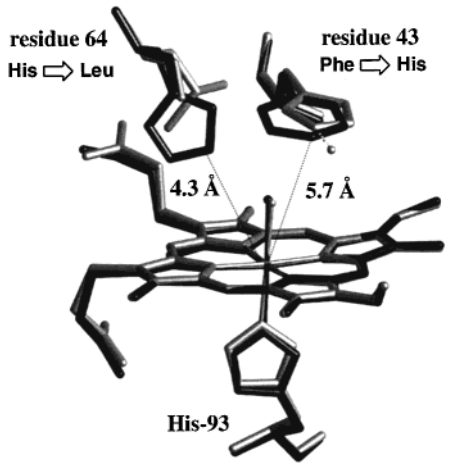


FIGURE 5. Comparison of wild-type Mb (black) and F43H/H64L Mb mutant (gray). Balls represent water molecules.

The reasons for the enhanced reactivity with H_2O_2 for the F43H/H64L mutant were further investigated using hydrogen cyanide (HCN) and cumene hydroperoxide (CHP).[39] Since the association of cyanide ion (CN^-) requires the deprotonation of HCN, the association rate constant (k_{CN}) to the ferric state would reflect the basicity of the distal histidine (Scheme 1).[42] The k_{CN} values of wild-type Mb and L29H/H64L Mb were 2.8- and 110-fold lower than that of the F43H/H64L mutant, respectively.[39] The results suggest that the distal histidine of F43H/H64L Mb functions as a general base, but His-29 in the L29H/H64L mutant cannot efficiently abstract a proton from HCN or H_2O_2.

Cumene hydroperoxide (CHP) has been used as a probe to examine the function of distal histidine as a general acid.[43] Heterolysis is known to produce compound I and cumyl alcohol (eq 1), whereas homolysis affords compound II and cumyloxy radical (eq 2). The subsequent elimination of a methyl radical from the cumyloxy radical yields acetophenone (eq 3). The ratio of heterolysis versus homolysis is determined by the yields of cumyl alcohol versus acetophenone.

$$Fe^{III}Por + PhC(CH_3)_2OOH \xrightarrow{\text{heterolysis}} Fe^{IV}{=}OPor^{+\bullet} + PhC(CH_3)_2OH \quad (1)$$

$$Fe^{III}Por + PhC(CH_3)_2OOH \xrightarrow{\text{homolysis}} Fe^{IV}{=}OPor + PhC(CH_3)_2O^{\bullet} + H^+ \quad (2)$$

$$PhC(CH_3)_2O^{\bullet} \rightarrow PhCOCH_3 + CH_3^{\bullet} \quad (3)$$

(Por = porphyrin)

The ratio of heterolysis for the L29H/H64L mutant does not significantly differ from that of the wild type. However, the F43H/H64L double mutation increases the heterolytic cleavage of the O−O bond to 85% of the total cleavage.[39] The results on the reaction with hydrogen cyanide and cumene hydroperoxide indicate that the distal histidine in F43H/H64L Mb functions as a general acid−base catalyst.

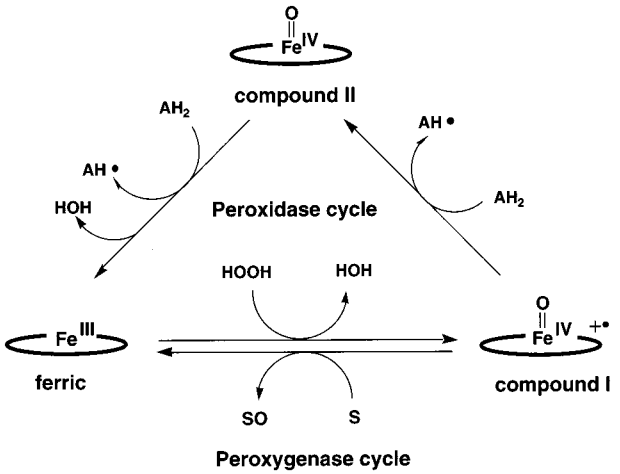
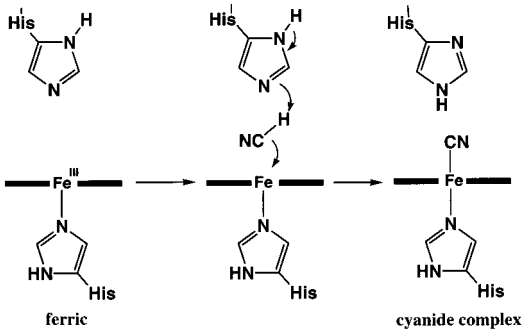


