ROLES OF DISTAL RESIDUES IN CATALYTIC OXIDATION BY HEME ENZYMES

Toshitaka Matsui

DOCTOR OF PHILOSOPHY

Department of Structural Molecular Science
School of Mathematical and Physical Science
The Graduate University for Advanced Studies

1997

CONTENTS

CONTENTS

PART I

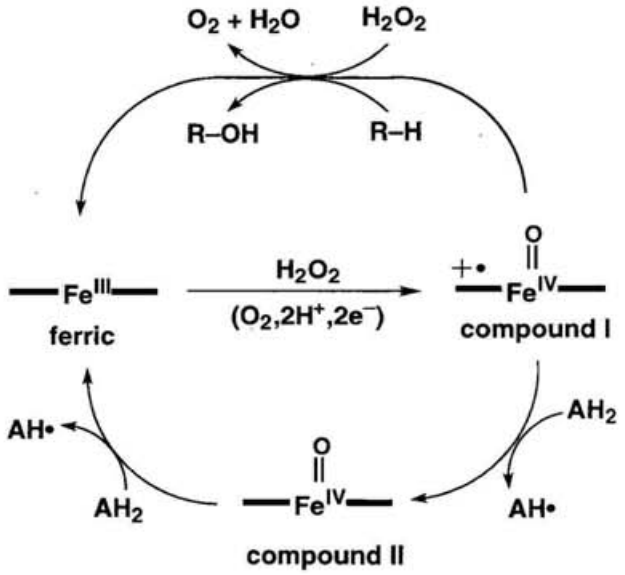
GENERAL INTRODUCTION

Hemoproteins play important roles in physiological systems and often associate with molecular oxygen. For instance, *hemoglobin* and *myoglobin*, which are located in red blood cells and muscle tissues, respectively, transport molecular oxygen. *Cytochrome c oxidase*, the last proton pump in the respiratory chain, reduces molecular oxygen to water with the use of electrons supplied by *cytochrome c*. *Cytochrome P-450* is a monooxygenase which reductively activates molecular oxygen to play essential roles in biosynthesis of sterols, metabolism of foreign compounds, and the detoxification of harmful substances. Half-reduced and toxic dioxygen, H_2O_2, is dismutated by *catalase* or further utilized by *peroxidase*. The diverse functions derived from the common heme prosthetic group should be induced, enhanced, and controlled mainly by their distinct active site structures. Many investigations have partially solved the structure-function relationships in hemoproteins; however, introduction of desired functions to hemoproteins is still difficult presumably because of the incomplete understanding.

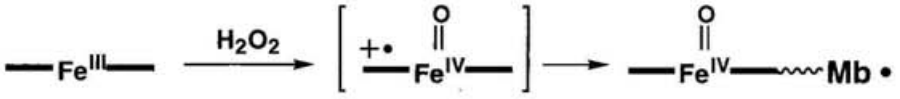
This thesis focuses on relationships between active site structures and catalytic oxidation reactions catalyzed by myoglobin (Mb), especially using H_2O_2 as an oxidant. A dioxygen carrier, Mb, has been one of the most intensively investigated hemoproteins as evident from the accumulated biochemical, biophysical, and spectroscopic data.[1,2] The heterologous overexpression system for recombinant sperm whale Mb in *Escherichia coli* has been developed,[3] and high resolution X–ray crystal structures of the wild type[4,5] as well as some Mb mutants[6] are available. In spite of the physiological function, Mb catalyzes a variety of H_2O_2-dependent oxidation reactions such as one-electron oxidation by peroxidase,[7-9] oxygenations by cytochrome P-450,[10-18] and dismutation of H_2O_2 by catalase[15,19,20], but less efficiently than these enzymes. Superposition of the active site structures of Mb and other hemoproteins enables us to utilize Mb as a heme enzyme model for the elucidation of structure–function relationships.

Oxidation reactions catalyzed by heme enzymes are normally associated with a ferryl porphyrin cation radical (Fe^{IV}=O $Por^{+\bullet}$), so called compound I.[20-22] Resting ferric peroxidase and catalase react with H_2O_2 at the rate of ~10^7 $M^{-1}s^{-1}$ to form compound I.[23-25] The peroxidase compound I is reduced to the ferric state by two

sequential one-electron oxidations via a ferryl species (Fe^{IV}=O Por), known as compound II (Scheme I).[9] The catalase compound I oxidizes another H_2O_2 molecule by two-electron to yield dioxygen and water (Scheme I).[20] Chloroperoxidase from *Caldariomyces fumago* also affords compound I in the presence of H_2O_2,[22] which shows versatile oxidation ability including one-electron oxidation,[26] H_2O_2 dismutation,[27] and oxygenation[28] in addition to chlorination, its physiological function.[29] The reactive intermediate responsible for oxygenations catalyzed by P-450 is also assumed to be compound I.[30,31] In contrast, ferric Mb is oxidized to a ferryl heme, equivalent of peroxidase compound II, paired with a transient protein radical (Scheme II).[19,32,33] Neither compound I nor its equivalents has been observed for Mb presumably due to its instability. Moreover, the reaction rate of ferric Mb with H_2O_2 is much slower (~10^2 $M^{-1}s^{-1}$) than the heme enzymes.[19] Thus, the most serious defects of Mb as an oxidation enzyme appear to be the poor reactivity with H_2O_2 and the instability of compound I.



Scheme I. General catalytic cycles of heme enzymes



Scheme II. Reactions of ferric Mb with hydrogen peroxide

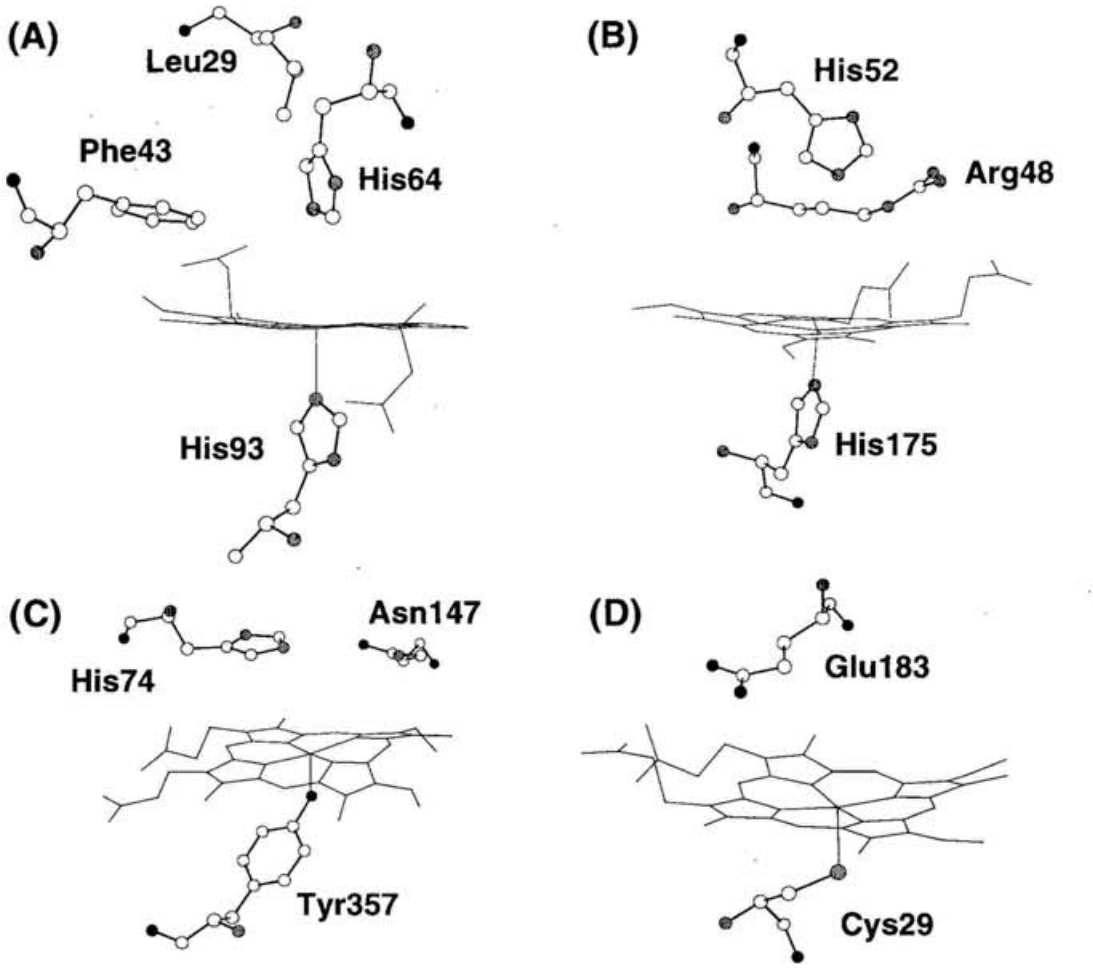
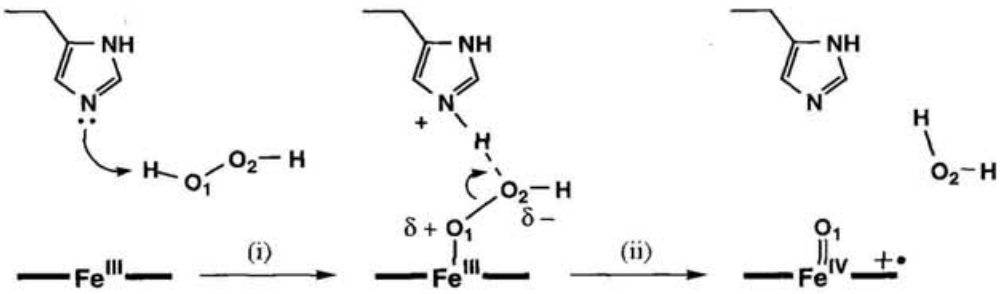


Figure 1. Active site structures of (A) sperm whale Mb, (B) cytochrome *c* peroxidase, (C) bovine liver catalase, and (D) chloroperoxidase from *Caldariomyces fumago*. Heme, axial ligand, and some selected distal residues are presented in the figure.

The critical residue for the rapid compound I formation in peroxidase and catalase has been shown to be an invariant histidine residue in the distal heme pocket (Figure 1B,C).[34-37] The distal histidines are believed to function as a general acid-base catalyst in the reaction with H_2O_2 (Scheme III);[38] i.e., the distal histidine (i) accelerates binding of H_2O_2 to the ferric heme iron by deprotonating the peroxide as a general base, and (ii) facilitates the heterolytic cleavage of the O–O bond of a plausible Fe^{III}–OOH complex by adding proton to a terminal oxygen (O_2) as a general acid. While distal histidine is also found in the active site of Mb (Figure 1A),[4,5] the distal histidine in Mb is considered to be a poor acid-base catalyst and has been suggested to be one of the initial radical site.[32,33,39]



Scheme III. Proposed formation mechanism of compound I in peroxidase and catalase

Comparison of crystal structures of sperm whale Mb and cytochrome *c* peroxidase (C*c*P) reveals that the distal histidine in Mb is more than 1 Å closer to the heme iron than that in peroxidase (Figure 1A,B).[5,40] The location of the catalytically indispensable histidine is expected to be important on the catalytic oxidation by peroxidase, especially in the formation of a plausible iron-peroxide-histidine ternary complex to enhance the heterolytic O-O bond cleavage (Scheme III). In part II, the distal histidine of Mb (Figure 1A, His64) is replaced by apolar Leu, and histidine is introduced at either Leu29 or Phe43 to elucidate the hypothesis (Figure 1A). The reactions of the resulted L29H/H64L and F43H/H64L Mb mutants with H_2O_2 are examined in chapter 1. Capability of distal histidine as a general acid-base catalyst is estimated by reactions with cyanide and cumene hydroperoxide. The effects of the location of distal histidine on one-electron oxidation activities supported by H_2O_2 are also examined in chapter 1. Chapters 2 and 3 focus on the H_2O_2-supported oxygenations (peroxygenations) of the Leu29 and Phe43 mutants, respectively. The peroxygenation mechanism is mainly investigated by tracer experiments using ^{18}O-labeled hydrogen peroxide. A ferryl porphyrin radical cation equivalent to compound I of peroxidase is observed for the double-mutated Mb in the reaction with *m*-chloroperbenzoic acid (*m*CPBA) for the first time.

Part III focuses on the novel compound I of Mb (Mb-I). In order to identify His64 (Figure 1A) as a critical residue to destabilize Mb-I, the reactions of His64 Mb mutants (H64A, H64S, and H64L) with *m*CPBA are performed in chapter 1. The reactivities of Mb-I, especially for the two-electron oxidations, have also been determined

from single-turnover kinetics by means of a double-mixing rapid-scan technique. The rapid reduction of Mb-I by H_2O_2 can rationalize the absence of Mb-I in the presence of H_2O_2, but there is no direct evidence for the formation of Mb-I when H_2O_2 is used as an oxidant. Thus, in order to establish the formation of compound I in the reaction with H_2O_2, the distal histidine of Mb is replaced by Asp to mimic the active site of chloroperoxidase for enhancing the reaction with H_2O_2 (chapter 2). Chloroperoxidase employs distinctive active site architecture from classical peroxidase (Figure 1D),[41] which is suggested to utilize distal glutamate as a general acid-base catalyst. Chapter 2 also deals with H_2O_2-supported oxidation activities catalyzed by Mb and the formation rate and stability of Mb-I.

REFERENCE

(1) Antonini, E.; Brunori, M. *Hemoglobin and Myoglobin in Their Reactions with Ligands*; North-Holland Publishing Co.: Amsterdam, 1971.

(2) Springer, B. A.; Sligar, S. G.; Olson, J. S.; Phillips, G. N., Jr. *Chem. Rev.* **1994**, *94*, 699-714.

(3) Springer, B. A.; Sligar, S. G. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 8961-8965.

(4) Takano, T. *J. Mol. Biol.* **1977**, *110*, 537-568.

(5) Phillips, G. N., Jr.; Arduini, R. M.; Springer, B. A.; Sligar, S. G. *Proteins: Struct. Funct. Genet.* **1990**, *7*, 358-365.

(6) For example, Quillin, M. L.; Arduini, R. M.; Olson, J. S.; Phillips, G. N., Jr. *J. Mol. Biol.* **1993**, *234*, 140-155.

(7) Everse, J.; Johnson, M. C.; Marini, M. A. *Methods in Enzymol.* **1994**, *231*, 547-561.

(8) Sievers, G.; Rönnberg, M. *Biochim. Biophys. Acta* **1978**, *533*, 293-301.

(9) *Peroxidases in Chemistry and Biology*; Everse, J.; Everse, K. E.; Grisham, M. B., Ed.; CRC Press: Boca Raton, 1991.

(10) Ortiz de Montellano, P. R.; Catalano, C. E. *J. Biol. Chem.* **1985**, *260*, 9265-9271.

(11) Catalano, C. E.; Ortiz de Montellano, P. R. *Biochemistry* **1987**, *26*, 8373-8380.

(12) Choe, Y. S.; Rao, S. I.; Ortiz de Montellano, P. R. *Arch. Biochem. Biophys.* **1994**, *314*, 126-131.

(13) Rao, S. I.; Wilks, A.; Ortiz de Montellano, P. R. *J. Biol. Chem.* **1993**, *268*, 803-809.

(14) Tschirret-Guth, R. A.; Ortiz de Montellano, P. R. *Arch. Biochem. Biophys.* **1996**, *335*, 93-101.

(15) Adachi, S.; Nagano, S.; Ishimori, K.; Watanabe, Y.; Morishima, I.; Egawa, T.; Kitagawa, T.; Makino, R. *Biochemistry* **1993**, *32*, 241-252.

(16) Matsui, T.; Nagano, S.; Ishimori, K.; Watanabe, Y.; Morishima, I. *Biochemistry* **1996**, *35*, 13118-13124.

(17) Levinger, D. C.; Stevenson, J.-A.; Wong, L.-L. *J. Chem. Soc., Chem. Commun.* **1995**, 2305-2306.

(18) *Cytochrome P450*; 2nd ed.; Ortiz de Montellano, P. R., Ed.; Plenum Press: New York, 1995.

(19) Yonetani, T.; Schleyer, H. *J. Biol. Chem.* **1967**, *242*, 1974-1979.

(20) Schonbaum, G. R.; Chance, B. In *The Enzymes*; 3rd ed.; P. D. Boyer, Ed.; Academic Press: New York, 1976; Vol. 13; pp 363-408.

(21) Dunford, H. B.; Stillman, J. S. *Coord. Chem. Rev.* **1987**, *19*, 187-251.

(22) Thomas, J. A.; Morris, D. R.; Hager, L. P. *J. Biol. Chem.* **1970**, *245*, 3135-3142.

(23) Loo, S.; Erman, J. E. *Biochemistry* **1975**, *14*, 3467-3470.

(24) Smith, A. T.; Sanders, S. A.; Thorneley, R. N.; Burke, J. F.; Bray, R. R. *Eur. J. Biochem.* **1992**, *207*, 507-519.

(25) Chance, B.; Greenstein, D. S.; Roughton, F. J. W. *Arch. Biochem. Biophys.* **1952**, *37*, 301-321.

(26) Lambeir, A.-M.; Dunford, H. B.; Pickard, M. A. *Eur. J. Biochem.* **1987**, *163*, 123-127.

(27) Araiso, T.; Rutter, R.; Palcic, M. M.; Hager, L. P.; Dunford, H. B. *Can. J. Biochem.* **1981**, *59*, 233-236.

(28) Casella, L.; Gullotti, M.; Ghezzi, R.; Poli, S.; Beringhelli, T.; Colonna, S.; Carrea, G. *Biochemistry* **1992**, *31*, 9451-9459.

(29) Griffin, B. W. In *Peroxidases in Chemistry and Biology*; J. Everse, K. E. Everse and M. B. Grisham, Ed.; CRC Press: Boca Raton, 1991; Vol. II; pp 85-137.

(30) White, R. E.; Coon, M. J. *Annu. Rev. Biochem.* **1980**, *49*, 315-350.

(31) Egawa, T.; Shimada, H.; Ishimura, Y. *Biochem. Biophys. Res. Commun.* **1994**, *201*, 1464-1469.

(32) King, N. K.; Winfield, M. E. *J. Biol. Chem.* **1963**, *238*, 1520-1528.

(33) King, N. K.; Looney, F. D.; Winfield, M. E. *Biochim. Biophys. Acta* **1967**, *133*, 65-82.

(34) Erman, J. E.; Vitello, L. B.; Miller, M. A.; Shaw, A.; Brown, K. A.; Kraut, J. *Biochemistry* **1993**, *32*, 9798-9806.

(35) Newmyer, S. L.; Ortiz de Montellano, P. R. *J. Biol. Chem.* **1995**, *270*, 19430-19438.

(36) Rodriguez-Lopez, J. N.; Smith, A. T.; Thorneley, R. N. F. *J. Bioinorg. Chem.* **1996**, *1*, 136-142.

(37) Obinger, C.; Maj, M.; Nicholls, P.; Loewen, P. *Arch. Biochem. Biophys.* **1997**, *342*, 58-67.

(38) Poulos, T. L.; Kraut, J. *J. Biol. Chem.* **1980**, *255*, 8199-8205.

(39) Allentoff, A. J.; Bolton, J. L.; Wilkis, A.; Thompson, J. A.; Ortiz de Montellano, P. R. *J. Am. Chem. Soc.* **1992**, *114*, 9744-9749.

(40) Poulos, T. L.; Freer, S. T.; Alden, R. A.; Edwards, S. L.; Skogland, U.; Takio, K.; Eriksson, B.; Xuong, N.-h.; Yonetani, T.; Kraut, J. *J. Biol. Chem.* **1980**, *255*, 575-580.

(41) Sundaramoorthy, M.; Terner, J.; Poulos, T. L. *Structure* **1995**, *3*, 1367-1377.

PART II

ROLES OF THE LOCATION OF DISTAL HISTIDINE IN OXIDATION REACTIONS CATALYZED BY MYOGLOBIN

CHAPTER 1.

Effects of the Location of Distal Histidine in the Reaction of Myoglobin with Hydrogen Peroxide

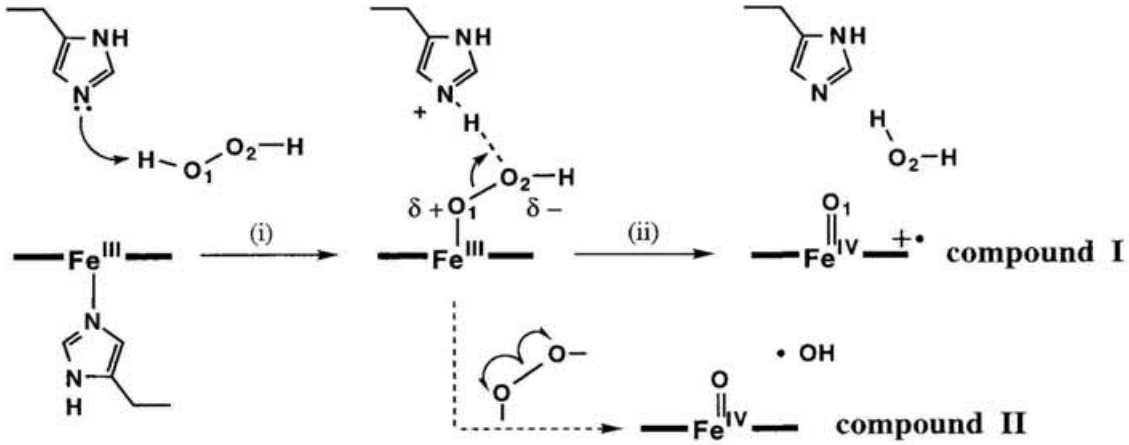
ABSTRACT: Distal histidine in peroxidase is believed to facilitate the reaction with hydrogen peroxide as a general acid-base catalyst in the formation of compound I whereas that in myoglobin (Mb) is considered to hardly help the reaction. In order to clarify the effects of the location of distal histidine especially on the reaction with H_2O_2, we have prepared L29H/H64L and F43H/H64L mutants of sperm whale Mb. While the L29H/H64L substitution was suggested to retard the reactivity of ferric Mb with H_2O_2 presumably due to too far location of His29 from the heme center, 11-fold rate increase versus wild type Mb was observed for F43H/H64L Mb. Although the formation of compound I was clearly observed for the mutants when *m*-chloroperbenzoic acid was used as an oxidant, the accumulation of compound I is not clear with H_2O_2 even in the F43H/H64L mutant because of the rapid reduction of compound I by H_2O_2. Since only the F43H/H64L mutation enhances the heterolytic O–O bond cleavage of cumene hydroperoxide, His43 has been suggested to function as a general acid. On the basis of these findings, the inability of the distal histidine in the wild type to help the heterolytic cleavage of the peroxide bond is partly attributed to its close location to the heme center.

ABBREVIATIONS

Mb	myoglobin
C*c*P	cytochrome *c* peroxidase
HRP	horseradish peroxidase
compound I	a ferryl porphyrin cation radical
compound II	a ferryl heme
Mb-I	compound I of myoglobin
Mb-II	compound II of myoglobin
*m*CPBA	*m*-chloroperbenzoic acid
CHP	cumene hydroperoxide
ABTS	2,2'-azino-*bis*(3-ethylbenzothiazoline-6-sulfonic acid)

1.1 INTRODUCTION

Peroxidase is a heme enzyme which catalyzes one-electron oxidations of a variety of substrates by utilizing H_2O_2 as an oxidant.[1,2] In the first step of a catalytic cycle of peroxidase, the resting ferric enzyme is oxidized by H_2O_2 to yield a ferryl porphyrin cation radical (Fe^{IV}=O $Por^{+\bullet}$) known as compound I.[3] Then, compound I is reduced by two sequential oxidations of substrates to the ferric states through a ferryl heme (Fe^{IV}=O Por) called compound II. An invariant histidine in the distal heme pocket[4-9] is a critical residue for the catalytic reaction of peroxidases, and its replacement by aliphatic residues retards the compound I formation by 5~6 orders of magnitude.[10-12] As shown in Scheme 1, the distal histidine is believed to (i) accelerate binding of H_2O_2 to the ferric heme iron by deprotonating the peroxide as a general base, and (ii) to facilitate the heterolytic cleavage of the O–O bond of a plausible Fe^{III}–OOH complex by adding proton to a terminal oxygen (O_2) as a general acid.[13] Heme-containing catalase, which catalyzes dismutation of H_2O_2, also employs distal histidine to enhance the formation of compound I.[14-16]



Scheme I. Proposed mechanism for the reaction of ferric heme proteins with H_2O_2

Myoglobin (Mb), a carrier of molecular oxygen, also possesses a distal histidine in the vicinity of the heme (Figure 1, His64),[17,18] and as well as peroxidase, can catalyze various oxidation reactions by using H_2O_2.[19-23] However, the reaction of ferric Mb with H_2O_2 is slower (~10^2 $M^{-1}s^{-1}$) than those of peroxidases (~10^7 $M^{-1}s^{-1}$). Furthermore,

the ferric Mb cleaves the O–O bond of the heme-bound peroxide both heterolytically and homolytically (Scheme 1), and is oxidized to a ferryl heme (Mb-II) paired with a transient protein radical.[24-26] The poor reactivity of Mb with H_2O_2 can be partly attributed to low ability of the distal histidine in Mb as a general acid-base catalyst. While the distal histidine in peroxidase is suggested to raise its basicity by a hydrogen bonding with adjacent asparagine,[27,28] the absence of such a hydrogen bonding is indicative of the less basicity of the distal histidine in Mb than that in peroxidase.[17,18] No participation of the distal histidine as a general acid catalyst for the heterolytic O–O bond cleavage has been pointed out.[29]

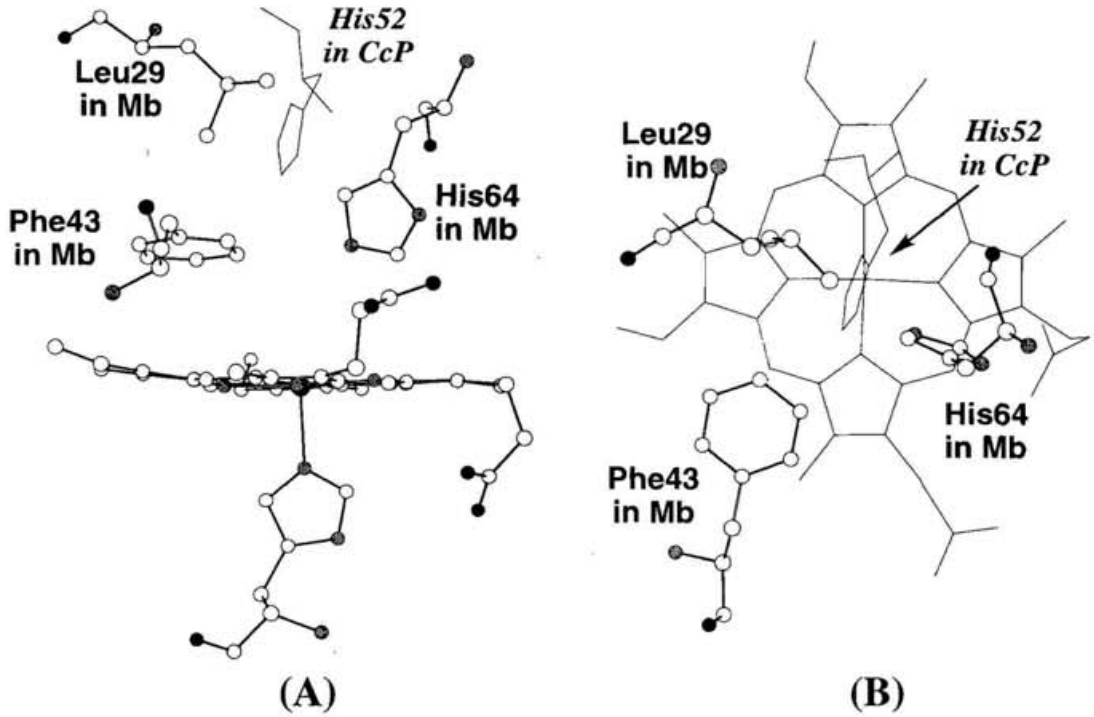


Figure 1. Superposition of the heme and some selected residues including distal histidine (His64 in Mb and His52 in C*c*P) in crystal structures of sperm whale Mb (thick line) and cytochrome *c* peroxidase (C*c*P) (thin line). Only heme in Mb is shown in this figure. (A) Side view. (B) Top view.