FIGURE 6. Peroxidase versus peroxygenase cycle. Circle represents heme. AH_2 and S indicate peroxidase substrates such as ABTS or guaiacol and peroxygenase substrates such as sulfide or styrene, respectively.

Scheme 1



B. The Distal Histidine Deletion Myoglobin Mutants H64L, H64A, H64S, and H64D. Prior to our investigations, compound I of wild-type Mb had never been observed due to rapid quenching of the porphyrin radical cation through the protein matrix.[44−47] The previously reported chemical modification during the treatment of Mb with H_2O_2 supports that His-64 is involved in leakage of oxidation equivalents from compound I.[48] To examine this premise, we have prepared a series of His-64 deletion mutants including H64L, H64A, H64S, and H64D Mb.[49,50] Since the absence of the distal acid−base catalyst causes the reaction with H_2O_2 to be inefficient, *meta*-chloroperbenzoic acid (*m*CPBA) was chosen as the oxidant. Compound I was successfully observed for the first time in *m*CPBA reactions of the distal histidine deletion mutants and distal histidine relocation mutants by stopped-flow experiments (Figure 7).[41,49,50] In contrast, wild-type Mb reacts with *m*-CPBA to give compound II. This proves that the deletion of His-64 considerably stabilizes compound I due to blockage of one of the putative radical leakage pathways.

The H64D mutant was constructed not only to remove a porphyrin radical leakage pathway but also to mimic the active site of chloroperoxidase (CPO). Unlike classical peroxidases, CPO has a glutamate which is the only potentially "catalytic" residue in the distal heme pocket.[27,50] It is generally believed that CPO utilizes the cysteinate

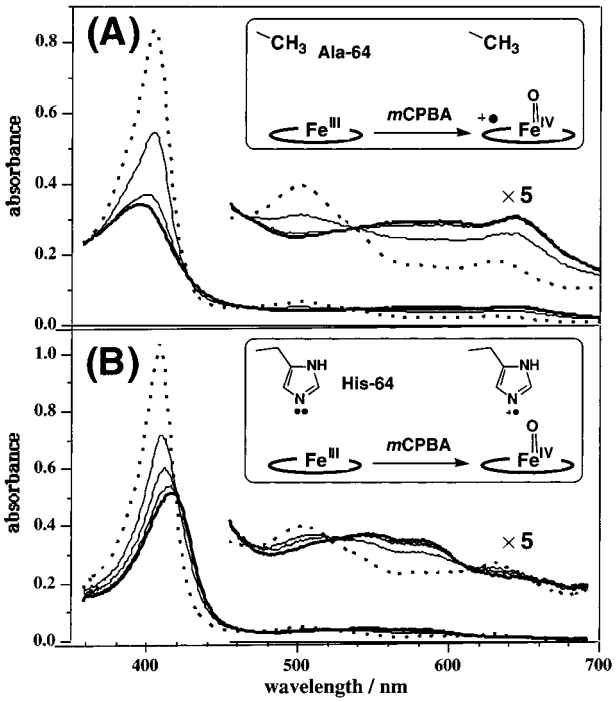


FIGURE 7. Absorption spectral changes of H64A (A) and wild-type Mb (B) upon addition of *m*CPBA. The dashed line spectrum is for the ferric state. Spectra were monitored at 16, 48, and 80 ms in (A) and 2, 4, 6, and 8 s in (B). Thick solid line spectra are for compound I (A) and compound II (B), respectively.

ligand for the heterolysis of peroxide.[29] While the cysteinate is expected to have a great push effect, the distal glutamate in CPO might also be important in the activation of H_2O_2 by CPO. Replacing His-64 in Mb with asparatate gives us an opportunity to examine potential functions of this residue in the active site.[50]

Upon addition of H_2O_2 to the ferric H64L, H64A, and H64S Mb, no evidence of formation of compound I nor II is observed. On the other hand, the reaction of H64D Mb with H_2O_2 affords approximately 40% of compound I accumulation.[50] This represents the first time that compound I of Mb could be transiently observed in reactions with H_2O_2.[50] Further kinetic analysis suggests that the H64D mutation enhances the rate of reaction with H_2O_2 by 50-fold with respect to the wild type, and the H64D replacement causes a 50−70-fold increase in peroxidation.[50] To further investigate the function of the distal aspartate, the association rate of cyanide (k_{CN}) and the cumene hydroperoxide-driven heterolysis/homolysis ratio were determined. An approximately 10-fold decrease in the cyanide association rate is observed for H64D relative to the wild type indicating that Asp-64 does not function efficiently as a general base.[50] The H64D mutant shows a slightly higher ratio of heterolysis than wild-type Mb; however, the improvement does not seem to be great enough to rationalize the 50-fold increase in the reactivity with H_2O_2.[50] At this point, the role of Asp-64 remains unclear. Possibly, the increased polarity of the distal site as a result of the H64D mutation plays a role in increasing the affinity of the distal site for H_2O_2.

Our investigations of the distal histidine deletion mutants clearly provide solid evidence that His-64 of wild-type Mb destabilizes compound I by providing a pathway for leakage of the porphyrin cation radical. Furthermore, Asp-64 appears to increase the reactivity with H_2O_2 and makes it possible to observe a partial accumulation of compound I of H64D Mb.

II. The Role of the Proximal Heme Iron Ligand

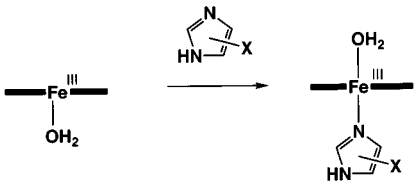
A. Investigations Based on the Replacement of Myoglobin's Proximal Histidine with Cysteine. The proximal cysteine thiolate ligand of the cytochrome P450 monooxygenases has long been thought to be crucial for efficient heterolysis of the O−O bond, the so-called push effect, prior to formation of compound I (Figure 2).[36,51] Model iron−porphyrin complexes with tethered thiolate proximal ligands have been shown to enhance heterolysis relative to the same iron−porphyrins with tethered imidazole ligands.[52,53] While it is an attractive hypothesis to explain the catalytic efficiency of the cytochrome P450s, the push effect has been notoriously difficult to confirm. There are presently no reported examples of a successful proximal ligand mutation or exchange for any of the cytochrome P450s. Fortunately, proximal ligand replacement via site-directed mutagenesis has proven much more successful for myoglobin than for cytochrome P450s. The His-93 has been successfully replaced with cysteine in horse heart,[54] human,[43,55,56] and sperm whale myoglobins.[57] The most extensive functional studies on the H93C mutants were undertaken by Watanabe, Morishima, and co-workers with human myoglobin.[43,55,56]

The absorption spectra of ferric H93C, H64G/H93C, and H64V/H93C human Mb resemble that of P450cam, which is in the thiolate-bound five-coordinate state.[43,55,56] The evidence of thiolate ligation has also been confirmed by EPR and resonance Raman studies.[43,56] However, upon reduction to the ferrous state, both in the presence and in the absence of CO, none of the H93C mutants are able to retain their thiolate ligation.[43,54,56]