Comparison of crystal structures of sperm whale Mb and cytochrome *c* peroxidase (C*c*P) leads us to propose that the distal histidine in Mb is too close to the heme center for enhancing the reaction with H_2O_2 as a general acid catalyst (Figure 1).[5,17,18] The

distance between N_ε of distal histidine and ferric heme iron is 4.3 and 5.6 Å in sperm whale Mb and C*c*P, respectively. In order to clarify the effects of the location of distal histidine on the catalytic oxidations, we have prepared L29H/H64L and F43H/H64L mutants of sperm whale Mb (Figure 1).[30,31] The distance between C_ζ of Phe43 and the heme iron is 5.2 Å,[18] and thus, His43 in the F43H/H64L can be expected to be located at a similar distance from the heme iron to the distal histidine in C*c*P. A recent crystal structural analysis on the ferric L29H/H64L mutant[32] reveals the distance of His29 from the iron to be 6.6 Å, which is additionally 1.0 Å far from the heme than that in C*c*P (Figure 2). In this chapter, effects of the rearrangement of distal histidine on the reaction with H_2O_2 and one-electron oxidation activity of Mb have been examined. The F43H/H64L double mutation raises the reactivity with H_2O_2, and more importantly, His43 is suggested to facilitate the heterolytic O–O bond cleavage of the heme-bound peroxide as a general acid catalyst.

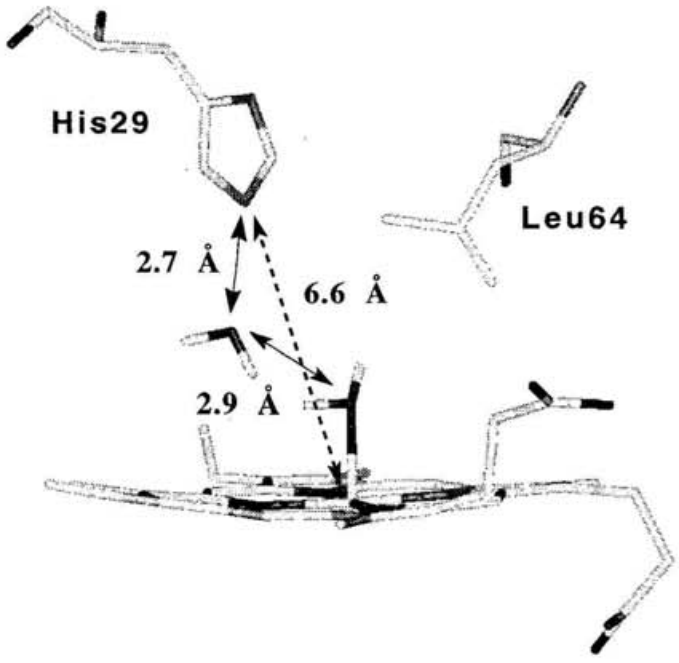


Figure 2. Active site structure of the ferric form of the L29H/H64L Mb mutant.[32]

1.2 EXPERIMENTAL PROCEDURES

Preparation of myoglobin mutants The expression vector for wild type and H64L mutant of sperm whale Mb are gifts from Olson.[33] L29H and F43H mutations, respectively with a new silent *Hin*fI and *Pvu*I site, were introduced by use of a PCR-based technique. The expression and purification of the mutants were performed according to the method described by Springer et al.[34], with some modifications. Briefly, *E. Coli* TB-1 harboring pUC19 inserted a sequence coding Mb between *Kpn* I–*Pst* I restriction sites was grown for 16 hours at 37°C in LB culture containing ampicillin (50 mg/litter). Typically, 60 g of cells was frozen at –80°C for 1 hour, thawed at room temperature, and suspended to 240 ml of 50 mM Tris•HCl (pH 8.0), 1 mM EDTA, 0.5 mM dithiothreitol. Then, 150 mg of lysozyme and 40 units of DNase I per ml were added to the solution, and left for an hour for lysis. The lysis buffer was 5-times sonicated for 30 sec on ice and the cell debris was removed by centrifugation. The blood-red supernatant was precipitated at 4°C by ammonium sulfate (60–95 % saturation), and then, dialyzed to 2 litter of 20 mM potassium phosphate buffer (pH 6.0). The crude Mb solution was loaded on a CM-52 cation-exchange column equilibrated with 20 mM potassium phosphate buffer (pH 6.0), washed with the same buffer, and eluted by a linear gradient with 50 mM potassium phosphate buffer (pH 9). The appropriate fractions were concentrated, and when necessary, ferricyanide was added for oxidizing ferrous-CO Mb to the ferric state. Then, the solution was passed through a G-25 gel filtration column equilibrated with 20 mM potassium phosphate buffer (pH 6.0), and re-loaded on the CM-52 column. This procedure resulted in a homogenous solution of Mb as monitored by SDS-PAGE and the purified Mb was stored at –80°C except for the L29H/H64L mutant (4°C).

Spectroscopy Electronic absorption spectra in 50 mM sodium phosphate buffer (pH 7.0) were recorded on a Shimadzu UV-2400 spectrophotometer and concentration of the samples was 10 μM. ^{1}H-NMR spectra in 0.1 M sodium phosphate buffer (pD 7.0) were recorded at 25°C and at 270 MHz on a JEOL EX-270 spectrometer. Chemical shifts were referenced to HDO.

Reaction with hydrogen peroxide All the reactions of ferric Mb with H_2O_2 were carried out in 50 mM sodium phosphate buffer, pH 7.0.

Formation rates of a ferryl heme in the wild type and F43H/H64L mutant (*see also RESULTS*) were determined at 20°C from the decay of absorbance at 407 nm. Bimolecular rate constants were given by the slope of a plot of the observed rates *versus* H_2O_2 concentration. The amounts of H_2O_2 were kept more than 10 molar equivalents over Mb for ensuring the pseudo-first-order condition. Whole spectral changes of rapid reactions were monitored by using a Hi-Tech SF-43 stopped-flow apparatus equipped with a MG 6000 diode array spectrophotometer.

Catalase activity of Mb was measured at 25°C from amounts of molecular oxygen formed with a Hansantech DW1 oxygen electrode. The reaction mixture contained 10 μM Mb and 1 mM H_2O_2.

Consumption rates of H_2O_2 by the L29H/H64L and H64L were determined at 20°C as follows. A reaction mixture of 10 μM Mb mutant and 50 μM H_2O_2 was incubated for 5~30 min. During the incubation, to a solution containing a trace amount of HRP (800 μl, pH 5.3) was added 200 μl of the incubated solution, and then, an excess amount of potassium iodide. The remaining H_2O_2 was quantified from the amounts of triiodide formed using absorbance changes at 353 nm ($\varepsilon_{353} = 2.62 \times 10^4$ $M^{-1}cm^{-1}$).[35] During the incubation, whole spectra of the mutants showed no considerable change including the absorbance at 353 nm.

Kinetic measurements for association of azide and cyanide The reactions of ferric L29H/H64L and F43H/H64L Mb mutants with azide or cyanide were performed at 20°C on a Hi-Tech stopped-flow apparatus in 50 mM sodium phosphate buffer, pH 7.0. The kinetic traces at 408 nm were used for determining pseudo-first-order rates. The association rate constants were given by the slope of a plot of the observed rates *versus* azide or cyanide concentration.

Reaction with cumene hydroperoxide A reaction mixture containing 10 μM Mb and 270 μM cumene hydroperoxide was incubated at 20°C in 50 mM sodium phosphate buffer, pH 7.0. Aliquots of the mixture were analyzed by a Shimadzu HPLC

system equipped with a Shimadzu CR-6A Data Module on a reverse phase column (Tosoh inertsil ODS). Phenethylalcohol was employed as an internal standard. The column was eluted with 50% water/50% methanol at a flow rate of 1.0 ml/min and the effluent was monitored at 210 nm. Assignment of the components was based on the retention time of authentic samples.

Measurements of oxidation activities One-electron oxidation activities were measured at 20°C in 50 mM sodium phosphate buffer, pH 7.0. At least two experiments were performed for each experimental point. Steady-state kinetic constants for the oxidation of guaiacol and ABTS were obtained by measuring the initial rates as varying the substrate concentration. A Hanes plot of [S]/*v versus* [S] was used to estimate the V_{max} and K_m values for the oxidations. The formation rate of the guaiacol oxidation product was determined from the increase in the absorbance at 470 nm using a molar extinction coefficient of 2.6×10^4 M^{-1} cm^{-1}.[36] The 1 ml final assay volume contained 1 μM Mb, 0.2 mM H_2O_2, and variable amounts of guaiacol (0.08–2.5 mM). The formation of ABTS cation radical was monitored at 730 nm where the absorption of Mb was negligible.[23] The absorption coefficient of the ABTS cation at 730 nm ($\varepsilon_{730} = 1.44 \times 10^4$ $M^{-1}cm^{-1}$) was calculated from that at 415 nm (3.6×10^4 $M^{-1}cm^{-1}$).[36] The reaction mixture contained 0.5 μM Mb, 0.2 mM H_2O_2, and ABTS (20–300 μM).

1.3 RESULTS

Spectroscopic Features of L29H/H64L and F43H/H64L Mb Mutants

Ferric F43H/H64L and L29H/H64L Mb mutants exhibit similar absorption spectra to that of the wild type including the Soret maxima centered around 408 nm (Figure 3), which are typical of hexa-coordinated ferric high-spin hemes.[17] The sixth ligand in the wild type is a water molecule stabilized by His64 through a hydrogen-bonding.[37] It has been shown that ferric H64L Mb loses the water ligation to cause a blue-shift of the Soret to 393 nm (Figure 3).[38] As shown in Figure 2, the L29H/H64L

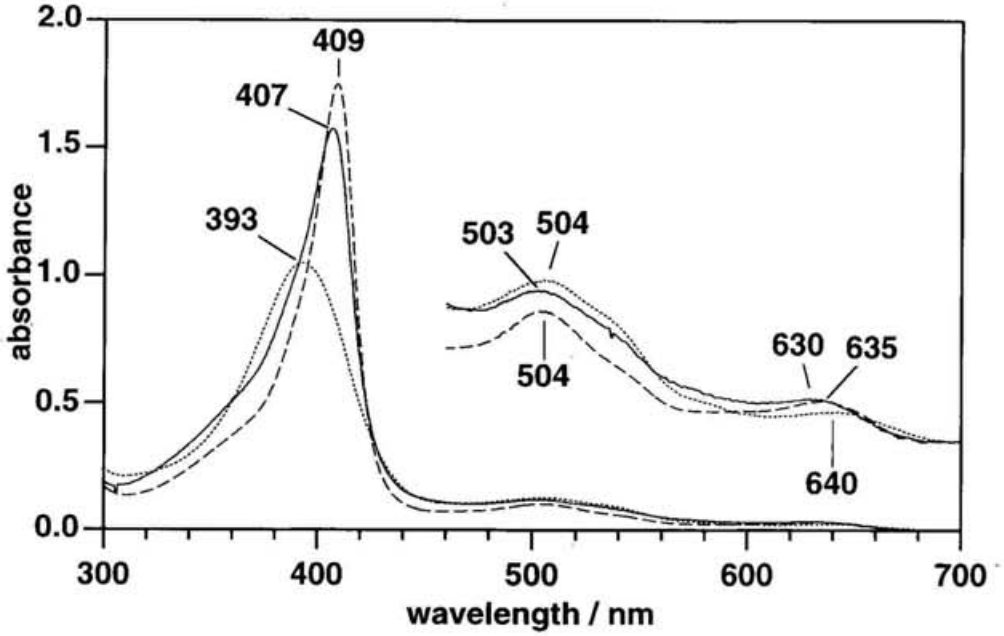


Figure 3. Absorption spectra of ferric forms of L29H/H64L (*solid line*), F43H/H64L (*broken line*), and H64L (*dotted line*) Mb mutants in 50 mM sodium phosphate buffer (pH 7.0).

Table 1. Wavelength of the absorption maxima of Mb mutants in 50 mM sodium phosphate buffer (pH 7.0)

		Soret / nm	visible / nm
L29H/H64L	ferric	409	504, 635
	ferric-CN	424	542
	ferrous	437	555
	ferrous-CO	426	545, 579
F43H/H64L	ferric	407	503, 630
	ferric-CN	421	540
	ferrous	431	558
	ferrous-CO	424	540, 575

mutant is also coordinated by a water molecule which is hydrogen-bonded with His29 through another water.[32] Although the crystal structure of the F43H/H64L mutant is not available at this point, it is likely that His43 stabilizes the heme-bound water through a direct or indirect hydrogen-bonding. The absorption maxima of ferric-CN, ferrous and

ferrous-CO forms of the L29H/H64L and F43H/H64L mutants are also summarized in Table 1 and essentially identical with the corresponding forms of the wild type and H64L mutant.

Figure 4 presents hyperfine-shifted ^{1}H-NMR spectra of ferric forms of wild type, L29H/H64L, and F43H/H64L Mb at pD 7.0. Four intense peaks in each spectrum are easily assigned to the heme methyl protons though some of them are not well resolved from other signals. While the double mutations do not greatly alter the whole spectra, the heme methyl protons of the double mutants are located at further down-field (mean methyl shift: wild type, 74.6 ppm; L29H/H64L, 77.3 ppm; F43H/H64L, 78.3 ppm). Similar shift of the heme methyl protons has been also reported for various His64 Mb mutants.[39,40] Therefore, the introduction of histidines at the positions of 29 and 43 does not appear to greatly alter electronic structures of the ferric heme.

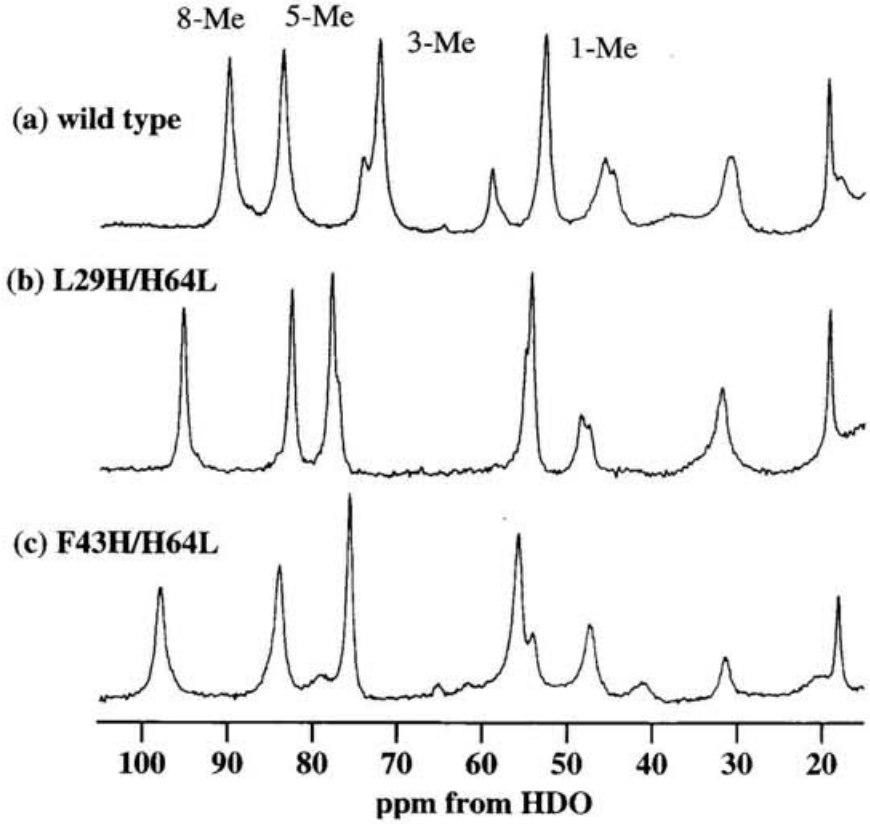


Figure 4. ^{1}H-NMR spectra of ferric forms of (a) wild type, (b) L29H/H64L, and (c) F43H/H64L Mb in 50 mM sodium phosphate buffer (pD 7.0) at 25°C.

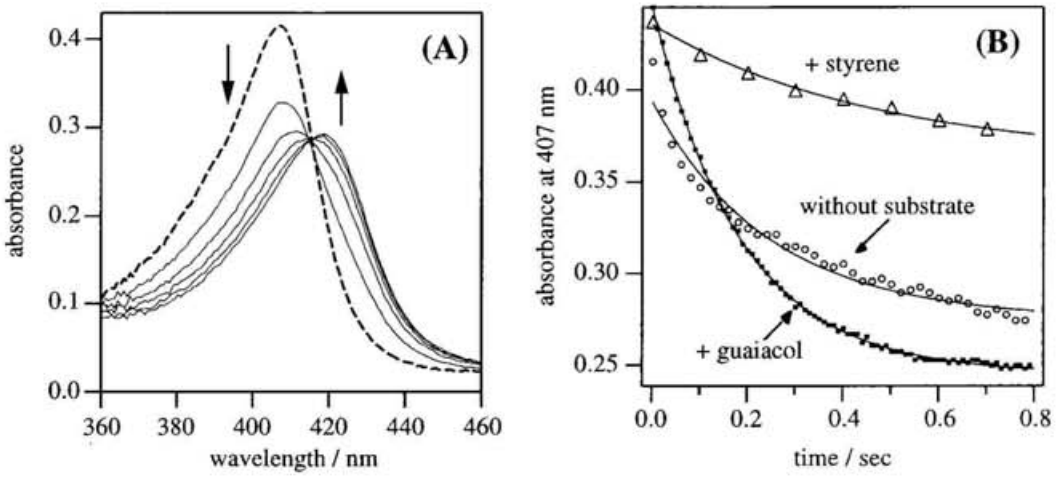


Figure 5. Spectral change of F43H/H64L Mb mutant in the reaction with 1 mM hydrogen peroxide. (A) Whole spectral change in the Soret region. The broken line represents the spectrum of the ferric Mb mutant. (B) Time-dependent absorbance change at 407 nm in the absence of substrate (E), presence of 50 μM guaiacol (•), and 0.5 mM styrene (C). The solid lines represent best-fittings of the absorbance changes to single-exponential functions.

Reaction of Ferric Myoglobin with H_2O_2

The ferric heme of wild type Mb was oxidized by H_2O_2 to a ferryl heme (Fe^{IV}=O Por) equivalent to compound II of peroxidase.[24-26] Another oxidizing equivalent has been shown to be stored as a protein radical,[24-26] rather than a porphyrin cation radical as observed for peroxidase compound I (Fe^{IV}=O $Por^{+\bullet}$).[3] The ferryl heme (Mb-II) formation in wild type Mb afforded distinct isosbestic points and obeyed pseudo-first-order kinetics. While the ferric F43H/H64L mutant also exhibited nearly isosbestic conversion to Mb-II (Figure 5A), a time-dependent decay of absorbance at 407 nm did not fit to a single exponential curvature (Figure 5B). This may be due to a slight accumulation of compound I of Mb (Mb-I) because the F43H/H64L mutant reacts with *m*-chloroperbenzoic acid (*m*CPBA) to give Mb-I.[31] When the reaction with H_2O_2 was performed in the presence of guaiacol, the F43H/H64L mutant as well as the wild type exhibited an isosbestic change to Mb-II with pseudo-first-order kinetics profiles (Figure 5B). The result is consistent with rapid reduction of Mb-I to Mb-II by guaiacol. The addition of styrene significantly retarded the Mb-II formation presumably due to the

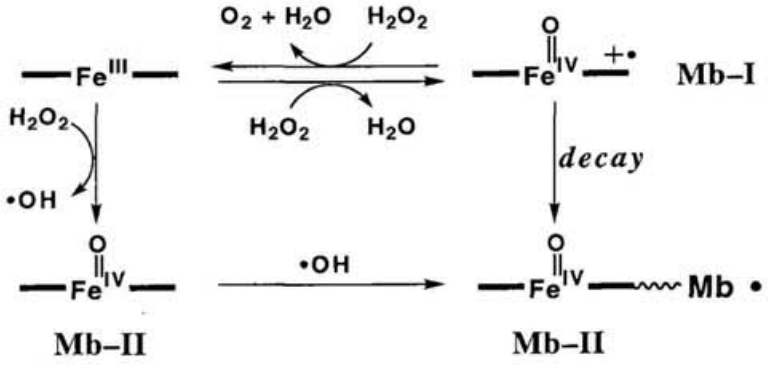
reduction of Mb-I by styrene (Figure 5B). We have shown that Mb-I was highly reactive with styrene to be reduced to the ferric state[41] while Mb-II was shown to be inactive with styrene.[42] In the case of the wild type, there is no spectral evidence of the Mb-I accumulation even in the presence of 20 mM of H_2O_2. Neither guaiacol nor styrene hardly affected the formation of wild type Mb-II.

A less accumulation of the F43H/H64L Mb-I with H_2O_2 than *m*CPBA can be mainly attributed to the rapid reduction of Mb-I by H_2O_2. We have also shown that Mb-I was immediately reduced to the ferric state by H_2O_2,[41] and dismutation activity of H_2O_2 measured based on the amount of molecular oxygen generated was 50-fold higher in the F43H/H64L mutant than the wild type (Table 2). Considering a possible direct formation of Mb-II via homolytic cleavage of the peroxide O–O bond (Scheme 1), the reaction scheme of Mb with H_2O_2 can be written as Scheme 2. The reaction rate of ferric F43H/H64L Mb with H_2O_2 was determined in the presence of a low concentration of H_2O_2 (5.0–20 μM). Under the condition, the Mb-II formation in the F43H/H64L mutant as well as the wild type obeyed pseudo-first order kinetics. It seems likely that Mb-I decays to Mb-II much faster than the regeneration of the ferric Mb through the catalase cycle at the conditions (Scheme 2). The bimolecular rate constant for the reaction of the ferric F43H/H64L with H_2O_2 was determined to be 5.6×10^3 $M^{-1}s^{-1}$, which is 11-fold higher than that of the wild type (Table 2).

Table 2. Reaction of Mb with hydrogen peroxide

	Mb-II formation [a]	O_2 evolution [b]
wild type	5.1×10^2	1.7
L29H/H64L	–	8.8
F43H/H64L	5.6×10^3	79

[a] The rate is determined at 20°C in the unit of $M^{-1}s^{-1}$. [b] The rate is determined at 25°C in the presence of 1.0 mM H_2O_2 in the unit of turnover/min^{-1}.



Scheme II. Plausible scheme for the reaction of ferric Mb with H_2O_2

On the contrary, the ferric L29H/H64L and H64L mutants showed little spectral changes upon the addition of H_2O_2 while ESR measurements revealed generation of a protein radical as reported for H64V Mb.[20] During the incubation, both L29H/H64L and H64L Mb slowly consumed H_2O_2 at the rate of 0.11 and 0.003 min^{-1}, respectively ($[H_2O_2] = 50\ \mu M$). There should be at least two pathways for the consumption: decay of Mb-I and Mb-II generated in the reaction with H_2O_2 and the oxidation of H_2O_2 by transiently formed Mb-I to produce molecular oxygen (Scheme 2, Table 2). Since most of the mutants were in the ferric states during the incubation, the consumption rates of H_2O_2 should be mainly dependent on the reactivity of the ferric Mb with H_2O_2 which can be estimated for the L29H/H64L and H64L mutants to be 3~6-fold and ~100-fold lower than that of the wild type, respectively. The results indicate that His64 in wild type Mb and His29 in L29H/H64L Mb enhance the reaction of ferric Mb with H_2O_2, and that the enhancement by the His64 is more effective than the His29.

Nevertheless, L29H/H64L Mb showed 5-fold higher catalase activity than wild type Mb (Table 2), suggesting longer life-time of Mb-I in the double mutant than that in the wild type. In fact, the L29H/H64L mutant reacted with *m*CPBA to afford Mb-I while the wild type Mb-I has never been observed. On the contrary, Mb-II of the L29H/H64L and H64L prepared by *m*CPBA decayed to the ferric states much faster than those of the wild type and F43H/H64L. Thus, the absence of Mb-II in the L29H/H64L and H64L mutants is due to its instability.

Reaction with Cumene Hydroperoxide

In order to examine the capability of the distal histidines as a general acid to facilitate heterolytic O–O bond cleavage of the heme-bound peroxide (Scheme 1), the reaction of ferric Mb with cumene hydroperoxide (CHP) was carried out. The heterolytic O–O bond cleavage of CHP is known to afford compound I (or its equivalent) and cumylalcohol (eq 1). On the other hand, homolysis of the O–O bond gives compound II and cumyloxy radical which subsequently eliminates methyl radical to afford acetophenone (eq 2,3). The general acid catalyst is expected to selectively enhance the heterolysis, which should raise the ratio of heterolysis over homolysis (cumylalcohol/acetophenone).

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

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

$$PhC(CH_3)_2O\bullet \longrightarrow PhCOCH_3 + CH_3\bullet \quad (3)$$

Table 3. Product ratio in the reaction with cumene hydroperoxide [a]

	cumylalcohol / acetophenone
wild type	3.3
H64L	3.3
L29H/H64L	2.8
F43H/H64L	5.4

[a] determined from the product ratio at 50 min.

As shown in Table 3, the cumylalcohol/acetophenone ratio was not affected by the His64→Leu substitution. The result suggests that His64 in the wild type Mb does not facilitate the heterolytic cleavage of the peroxide bond as reported earlier.[29] While Leu29→His replacement did not alter the ratio greatly, the F43H/H64L mutant showed higher cumylalcohol/acetophenone ratio than the other (Table 3). Thus, only His43 in

the F43H/H64L is able to facilitate the heterolytic O–O bond cleavage of the heme-bound CHP possibly as a general acid.

Association Rates of Cyanide and Azide

In order to evaluate the roles of the distal histidines in the binding of H_2O_2 (Scheme 1), we have examined association rates of cyanide (k_{CN}) to the ferric heme iron at pH 7 (Table 4). Most of cyanide is protonated at neutral pH (pKa ~9), and the crucial step for cyanide association has been shown to be the deprotonation of HCN in the distal heme pocket[43] as suggested for the binding of H_2O_2 (pKa 11.6). The 160-fold decrease in the k_{CN} upon the His64→Leu mutation was attributed to the loss of a general base (His64).[43] We have also measured association rates of azide (k_{N3}) to the ferric heme. In contrast to the cyanide, azide is negatively charged (pKa 4.5) at neutral pH, and the k_{N3} value was reported to largely depend on accessibility to the ferric heme center.[43] The H64L Mb showed 12-fold increase in k_{N3} versus wild type due to the more open distal heme pocket.

Table 4. Association rate constants of azide and cyanide at 20°C in 50 mM sodium phosphate buffer (pH 7.0) / $mM^{-1}s^{-1}$

	azide	cyanide	ref.
wild type	2.9	0.32	a
H64L	34	0.002	a
L29H/H64L	3.5	0.0081	this study
F43H/H64L	31	0.88	this study

[a] data taken from Brancaccio et al.[43]

Although the Phe43→His replacement on H64L Mb did not affect on the k_{N3} value, the F43H/H64L mutant showed a similar k_{CN} value to that of the wild type (Table 4). The result suggests that His43 as well as His64 facilitates the cyanide binding as a general base. On the other hand, the Leu29→His mutation on H64L Mb seems to suppress the azide binding by 10-fold, but L29H/H64L still reacted with azide anion as

fast as the wild type (Table 4). Nevertheless, the k_{CN} value of the L29H/H64L mutant was 40-fold lower than the wild type. Thus, His29 in L29H/H64L appears to be a less effective base to help the cyanide binding than His64 and His43.

Table 5. One-electron oxidations catalyzed by Mb mutants at 20°C in 50 mM sodium phosphate buffer, pH 7.0 [a]

	guaiacol		ABTS	
	V_{max}	K_m	V_{max}	K_m
wild type	0.96	570	26	77
L29H/H64L	0.27	76	3.1	11
F43H/H64L	6.2	99	150	66

[a] The units are as follows: V_{max}, nmol product / nmol Mb • min: K_m, μM.

Oxidation Activities of Wild Type Mb and Its Mutants

One-electron oxidations of guaiacol and ABTS were examined at pH 7 by using H_2O_2 as an oxidant. The initial oxidation rates of these substrates exhibited hyperbolic dependence on the concentrations of the substrates at the condition employed. Table 5 summarizes V_{max} and K_m values for the oxidations. The F43H/H64L Mb exhibited approximately 6-fold higher V_{max} values than the wild type both in the guaiacol and ABTS oxidations. In contrast, the L29H/H64L mutant gave one-fourth or less V_{max} values, and we could not find any considerable activities for the H64L at the condition employed. These changes in the V_{max} values are well correlated to those observed for the reactivities of ferric mutants with H_2O_2; i.e., 11-fold higher and 3–6-fold lower reactivities of F43H/H64L (Table 2) and L29H/H64L Mb than wild type Mb, respectively. Steady-state absorption spectra of the wild type Mb during the guaiacol oxidation (Figure 6) showed that most of the wild type existed as Mb-II in the presence of 40 μM guaiacol but as the ferric form with 4 mM guaiacol. The result reveals that the saturation of the oxidation activities is due to the transition of the rate-determining step,

and that a major rate-determining step in the presence of a large excess amount of guaiacol, where the rate is almost identical with the V_{max} values, is the reaction with H_2O_2 to form high valent hemes.