The ratio of heterolysis/homolysis evaluated from the reaction with CHP (eqs 1−3) increases from 69% to 89% for the H93C single mutation, and further substitution of the distal histidine with a glycine or valine residue does not alter the ratio significantly.[56] Thus, our results have supported the hypothesis that the electron donation of the proximal ligand enhances heterolysis. Peroxygenase activities of the H93C mutants were measured using a peroxide as an oxidant.[56] The initial reaction rate of the sulfoxidation of *p*-tolyl sulfide by H93C Mb is improved by 15-fold relative to the wild type; however, the rates for H64G/H93C and H64V/H93C Mb were slightly slower than that for wild-type Mb. It appears that the hydrophobic distal heme pocket for H64G/H93C and H64V/H93C Mb decreases the affinity for H_2O_2.

B. Investigations Based on the Replacement of Myoglobin's Proximal Histidine with Exogenous Substituted Imidazoles. The myoglobin cavity mutant H93G designed by Barrick[58] has its proximal histidine ligand replaced by

Scheme 2



glycine. Removal of the histidine has the effect of creating a cavity on the proximal side that can accommodate a variety of unnatural proximal ligands. Boxer and co-workers[59] have developed and characterized this ligand-exchange system to investigate proximal ligand effects on various properties of heme proteins, such as the trans effects of binding of CO[60] and NO[30] to the distal coordination site. Dawson and co-workers have shown that reaction of H93G, having an imidazole proximal ligand (H93G(Im)), with H_2O_2 formed a stable compound II species which could be characterized by magnetic circular dichroism spectroscopy.[61]

We chose to expand upon this result and have investigated proximal ligand effects on peroxide reactivity of complexes of H93G with substituted imidazole proximal ligands (H93G(Im-X)) (Scheme 2).[57] The electron-donating capability of each substituted imidazole ligand may be assessed by the pK_a value of the N-3 nitrogen. The higher the pK_a, the greater the extent of electron localization on the N-3 nitrogen. We do not expect to observe formation of compound I in reactions of H93G(Im-X) Mb because of the presence of His-64. However, the rate of compound II formation should reflect the efficiency of the O−O bond cleavage process because the decay of compound I to II is extremely fast. The rates of formation of compound II of H93G(Im-X) were determined under various H_2O_2 concentrations, and a correlation between the apparent rate of formation of compound II and the N-3 pK_a of the substituted imidazoles was observed.[62] For each increase of 1 pK_a unit, the rate of compound II formation increases 1.7-fold. To investigate the possibility that the increase in the apparent rate of compound II formation is due to enhanced binding affinity prior to O−O bond cleavage, the observed rates of azide (N_3^-) binding were determined for the same series of H93G(Im-X). Contrary to the results obtained for the H_2O_2 reactions, a correlation between the binding rates of azide and N-3 pK_a was not observed. This is an indication that trans binding effects are not contributing significantly to the correlation observed for H_2O_2 reactivity and that O−O bond cleavage is directly affected by the electron-donating capability of the proximal heme iron ligand. However, this 1.7-fold increase in compound II formation per pK_a unit is a relatively small effect.

C. Investigations Based on the Replacement of Myoglobin's Proximal Histidine with Tyrosine, Exogenous Phenolate, and Benzoate Ligands. Although there are several examples of amino acid side chains with oxygen atoms that can act as potential heme iron ligands, the occurrence of such oxygen donor ligation in natural protein systems is quite rare. A notable exception is the tyrosinate proximal ligand in a five-coordinate heme iron structure of the catalases, which are responsible for dismutation of H_2O_2 to H_2O and O_2.[25]