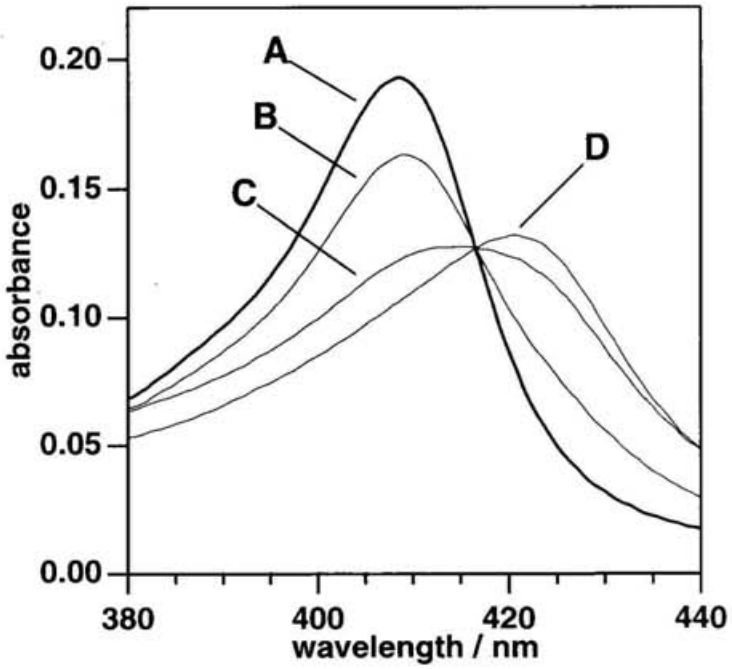


Figure 6. Steady-state absorption spectra of wild type Mb during guaiacol oxidation. (A) the spectrum of the ferric state and the steady-state spectra in the presence of (B) 4 mM, (C) 400 μM, and (D) 40 μM guaiacol.

1.4 DISCUSSION

Roles of Distal Histidine in the Reaction of Hydrogen Peroxide

We have prepared L29H/H64L and F43H/H64L mutants of sperm whale Mb in which novel distal histidines are expected to be farther from the heme iron than that (His64) in the wild type Mb (Figure 1). The spectral features of the mutants did not greatly differ from those of the wild type (Figures 3,4), suggesting similar coordination and electronic states of the heme chromophore. Especially, the crystal structure of the L29H/H64L mutant (Figure 2)[32] reveals that His29, 6.6 Å above the heme iron, is directed to the heme center and stabilizes a heme-bound water molecule by a hydrogen-

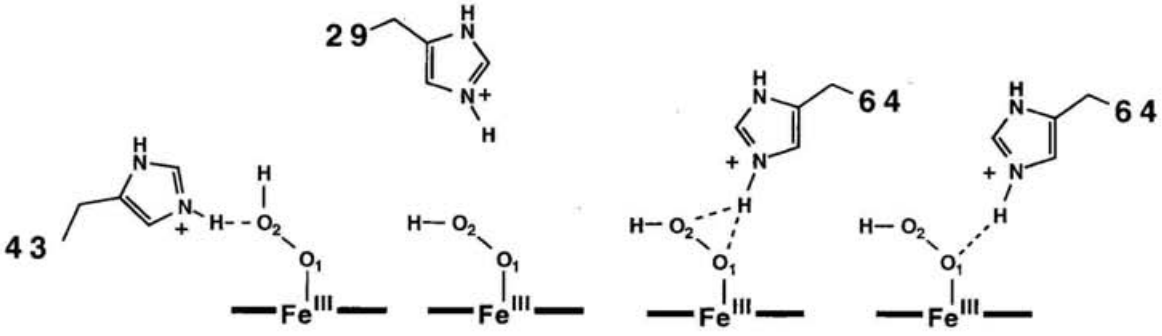
bonding through another water in the heme pocket. The ferric F43H/H64L mutant should also contain an *aquo*-ligand as well as the wild type.

On the basis of the consumption rate of H_2O_2, ferric H64L Mb was suggested to react with H_2O_2 about 100-times lower than wild type Mb. The result clearly indicates that His64 in the wild type enhances the reaction with H_2O_2. In order to estimate the capability of His64 as a general acid (Scheme 1), we have carried out the reaction with cumene hydroperoxide (CHP). The ratio of the heterolytic over homolytic O–O bond cleavage was not affected by the His64 → Leu substitution (Table 3). A similar result was reported for H64V Mb mutant using another organic peroxide.[29] Thus, His64 does not appear to enhance the reaction with H_2O_2 as a general acid catalyst. On the contrary, His64 in wild type Mb has been shown to enhance the association of cyanide to the ferric heme iron presumably by abstracting proton as a general base.[43] Therefore, the role of His64 should be the enhancement of the binding of H_2O_2 to the ferric heme but not acceleration of the O–O bond cleavage of the heme-bound peroxide (Scheme 1).

While L29H/H64L Mb consumed H_2O_2 about 40-fold faster than H64L Mb, the reactivity of the ferric L29H/H64L mutant with H_2O_2 seems still 3–6-fold lower than that of the wild type. The result indicates that His29 in the double mutant can facilitate the reaction but less efficiently than His64 in the wild type. The distance of His29 in L29H/H64L Mb from the iron is 6.6 Å, which is 1.0 Å additionally far from the heme than that in C*c*P (Figures 1,2). Superposition of ferric L29H/H64L Mb (Figure 2) and oxy-Mb (Fe^{II}–O_2),[44] a model of the ferric-peroxide complex (Fe^{III}–OOH), shows that the distance between N_ε of His29 and terminal oxygen of the heme-bound oxygen is greater than 3.9 Å. Thus, the location of His29 in L29H/H64L Mb seems too far from heme center to enhance the heterolytic O–O bond cleavage of CHP (Table 3) and to help the binding of cyanide efficiently (Table 4). In comparison with the wild type, the lower reactivity of ferric L29H/H64L Mb with H_2O_2 can be attributed to the poor ability of His29 as a general base catalyst to facilitate the binding of H_2O_2 to the heme iron.

On the other hand, F43H/H64L Mb showed 11-fold rate increase in the reaction with H_2O_2 *versus* wild type Mb (Table 2). The F43H/H64L double mutation maintains

the high reactivity of ferric Mb with cyanide and raises the heterolytic O–O bond cleavage of CHP (Tables 3,4). These results indicate that His43 serves as a general acid-base catalyst to improve the reactivity with H_2O_2 though it should be still less effective than the distal histidine in peroxidase. The capability of His43 as a general acid suggests that His64 in wild type Mb is too close to help the heterolytic cleavage of the peroxide bond. In the ferric state, His64 forms a direct hydrogen bonding with a heme-bound water.[18,37] While His64 in a ferrous-oxy Mb is shown to be hydrogen bonded only to the terminal oxygen (2.8 Å from N_ε of the His64),[45] the other oxygen bound to the iron is also in similar distance (3.0 Å).[44] As shown in Scheme 3, the distal histidine in the wild type may interact with only O_1 or both oxygen atoms of the heme-bound peroxide. In neither case, the charge separation of the transition state for the heterolysis is stabilized by the distal histidine. In addition, the interaction with O_1 may result in re-protonation of the bound peroxide or stabilize the bound peroxide.



Scheme III. Possible interaction between distal histidines and heme-bound peroxide

Effects of the Location of Distal Histidine on Their Oxidation Activities

Ferric wild type Mb is known to react with peroxides to afford a ferryl heme (Mb-II) coupled with a transient protein radical. A ferryl porphyrin cation radical (Mb-I) of the wild type has not been identified yet. We have recently succeeded in direct observation of Mb-I in the F43H/H64L mutant[31] and three His64 mutants[41] including H64L Mb when *m*CPBA was used as an oxidant. Thus, His64 in the wild type was

found to substantially destabilize Mb-I to decay to Mb-II and a protein radical. The L29H/H64L mutation also allowed us to see Mb-I in the oxidation by *m*CPBA.

When H_2O_2 was used as an oxidant, however, the F43H/H64L mutant exhibited essentially isosbestic conversion to Mb-II (Figure 5A) while the kinetic trace of the Soret absorbance and its behavior in the presence of substrates are consistent with a slight accumulation of Mb-I (Figure 5B). The L29H/H64L mutant also did not afford Mb-I with H_2O_2. These observations can be rationalized by rapid reduction of Mb-I by H_2O_2 (Scheme 2). During the incubation, both double mutants produced molecular oxygen (Table 2). While Mb-II does not react with H_2O_2, we have shown that Mb-I in H64A and H64S Mb mutants were reduced to the ferric state by H_2O_2 at the rate of ~6×10^3 $M^{-1}s^{-1}$.[41] Although needs of large excess peracid for preparing Mb-I prevent us from determining the rates in the double mutants at this point, both F43H/H64L and L29H/H64L Mb-I were also highly reactive with H_2O_2.

As compared to wild type Mb, the catalase-like activity of F43H/H64L and L29H/H64L are higher than expected from the varied rates for the formation of Mb-I by H_2O_2; i.e., L29H/H64L Mb showed 5-times higher activity than the wild type in spite of the lower reactivity of the ferric mutant with H_2O_2 (Table 2). The 46-fold increase of the F43H/H64L mutant in the catalase activity versus wild type Mb is greater than the improvement in the reaction rate of the ferric mutant with H_2O_2 (Table 2). The additional increase in the double mutants should be due to the prolonged life-time of Mb-I.[30,31] Although the formation of Mb-I using H_2O_2 is much faster with the wild type than the L29H/H64L mutant, the wild type Mb-I seems to preferentially oxidize His64 rather than the exogenous substrates.

On the contrary, the life-time of Mb-I appeared to have a subtle effect on the one-electron oxidation activities (Table 5). On the basis of the steady-state spectra (Figure 6), the major rate-determining step for the guaiacol oxidation was revealed to be the formation of high valent heme in the presence of a large excess amount of guaiacol. The V_{max} values for the guaiacol and ABTS oxidations were varied roughly along with the changes in the reactivities of the ferric Mb with H_2O_2 (Tables 2,5). This is not

surprising because Mb-I was reduced to Mb-II upon the one-electron oxidation of these substrates. Thus, the effective use of Mb-I for the one-electron oxidations should cause at most 2-fold increase in the activities. In addition, while wild type Mb reacts with H_2O_2 to form a Mb-dimer due to the coupling of the protein radical, there is no evidence of the dimerization during the guaiacol oxidation (data not shown). This result suggests that the wild type also utilizes the oxidizing equivalent efficiently for guaiacol oxidation. Therefore, the changes in the guaiacol and ABTS oxidation activities should be mainly caused by the varied reactivities of the ferric Mb with H_2O_2.

In summary, we have prepared L29H/H64L and F43H/H64L mutants of sperm whale Mb in order to clarify the effects of the location of distal histidines in the reaction with H_2O_2. The rearrangement to the position 43 is relevant to enable the distal histidine in Mb to facilitate the reaction with H_2O_2 especially as a general acid catalyst. Although the rearrangement of the distal histidine also prolonged the life-time of Mb-I, the accumulation of Mb-I was not apparent in the reactions of the double mutants with H_2O_2 due to the concomitant reduction of Mb-I by H_2O_2. Along with the varied reactivity with H_2O_2, one-electron oxidation activity in terms of V_{max} was altered upon the mutations.

1.5 REFERENCE AND NOTES

(1) *Peroxidases in Chemistry and Biology*; Everse, J.; Everse, K. E.; Grisham, M. B., Ed.; CRC Press: Boca Raton, 1991.

(2) Hewson, D. W.; Hager, L. P. In *The Porphyrins*; D. Dorphin, Ed.; Academic Press: New York, 1978; Vol. VII; pp 295-332.

(3) Dunford, H. B.; Stillman, J. S. *Coord. Chem. Rev.* **1987**, *19*, 187-251.

(4) Edwards, S. L.; Raag, R.; Wariishi, H.; Gold, M. H.; Poulos, T. L. *Proc. Natl. Acad. Sci. U. S. A.* **1993**, *90*, 750-754.

(5) Poulos, T. L.; Freer, S. T.; Alden, R. A.; Edwards, S. L.; Skogland, U.; Takio, K.; Eriksson, B.; Xuong, N.-h.; Yonetani, T.; Kraut, J. *J. Biol. Chem.* **1980**, *255*, 575-580.

(6) Patterson, W. R.; Poulos, T. L. *Biochemistry* **1995**, *34*, 4331-4341.

(7) Sundaramoorthy, M.; Kishi, K.; Gold, M. H.; Poulos, T. L. *J. Biol. Chem.* **1994**, *269*, 32759-32767.

(8) Kunishima, N.; Amada, F.; Fukuyama, K.; Kawamoto, M.; Matsunaga, T.; Matsubara, H. *FEBS Lett.* **1996**, *378*, 291-294.

(9) Finzel, B. C.; Poulos, T. L.; Kraut, J. *J. Biol. Chem.* **1984**, *259*, 13027-13036.

(10) Erman, J. E.; Vitello, L. B.; Miller, M. A.; Shaw, A.; Brown, K. A.; Kraut, J. *Biochemistry* **1993**, *32*, 9798-9806.

(11) Newmyer, S. L.; Ortiz de Montellano, P. R. *J. Biol. Chem.* **1995**, *270*, 19430-19438.

(12) Rodriguez-Lopez, J. N.; Smith, A. T.; Thorneley, R. N. F. *J. Bioinorg. Chem.* **1996**, *1*, 136-142.

(13) Poulos, T. L.; Kraut, J. *J. Biol. Chem.* **1980**, *255*, 8199-8205.

(14) Fita, I.; Rossmann, M. G. *J. Mol. Biol.* **1985**, *185*, 21-37.

(15) Fita, I.; Rossmann, M. G. *Proc. Natl. Acad. Sci. USA.* **1985**, *82*, 1604-1608.

(16) Obinger, C.; Maj, M.; Nicholls, P.; Loewen, P. *Arch. Biochem. Biophys.* **1997**, *342*, 58-67.

(17) Takano, T. *J. Mol. Biol.* **1977**, *110*, 537-568.

(18) Phillips, G. N., Jr.; Arduini, R. M.; Springer, B. A.; Sligar, S. G. *Proteins: Struct. Funct. Genet.* **1990**, *7*, 358-365.

(19) Ortiz de Montellano, P. R.; Catalano, C. E. *J. Biol. Chem.* **1985**, *260*, 9265-9271.

(20) Rao, S. I.; Wilks, A.; Ortiz de Montellano, P. R. *J. Biol. Chem.* **1993**, *268*, 803-809.

(21) Adachi, S.; Nagano, S.; Ishimori, K.; Watanabe, Y.; Morishima, I.; Egawa, T.; Kitagawa, T.; Makino, R. *Biochemistry* **1993**, *32*, 241-252.

(22) Matsui, T.; Nagano, S.; Ishimori, K.; Watanabe, Y.; Morishima, I. *Biochemistry* **1996**, *35*, 13118-13124.

(23) Everse, J.; Johnson, M. C.; Marini, M. A. *Methods in Enzymol.* **1994**, *231*, 547-561.

(24) King, N. K.; Winfield, M. E. *J. Biol. Chem.* **1963**, *238*, 1520-1528.

(25) Yonetani, T.; Schleyer, H. *J. Biol. Chem.* **1967**, *242*, 1974-1979.

(26) Fenwick, C. W.; English, A. M. *J. Am. Chem. Soc.* **1996**, *118*, 12236-12237.

(27) Nagano, S.; Tanaka, M.; Ishimori, K.; Watanabe, Y.; Morishima, I. *Biochemistry* **1996**, *35*, 14251-14258.

(28) Mukai, M.; Nagano, S.; Tanaka, M.; Ishimori, K.; Morishima, I.; Ogura, T.; Watanabe, Y.; Kitagawa, T. *J. Am. Chem. Soc.* **1997**, *119*, 1758-1766.

(29) Allentoff, A. J.; Bolton, J. L.; Wilkis, A.; Thompson, J. A.; Ortiz de Montellano, P. R. *J. Am. Chem. Soc.* **1992**, *114*, 9744-9749.

(30) Ozaki, S.; Matsui, T.; Watanabe, Y. *J. Am. Chem. Soc.* **1996**, *118*, 9784-9785.

(31) Ozaki, S.; Matsui, T.; Watanabe, Y. *J. Am. Chem. Soc.* **1997**, *119*, 6666-6667.

(32) Personal communication from Phillips, G. N., Jr. (Rice University). The R and R-free values are 16.7% and 21.6%, respectively. The RMS deviation of bond length and bond angle are 0.010 Å and 1.825 degrees, respectively. The crystal structure of L29H/H64L Mb is essentially the same with that of wild type Mb except for residues at the position 29 and 64.

(33) Springer, B. A.; Sligar, S. G. *Proc. Natl. Acad. Sci. U. S. A.* **1987**, *84*, 8961-8965.

(34) Springer, B. A.; Egeberg, K. D.; Sligar, S. G.; Rohlfs, R. J.; Mathews, A. J.; Olson, J. S. *J. Biol. Chem.* **1989**, *264*, 3057-3060.

(35) Ramette, R. W.; Sandford, R. W. J. *J. Am. Chem. Soc.* **1965**, *87*, 5001-5005.

(36) Ozaki, S.; Ortiz de Montellano, P. R. *J. Am. Chem. Soc.* **1995**, *117*, 7056-7064.

(37) Antonini, E.; Brunori, M. *Hemoglobin and Myoglobin in Their Reactions with Ligands*; North-Holland Publishing Co.: Amsterdam, 1971, pp 10-11.

(38) Quillin, M. L.; Arduini, R. M.; Olson, J. S.; Phillips, G. N., Jr. *J. Mol. Biol.* **1993**, *234*, 140-155.

(39) Rajarathnam, K.; La Mar, G. N.; Chiu, M. L.; Sligar, S. G.; Singh, J. P.; Smith, K. M. *J. Am. Chem. Soc.* **1991**, *113*, 7886-7892.

(40) Ikeda-Saito, M.; Hori, H.; Andersson, L. A.; Prince, R. C.; Pickering, I. J.; George, G. N.; Sanders, C. R.; Lutz, R. S.; McKelvey, E. J.; Mattera, R. *J. Biol. Chem.* **1992**, *267*, 22843-22852.

(41) Matsui, T.; Ozaki, S.; Watanabe, Y. *J. Biol. Chem.* **1997**, *272*, 32735-32738.

(42) Choe, Y. S.; Rao, S. I.; Ortiz de Montellano, P. R. *Arch. Biochem. Biophys.* **1994**, *314*, 126-131.

(43) Brancaccio, A.; Cutruzzola, F.; Allocatelli, C. T.; Brunori, M.; Smerdon, S. J.; Wilkinson, A. J.; Dou, Y.; Keenan, D.; Ikeda-Saito, M.; Robert E. Brantley, J.; Olson, J. S. *J. Biol. Chem.* **1994**, *269*, 13843-13853.

(44) Phillips, S. E. *J. Mol. Biol.* **1980**, *142*, 531-534.

(45) Phillips, S. E.; Schoenborn, B. P. *Nature* **1981**, *292*, 81-82.

CHAPTER 2.

Highly Stereospecific Peroxygenations by a L29H/H64L Myoglobin Mutant.*

* published in *J. Am. Chem. Soc.* **1996**, *118*, 9784-9785.

Conversion of Myoglobin into a Highly Stereospecific Peroxygenase by the L29H/H64L Mutation.

Shin-ichi Ozaki, Toshitaka Matsui, and Yoshihito Watanabe

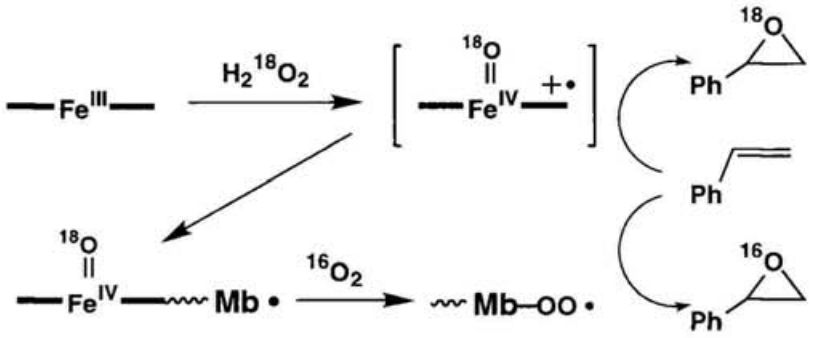
ABSTRACT: The comparison of the X-ray structures of cytochrome *c* peroxidase (C*c*P) and sperm whale myoglobin (Mb) suggests that the Leu29 → His and His64 → Leu double mutation of Mb would create a heme crevice similar to the active site structure of C*c*P, and enhance hydrogen peroxide supported peroxygenation activity. We report here that L29H/H64L Mb significantly increases the rate for the oxidation of both thioanisole and styrene, and more importantly the enantioselectivity. The 22-fold rate increase *versus* wild type Mb and 97 % incorporation of ^{18}O from $H_2{}^{18}O_2$ into the sulfoxide with 97 % ee for *R* isomer have been observed for thioanisole oxidation by L29H/H64L Mb. Regardless of the size and electronic properties for *para*-substituents of thioanisole, the L29H/H64L mutant oxidizes sulfides faster than the wild type with higher enantioselectivity. A great improvement of enantioselectivity from 9 to 80 % is seen for styrene epoxidation by L29H/H64L Mb, and 94 % of oxygen atom of epoxide formed is derived from peroxide. The removal of His64 improves the efficiency of the ferryl oxygen transfer to styrene. The roles of His29 in the double mutant can be considered to compensate the reactivity with hydrogen peroxide upon the loss of His64 and to regulate the binding mode of the substrates.

ABBREVIATIONS

Mb	myoglobin
C*c*P	cytochrome *c* peroxidase
HRP	horseradish peroxidase
CPO	chloroperoxidase from *Caldariomyces fumago*
$H_2{}^{18}O_2$	^{18}O-labeled hydrogen peroxide
ee	enantiomer excess

2.1 INTRODUCTION

Myoglobin (Mb), normally a carrier for molecular oxygen, can catalyze hydrogen peroxide supported oxygenation (peroxygenation) of a variety of substrates, including olefin epoxidation and thioether sulfoxidation.[1] However, the turnover numbers for sulfoxidation by Mb are less than the values obtained by the incubation with peroxidases.[2] In contrast to the oxidation of alkene mediated by cytochrome *c* peroxidase (C*c*P), horseradish peroxidase (HRP) mutants, and chloroperoxidase (CPO) from the fungus *Caldariomyces fumago*, olefin epoxidation by Mb results in low yield incorporation of peroxide oxygen due to the competitive molecular oxygen incorporation (Scheme I).[2,3] Catalysis by Mb is presumably associated at least partly with an intermediate equivalent to compound I of peroxidase; however, the exact location of one of the two oxidation equivalents has not clearly been defined yet.[4]



Scheme I. Ferryl oxygen transfer *versus* co-oxidation mechanisms

In order to identify active site residues controlling ferryl oxygen transfer reactions, we have performed site directed mutagenesis studies of sperm whale myoglobin. Previous studies indicate that the replacement of His64, 4.3 Å above the heme iron,[5] by unoxidizable amino acids such as a valine prevents protein-peroxy radical mediated epoxidation,[3b] but the formation of ferryl species with hydrogen peroxide appears to be slower than that for wild type Mb. The comparison of the X-ray structures of C*c*P[6] and Mb[5] suggests that the Leu29 → His and His64 → Leu double mutation of Mb would create a heme crevice similar to the active site structure of C*c*P, of which the distal histidine lies 5.6 Å above the heme iron, and we expect the L29H/H64L mutant would

transfer the ferryl oxygen to the substrates efficiently (Figure 1). Thus, we have constructed L29H, H64L, and L29H/H64L mutants and found that L29H/H64L Mb significantly increases the rate for the oxidation of both thioanisole and styrene, and more importantly the enantioselectivity. In addition, the oxygen atom of epoxide formed by the incubation with the L29H/H64L mutant is mostly derived from peroxide.

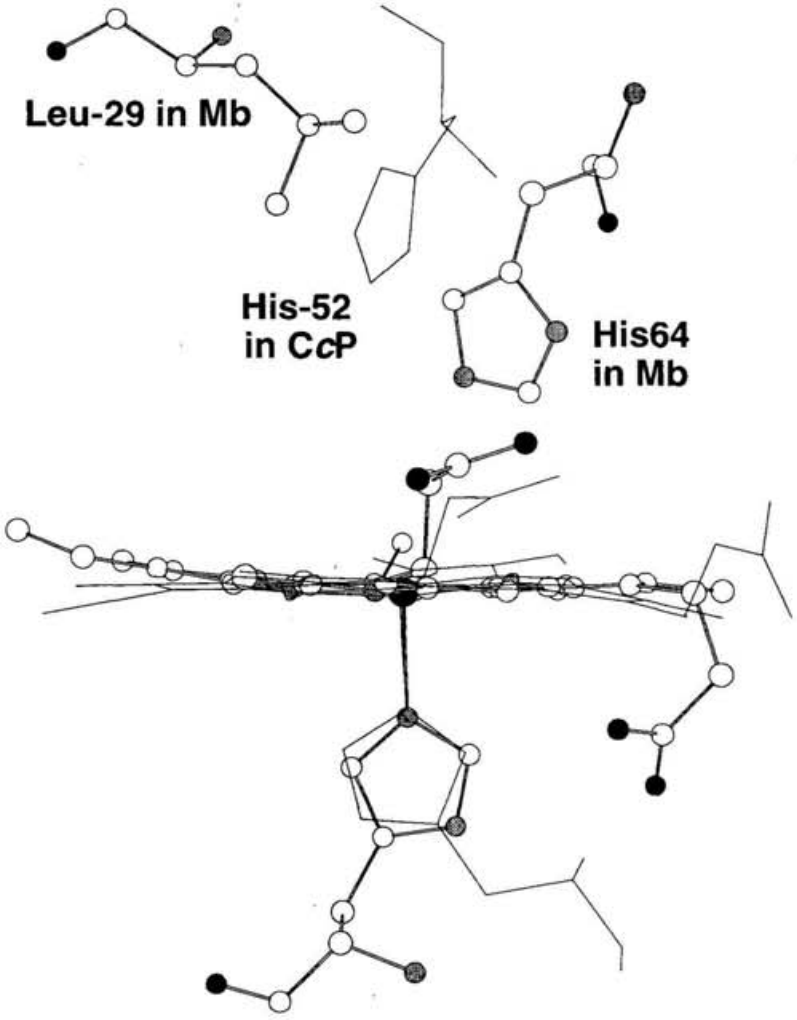


Figure 1. Superposition of the heme and some selected residues including proximal histidine (His175 in C*c*P, and His93 in Mb) in the crystal structures of cytochrome *c* peroxidase (C*c*P) (thin line) and myoglobin (Mb) (thick line). The distance between N_{ε} of distal histidine and iron is 5.6 Å and 4.3 Å in C*c*P and Mb, respectively.

2.2 EXPERIMENTAL PROCEDURE

Construction of mutant genes, expression and purification of Mb mutants were described in chapter 1 of this part.

Assay of Thioanisole Sulfoxidation 1 mM H_2O_2 was added to a solution of either 5 μM Mb or the Mb mutants and 1 mM thioether in 0.5 ml of 50 mM sodium phosphate buffer (pH 7.0) at 25°C. Acetophenone was added as an internal standard, and the mixture was extracted with dichloromethane for HPLC analysis on a Daicel OD chiral-sensitive column installed on a Shimadzu SPD-10A spectrophotometer equipped with a Shimadzu LC-10AD pump system.[2] Standard curves prepared with synthetic authentic sulfoxides were used for quantitative analysis, and the absolute stereochemistry was determined based on a retention time. In all cases, the dominant isomer was *R*. A linear relationship between time *versus* product formation was observed at least 15 minutes. The sulfoxide formed in control incubations without enzyme was subtracted when necessary. For the determination of K_m and V_{max}, the amounts of thioanisole varied.