H93Y Mb has been prepared for horse heart,[63] human,[43,55] and sperm whale[57] myoglobins. An extensive structural characterization of horse heart H93Y indicates unequivocally that the heme iron is in a five-coordinate tyrosinate-ligated configuration.[63] UV−vis spectra of ferric human[43,55] and sperm whale[57] H93Y myoglobins are essentially identical to those of ferric horse heart Mb H93Y, indicating that these also have the same five-coordinate tyrosinate-ligated structure as horse heart Mb H93Y. In human myoglobin, the H93Y mutation had the unexpected effect of decreasing the catalase activity by approximately 50% relative to the wild type.[43]

To expand upon the studies of H93Y myoglobin, complexes of ferric H93G with phenolate (PhO^-) and benzoate (BzO^-) as proximal ligands were studied.[64] The coordination structures of H93G(PhO^-) and H93G(BzO^-) were investigated by magnetic circular dichrosim and UV−vis spectroscopies.[64] The H93G(PhO^-) was found to have a five-coordinate heme iron structure. On the other hand, H93G(BzO^-) has a six-coordinate high-spin structure similar to that of wild-type myoglobin. Two lines of evidence indicate that, compared to the phenolate ligand, the benzoate ligand is weakly bound to the heme iron of H93G. The dissociation constant for binding of benzoate to H93G is 700 times higher than that of phenolate. Second, the benzoate ligand dissociates from H93G at alkaline pH, whereas the phenolate ligand remains bound.

H93G(BzO^-) undergoes heme destruction upon reaction with H_2O_2 at a rate similar to that of exogenous ligand-free H93G, while the heme bleaching reaction of H93G(PhO^-) with H_2O_2 is very slow. In addition, a similar oxo-ferryl intermediate is partially observed in the exogenous ligand-free H93G and H93G(BzO^-) reactions which is not observable in the H93G(PhO^-) reaction. The best explanation for the results observed for exogenous ligand-free H93G and H93G(PhO^-) is that the weakly bound benzoate ligand is quickly displaced upon reaction with H_2O_2. On the other hand, the poor reactivity of H93G(PhO^-) with H_2O_2 could be an indication that a tightly bound proximal ligand with strong electron-donating ability discourages the efficient binding and, hence, the reactivity of the complex with H_2O_2. This would also explain the poor reactivity of H93Y with H_2O_2.

Conclusions

We have demonstrated that a close examination of active site structures and reaction mechanism enables us to introduce catalytic activity by a rational design principle. The cases of rational design of a peroxidase enzyme from Mb presented here have achieved results comparable to those obtained by in vitro directed evolution.[32] The increase of the oxidation activities of F43H/H64L Mb indicates that the location of the distal histidine relative to the heme iron is important to accelerate compound I formation. The results are consistent with the hypothesis that the distal histidine in peroxidase functions as a

general acid−base catalyst to promote H_2O_2 activation. However, the reaction of F43H/H64L Mb with H_2O_2 is still slower than that of peroxidases. Further manipulations of distal side residues (e.g., the introduction of a charged residue to promote the polarization of the O−O bond or the increase of basicity by forming a hydrogen bond with the distal histidine[65,66]) may yield improvements. It has also been proven that the replacement of His-64 with an unoxidizable residue prolongs the lifetime of compound I and allows observation of a Mb ferryl porphyrin radical cation for the first time. H64D Mb can react with H_2O_2 efficiently, and compound I is transiently observed. Although the role of a negatively charged residue in the distal side remains to be confirmed, our results suggest that the distal carboxylate is important for the catalysis by chloroperoxidase.

The replacement of proximal histidine with cysteine accelerates the heterolytic O−O bond cleavage. The combination of H93G myoglobin with various substituted imidazole proximal ligands as a modular peroxide O−O bond cleavage model system has also indicated that a push effect can be discerned in the rates of formation of compound II upon reaction with H_2O_2.[62] The magnitude of the effect was observed to be quite low, however. This might indicate that the precise nature of the proximal ligand is not crucial for obtaining a high rate of peroxide O−O bond cleavage. A clear understanding of the absolute role of the proximal heme iron ligand in obtaining a clean and efficient O−O bond cleavage process and controlling the overall polarity of the heme environment remains elusive.

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