Assay of Styrene Epoxidation The wild type or mutants (10 μM) in 0.5 ml of 50 mM sodium phosphate buffer (pH 7.0) was incubated with 0.5 μl of neat styrene and 1 mM H_2O_2 at 25°C. 2-Phenyl-2-propanol was added as an internal standard, and the dichloromethane extracts were analyzed by GC (Shimadzu GC-14B) equipped with a Chiraldex G-TA capillary column. The standard curve was prepared for quantitative analysis, and the absolute stereochemistry was determined based on a retention time of the authentic 1*S* or 1*R* epoxide. The rates were determined from the linear portion of the product *versus* time plot. For the determination of K_m and V_{max}, the amounts of styrene varied.

Determination of Oxygen Source Similar incubations were performed at 25°C with $H_2{}^{18}O_2$ in 50 mM sodium phosphate buffer (pH 7.0) pretreated with Chelex-100 (Bio-Rad). $H_2{}^{18}O_2$ was prepared from $^{18}O_2$ as described by Sawaki and Foote.[7] The ^{18}O-content of the peroxide was determined to be 92 % by alkaline epoxidation of menadione.[1a] The dichloromethane extracts of the incubation mixtures were analyzed by

GC/MS (Shimadzu GC-17A/GCMS-QP5000) equipped with a Shimadzu CBP1 capillary column.

Reaction with Phenylhydrazine Reaction of wild type and L29H/H64L Mb with phenylhydrazine was carried out as described by Kunze et al.[8] with monitoring spectral changes on a Shimadzu UV-2400 spectrophotometer. The UV spectra of the wild type and mutant were identical, and the spectrum of σ-bonded phenyl-iron complex for the wild type agreed with that previously reported.

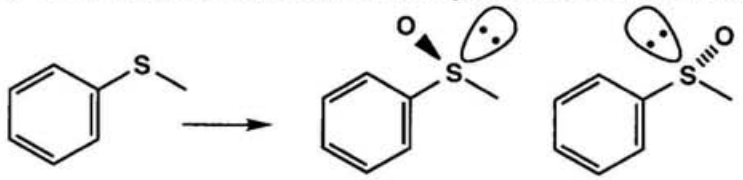
2.3 RESULTS AND DISCUSSION

Mutating Leu29 to histidine improves the rate and enantioselectivity for the oxidation of thioanisole (Table 1). The rate increases versus wild type Mb are 15-fold and 22-fold for L29H and L29H/H64L Mb, respectively. On the contrary, the elimination of His64 in the distal pocket causes about 70 % decrease in the oxidation rate with respect to the recombinant wild type. The L29H/H64L double mutation enhances the enantiomeric excess from 25 % to 97 %. The dominant formation of *R* by the mutation is in contrast with 97 % ee for the *S* enantiomer given by F41L HRP.[2] In order to compare the efficiency as a sulfoxidation catalyst, we have determined the K_m and V_{max} values. The kinetic constants for thioanisole oxidation by wild type and L29H/H64L Mb are K_m = 0.36 mM V_{max} = 0.52 turnover/min and K_m = 0.17 mM V_{max} = 9.5 turnover/min, respectively. The V_{max} of L29H/H64L Mb is half of that for F41L HRP, but approximately 3-fold higher than the previously reported V_{max} for native HRP.[2]

The extremely high stereoselectivity with 97 % incorporation of ^{18}O from $H_2{}^{18}O_2$ into the sulfoxide for L29H/H64L Mb clearly indicates the ferryl oxygen transfer to thioanisole (Scheme I) and rules out the involvement of molecular oxygen and hydroxyl radical.[9] Although the enantioselectivity is low for wild type and H64L Mb, $H_2{}^{18}O_2$-labeling experiments resulted in approximately 90 % incorporation of the labeled oxygen

into the sulfoxide. As shown in chapter 1 of this part, ferric L29H/H64L Mb is less reactive with H_2O_2 to yield reactive intermediates than wild type Mb. Thus, the enhancement of H_2O_2-supported peroxygenation activity by the L29H/H64L mutant *versus* wild type Mb is not due to the rate increase in the formation of a ferryl radical cation species (Fe^{IV}=O $Por^{+\bullet}$), equivalent to compound I of peroxidase.

Table 1. Thioanisole sulfoxidation activity of Mb and its mutants



S ***R***

	ee (%) [a]	rate (min^{-1})	^{18}O incorporation from $H_2{}^{18}O_2$ (%)
wild type	25	0.25	92
H64L	27	0.072	89
L29H	91	3.9	100
L29H/H64L	97	5.5	97

[a] The absolute stereochemistry of the dominant isomer is *R*.

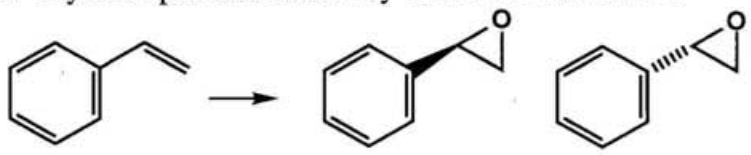
Regardless of the size and electronic properties for *para*-substituents of thioanisole, the L29H/H64L mutant oxidizes sulfides faster than the wild type with higher enantioselectivity (Table 2). However, in the case of sulfoxidation by L29H/H64L Mb, the methoxy group at the *para* position drops the rate and enantiomeric specificity with respect to thioanisole. The L29H/H64L mutant oxidizes benzyl methyl sulfide and ethyl phenyl sulfide at the rate comparable with that for thioanisole. The size of alkyl group and the distance between aromatic group and sulfur atom do not seem to be critical for enantioselective sulfoxidation by the L29H/H64L mutant.

Table 2. Oxidation of thioether, R--S–R'

		wild type		L29H/H64L	
R	R'	ee (%) [a]	rate [b]	ee (%) [a]	rate [b]
p-chlorophenyl	methyl	13	0.19	87	3.5
phenyl	methyl	25	0.25	97	5.5
p-methylphenyl	methyl	11	0.54	87	10
p-methoxyphenyl	methyl	2.8	1.1	45	1.7
benzyl	methyl	13	0.66	86	4.0
phenyl	ethyl	7.6	0.46	95	6.5

[a] The absolute stereochemistry of the dominant isomer is *R*. [b] The unit for rate is turnover/min.

Table 3. Styrene epoxidation activity of Mb and its mutants



1*R* **1*S***

	ee (%) [a]	rate (min^{-1})	^{18}O incorporation from $H_2{}^{18}O_2$ (%)
wild type	9	0.015	20
H64L	34	0.020	73
L29H	2 (*S*)	0.093	53
L29H/H64L	80	0.14	94

[a] The absolute stereochemistry of the dominant isomer is *R* unless noted.

Styrene epoxidation by H64L Mb was found to proceed at a similar rate for wild type Mb (Table 3). In comparison with wild type Mb, the L29H mutant oxidizes six-times faster, and 9-fold enhancement with an improvement of enantioselectivity from 9 to 80 % is seen for L29H/H64L Mb. Interestingly, preferred formation of the *R* configurations for methyl phenyl sulfoxide and styrene oxide requires the opposite

orientation of the phenyl group and the side chain with respect to ferryl oxygen; however, the structural information on the transition state can not be deduced at this point. The kinetic constants for styrene oxidation by wild type and L29H/H64L Mb were found to be K_m = 6 mM V_{max} = 0.031 turnover/min and K_m = 9 mM V_{max} = 0.74 turnover/min, respectively. The V_{max} of styrene oxidation by the L29H/H64L mutant is 2 order of magnitude greater than the previously reported values for F41L and F41T HRP.[2]

The oxidation of styrene in the presence of $H_2{}^{18}O_2$ by wild type Mb produces epoxide with 20 % ^{18}O-labeled oxygen (Table 3). The result is consistent with the competition of at least two mechanisms for epoxidation by wild type Mb (Scheme I): one that incorporates an oxygen atom from hydrogen peroxide employed and another incorporates an atom of molecular oxygen.[1b,3b] On the contrary, the incubation of styrene and $H_2{}^{18}O_2$ with L29H/H64L resulted in incorporation of 94 % of ^{18}O in the epoxides, suggesting the dominant ferryl oxygen transfer for the epoxidation by the double mutant (Scheme I). The wild type as well as the double mutant seems to have an active site large enough for the substrate to access because wild type and L29H/H64L Mb can form phenyl-iron complex in the presence of phenylhydrazine.[8]

The H64L and L29H single mutants also showed greater ratios for the incorporation of a peroxide oxygen in the epoxide than the wild type (Table 3). Upon the His64 → Leu replacement, the rate of ferryl oxygen transfer (activity × %^{18}O ÷ 100) increases from 0.003 min^{-1} in wild type Mb to 0.015 min^{-1} in H64L Mb while the co-oxidation rate (total rate minus ferryl oxygen transfer rate) decreases from 0.012 to 0.005 min^{-1} (Table 3). The oxidation equivalents of the heme could be easily given to His64, which lies closer to the heme iron than His29 in L29H/H64L Mb (Figure 1), as well as to the substrate.[10,11] Thus, the removal of His64 should improve the efficiency of the ferryl oxygen transfer to styrene. In contrast, L29H Mb enhances both the ferryl oxygen transfer and co-oxidation (0.049 and 0.044 min^{-1}, respectively) than wild type Mb (Table 3). The L29H/H64L mutant as well as the H64L mutant selectively facilitates the ferryl oxygen transfer but not the co-oxidation (0.13 and 0.008 min^{-1}, respectively). Therefore, the histidine residues at the position 29 in L29H and L29H/H64L Mb appear

to enhance the epoxidation in different ways. Although the mechanism for the rate enhancement by the L29H single mutant is unclear, this is not due to the high reactivity of the L29H mutant with hydrogen peroxide.[12]

In summary, L29H/H64L Mb significantly increases the rate for the oxidation of both thioanisole and styrene, and more importantly the enantioselectivity. Moreover, the oxygen in the products incorporated by L29H/H64L is mostly derived from peroxide. These observations can be partly attributed to the replacement of His64 by Leu; however, His29 in the double mutant must play an important role in improvement both in the rates and enantioselectivity. Since we have recently clarified that the step of the reaction with hydrogen peroxide is involved in the rate-determining step of the peroxygenations,[13] the roles of His29 in the double mutant can be considered to compensate the reactivity with hydrogen peroxide upon the loss of His64 and regulation of the binding mode of the substrates.

2.4 REFERENCE AND NOTES

(1) (a) Ortiz de Montellano, P. R.; Catalano, C. E. *J. Biol. Chem.* **1985,** *260,* 9265-9271.

(b) Catalano, C. E.; Ortiz de Montellano, P. R. *Biochemistry,* **1987,** *26,* 8373-8380.

(c) Adachi, S.; Nagano, S.; Ishimori, K.; Watanabe, Y.; Morishima, I. *Biochem. Biophys. Res. Commun.* **1991,** *180,* 138-144.

(d) Alvarez, J. C.; Ortiz de Montellano, P. R. *Biochemistry,* **1992,** *31,* 8315-8322.

(e) Adachi, S.; Nagano, S.; Ishimori, K.; Watanabe. Y.; Morishima, I.; Egawa, T.; Kitagawa, T.; Makino, R. *Biochemistry,* **1993,** *32,* 241-252.

(f) Matsui, T.; Nagano, S.; Ishimori, K.; Watanabe, Y.; Morishima, I. *Biochemistry* **1996,** *35,* 13118-13124.

(2) Ozaki, S.; Ortiz de Montellano, P. R. *J. Am. Chem. Soc.* **1995,** *117,* 7056-7064.

(3) (a) Miller, V. P.; DePhillis, G. D.; Ferrer, J. C.; Mauk, A. G.; Ortiz de Montellano, P. R. *J. Biol. Chem.* **1992,** *267,* 8936-8942.

(b) Rao, S. I.; Wilks, A. ; Ortiz de Montellano, P. R. *J. Biol. Chem.* **1993,** *268,* 803-809.

(c) Allain, E. J.; Hager, L. P.; Deng, L.; Jacobsen, E. N. *J. Am. Chem. Soc.* **1993,** *115,* 4415-4416.

(d) Collonna, S.; Gagerro, N.; Casella, L.; Carrea, G.; Pasta, P. *Tetrahedron: Asymmetry* **1993**, *4*, 1325-1330.

(e) Zaks, A.; Dodds, D. R. *J. Am. Chem. Soc.* **1995,** *117,* 10419-10424.

(4) (a) King, N. K.; Winfield, M. E. *J. Biol. Chem.* **1963,** *238,* 1520-1528.

(b) Yonetani, T.; Schleyer, H. *J. Biol. Chem.* **1967,** *242,* 1974-1979.

(c) Wilks, A. ; Ortiz de Montellano, P. R. *J. Biol. Chem.* **1992,** *267,* 8827-8833.

(5) Phillips, G. N., Jr.; Arduini, R. M.; Springer, B. A.; Sligar, S. G. *Proteins: Struct. Funct. Genet.* **1990**, *7*, 358-365.

(6) Poulos, T.; Freer, S. T.; Alden, R. A.; Edwards, S. L.; Skogland, U.; Tokio, K.; Eriksson, B.; Xuong, N.; Yonetani, T.; Kraut, J. *J. Biol. Chem.* **1980,** *255,* 575-580.

(7) Sawaki, Y.; Foote, C. S. *J. Am. Chem. Soc.* **1979,** *101,* 6292-6296.

(8) Kunze, K. L.; Ortiz de Montellano, P. R. *J. Am. Chem. Soc.* **1983**, *105*, 1380-1381.

(9) Gilbert, B.; Hodgeman, K. C.; Norman, R. O. C. *J. C. S. Perkin II* **1973**, 1748-1752.

(10) (a) King, N. K.; Winfield, M. E. *J. Biol. Chem.* **1963**, *238*, 1520-1528.

(b) Fox, J. B.; Nicholas, R. A.; Ackerman, S. A.; Swift, C. E. *Biochemistry* **1974,** *13,* 5178-5186.

(11) As shown in part III in this thesis, His64 in wild type Mb plays a crucial role in destabilizing a ferryl porphyrin cation radical of Mb (Mb-I). The L29H/H64L mutant reacted with *m*-chloroperbenzoic acid to afford Mb-I.

(12) The guaiacol oxidation activity of L29H Mb (0.48 min^{-1}) is slightly higher than that of L29H/H64L Mb (0.27 min^{-1}) but half of that of the wild type (0.96 min^{-1}) in the presence of 0.2 mM H_2O_2 and 1.6 mM guaiacol at pH 7.0 and 20°C. As described in chapter 1 in part II and chapter 2 in part III, the one-electron oxidation activity should be mainly dependent on the reactivity of ferric Mb with H_2O_2.

(13) The peroxygenation activities of His64 Mb mutants are well-correlated with the reactivity with H_2O_2 based on one-electron oxidation activities of the mutants.

CHAPTER 3.

Conversion of Myoglobin into a Peroxygenase: A Catalytic Intermediate of Sulfoxidation and Epoxidation by the F43H/H64L Mutant*

* published in *J. Am. Chem. Soc.* **1997**, *119*, 6666-6667.

Conversion of Myoglobin into a Peroxygenase: a Catalytic Intermediate of Sulfoxidation and Epoxidation by the F43H/H64L Mutant.

Shin-ichi Ozaki, Toshitaka Matsui, and Yoshihito Watanabe

ABSTRACT: Comparison of the X-ray crystal structures of sperm whale myoglobin (Mb) and cytochrome *c* peroxidase (C*c*P) allows us to design Mb mutants to mimic the active site of peroxidase. We have found that F43H/H64L Mb oxidizes thioanisole and styrene 200- and 300-fold faster than the wild type, respectively. The peroxygenase activity for the mutant is better than those for L29H/H64L Mb and horseradish peroxidase (HRP). More intriguingly, a ferryl radical cation like species of the Mb mutant has been identified as the catalytic intermediate for the first time. The results clearly indicate that the alignment of the distal histidine is important for the reactivity as well as the direct observation of the transient catalytic species for Mb.

ABBREVIATIONS

Mb	myoglobin
C*c*P	cytochrome *c* peroxidase
HRP	horseradish peroxidase
*m*CPBA	*m*-chloroperbenzoic acid
compound I	a ferryl porphyrin cation radical
compound II	a ferryl heme

3.1 INTRODUCTION

Myoglobin (Mb), normally a carrier for molecular oxygen, can catalyze hydrogen peroxide supported oxygenation (peroxygenation) of a variety of substrates, including olefin epoxidation and thioether sulfoxidation.[1-4] However, the oxidation of the ferric Mb by hydrogen peroxide affords a ferryl heme (Fe^{IV}=O Por) coupled with a transient protein radical.[5,6] A ferryl porphyrin cation radical (Fe^{IV}=O $Por^{+\bullet}$), equivalent to compound I of peroxidase,[7] has not been identified yet, and the turnover numbers for the sulfoxidation by Mb are less than the values obtained by the incubation with peroxidases.[8]

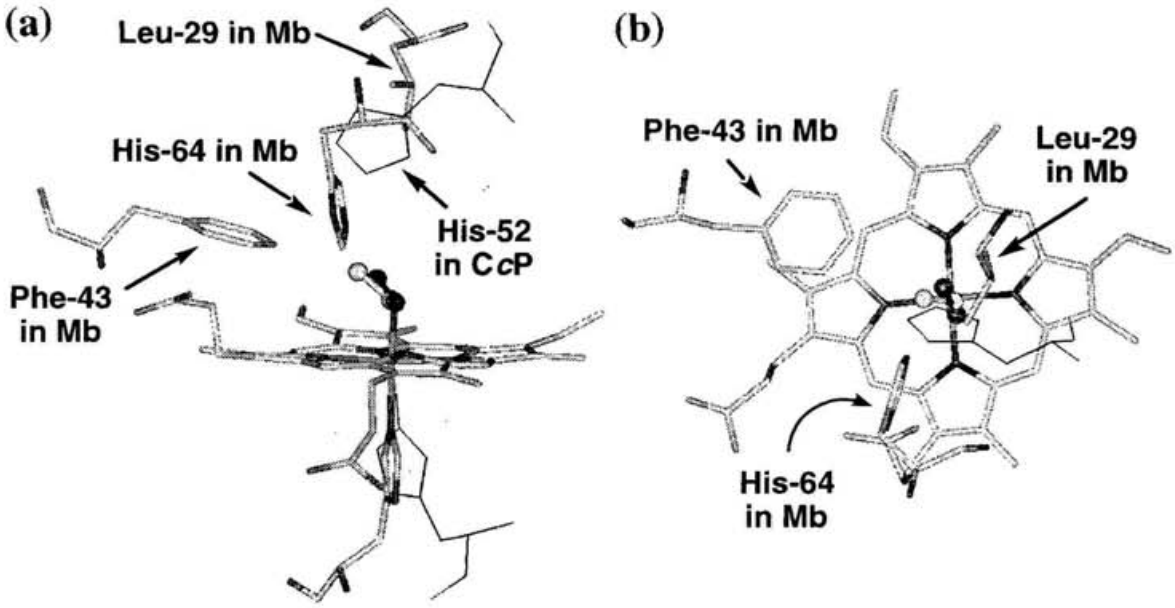


Figure 1. Superposition of the heme and some selected residues including distal histidine (His64 in Mb and His52 in C*c*P) in crystal structures of ferrous-oxy forms of sperm whale Mb (thick line) and cytochrome *c* peroxidase (C*c*P) (thin line). Black and gray balls indicate oxygen molecule bound to heme iron in Mb and C*c*P, respectively. The distance between N_{ε} of distal histidine and iron is 4.3 and 5.6 Å in Mb and C*c*P, respectively. Only heme in Mb is shown in this figure. (a) Side view. (b) Top view.

We have engineered sperm whale Mb based on the comparison of crystal structures of oxymyoglobin[9] and an oxy-form of cytochrome *c* peroxidase (C*c*P)[10,11] (Figure 1). The Leu29 → His and His64 → Leu double replacement of Mb seems to

create a peroxidase-like active site, and the L29H/H64L mutant significantly increases the rate and enantioselectivity for the peroxygenation of thioanisole and styrene.[12] We have attributed the rate increase both to the removal of His64 improving efficiency of the ferryl oxygen transfer, and to the introduction of His29 compensating the decreased reactivity with hydrogen peroxide upon the loss of His64. As shown in chapter 1 of this part, however, the imidazole in L29H/H64L Mb, 6.6 Å above the heme plane,[13] is located too far from the heme iron to enhance the reaction with hydrogen peroxide. Thus, we have mutated Phe43 to a histidine residue because the predicted distance between His43 and the heme iron is approximately equal to that of C*c*P. The novel F43H/H64L Mb mutant oxidizes sulfide and styrene more efficiently than peroxidase. More intriguingly, we have identified compound I-like species of the Mb mutant as the catalytic intermediate for the first time.

3.2 EXPERIMENTAL PROCEDURES

Materials Construction of mutant gene, expression, and purification of myoglobin mutants were described in chapter 1 of this part. All the reagents were purchased mainly from Wako and Nakarai Tesque, and used without further purification.

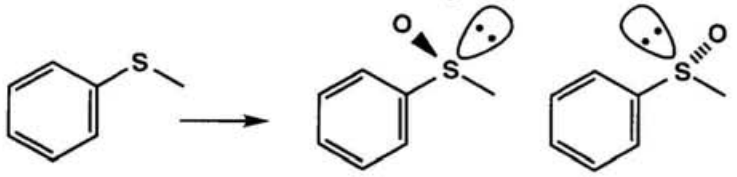
Assay of the Oxidation of Styrene and Thioanisole Peroxygenations of thioanisole and styrene by wild type Mb and the mutants were performed at 20°C in 50 mM sodium phosphate buffer (pH 7.0). The reaction mixture contained 10 μM Mb, 1 mM H_2O_2, and either 1 mM thioanisole or 8.7 mM styrene. Details for the incubation and work up were described in chapter 2 of this part.

Reaction with *m*CPBA Transient optical spectra in the reaction with *m*CPBA were measured by a Hi-Tech SF-43 stopped-flow apparatus equipped with a MG 6000 diode array spectrophotometer. All the measurements were performed at 5.0°C in 50 mM sodium acetate buffer (pH 5.3), and 96 transient spectra were recorded for each reaction with a 2 msec accumulation time.

3.3 RESULTS AND DISCUSSION

The replacement of Phe43 in the wild type with a histidine residue increases the rate of thioanisole oxidation by 14-fold, and the mutation of His64 → Leu in F43H Mb further enhances the sulfoxidation rate by 13-fold (Table 1). The enantiomeric excess is improved from 25 % to 85 % by the His64 → Leu and Phe43 → His double mutation of Mb, and the dominant enantiomer is *R*. More than 92 % of ^{18}O incorporation in the sulfoxide from $H_2{}^{18}O_2$ in the oxidation by wild type, F43H, and F43H/H64L Mb indicates that the ferryl oxygen is transferred to thioether. While the sulfoxidation rate of F43H Mb is similar to that of L29H Mb (3.9 min^{-1}), F43H/H64L Mb can oxygenate to thioanisole 9-times faster than L29H/H64L Mb (5.5 min^{-1}).[12] In contrast, the enantioselectivity is slightly higher with L29H/H64L Mb (97 %) than F43H/H64L Mb.[12]

Table 1. Thioanisole sulfoxidation activity of Mb and its mutants



S *R*

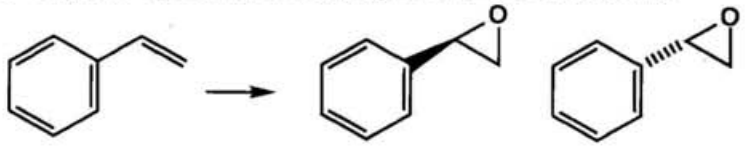
	rate (min^{-1})	ee (%) [a]	^{18}O incorporation from $H_2{}^{18}O_2$ (%)
wild type	0.25	25	92
F43H	3.5	59	97
F43H/H64L	47	85	96

[a] The absolute stereochemistry of the dominant isomer is *R*.

The F43H/H64L mutant oxidizes styrene 300-times faster than the wild type with an improvement of enantioselectivity from 9 to 68 % (Table 2).[8] Even in comparison with L29H/H64L Mb (0.14 min^{-1}),[12] the epoxidation rate of F43H/H64L Mb is 30-fold greater. Incubations of styrene and $H_2{}^{18}O_2$ with wild type and F43H/H64L Mb resulted in incorporation of 20 % and 94 % of ^{18}O in epoxide, respectively. The low ^{18}O incorporation into the epoxide in the presence of the wild type and $H_2{}^{18}O_2$ could be

rationalized by the competition of the ferryl oxygen transfer and co-oxidation mechanism (Scheme I). The co-oxidation mechanism requires protein radical formation followed by binding of molecular oxygen to generate a protein-peroxy radical, and His64 was suggested as the initial radical site.[2,5,6,14-16] The replacement of His64 with an unoxidizable leucine residue could prevent generation of the protein radical and improve the efficiency of the ferryl oxygen transfer. Nevertheless, the peroxygenation activities of the H64L mutant are very similar to those of the wild type presumably because the His64 → Leu replacement significantly decreases the reactivity of ferric Mb with hydrogen peroxide. In chapter 1 of this part, the author has shown that additional Leu29 → His replacement can partially compensate the loss of His64. Since the F43H/H64L mutant shows 11-fold higher reactivity with hydrogen peroxide than the wild type, the great improvement in the peroxygenation activity by the F43H/H64L mutation can be attributed to the efficient enhancement of the reaction with hydrogen peroxide by His43 together with the removal of the oxidizable histidine residue at the position 64.

Table 2. Styrene epoxidation activity of Mb and its mutants



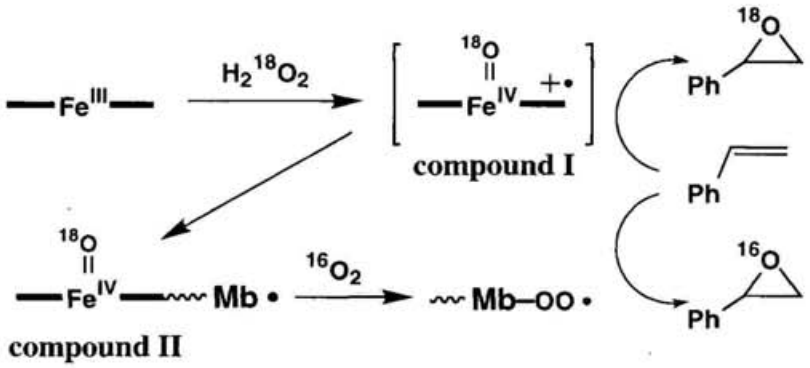
1*R* **1*S***

	rate (min^{-1})	ee (%) [a]	^{18}O incorporation from $H_2{}^{18}O_2$ (%)
wild type	0.015	9	20
F43H	0.091	41	54
F43H/H64L	4.5	68	94

[a] The absolute stereochemistry of the dominant isomer is *R*.

The value of ^{18}O incorporation from $H_2{}^{18}O_2$ for the F43H mutant, bearing two histidines in the active site, is 54 %, which is between the values for F43H/H64L and wild type Mb.[8] The epoxidation rate and the ratio of ^{18}O-incorporation of the F43H

mutant are very similar to those of the L29H mutants (0.093 min^{-1} and 53 %, respectively).[12] As discussed for L29H Mb in the previous chapter, F43H Mb seems to enhance the epoxidation both through the co-oxidation and the ferryl oxygen transfer. Since the visible band of compound II in F43H Mb was distinct from those in wild type and F43H/H64L Mb,[17] electronic states of the heme in F43H Mb may differ from the others having one histidine in their active sites.



Scheme I. Ferryl oxygen transfer *versus* co-oxidation mechanisms

We have attempted to identify the catalytic species of F43H/H64L Mb responsible for two-electron oxidations of thioanisole and styrene. As shown in chapter 1 of this part, the horseradish peroxidase compound I-like spectrum is not observed by monitoring the changes in absorption spectra of the incubation mixture containing the mutant and hydrogen peroxide.[6,7] However, the mixing of F43H/H64L Mb and *m*-chloroperbenzoic acid (*m*CPBA) causes the decrease in absorbance at 406 nm, followed by the shift to longer wavelength by 12 nm (Figure 2a). The formation of the first intermediate proceeds at the rate of $k_{obs1} = 110 \pm 3.8\ s^{-1}$, and the Soret shifts to 418 nm at the rate of $k_{obs2} = 9.2 \pm 0.38\ s^{-1}$ (at pH 5.3 and 5°C, [*m*CPBA] = 0.5 mM). Decrease in the absorbance of the Soret and intense absorbance around 500–700 nm are characteristic for the conversion of ferric to a ferryl porphyrin radical cation, and the following Soret shift is due to the decay of compound I (Fe^{IV}=O $Por^{+\cdot}$) to compound II (Fe^{IV}=O Por) as observed for other heme containing peroxidases.[18-20] Upon the

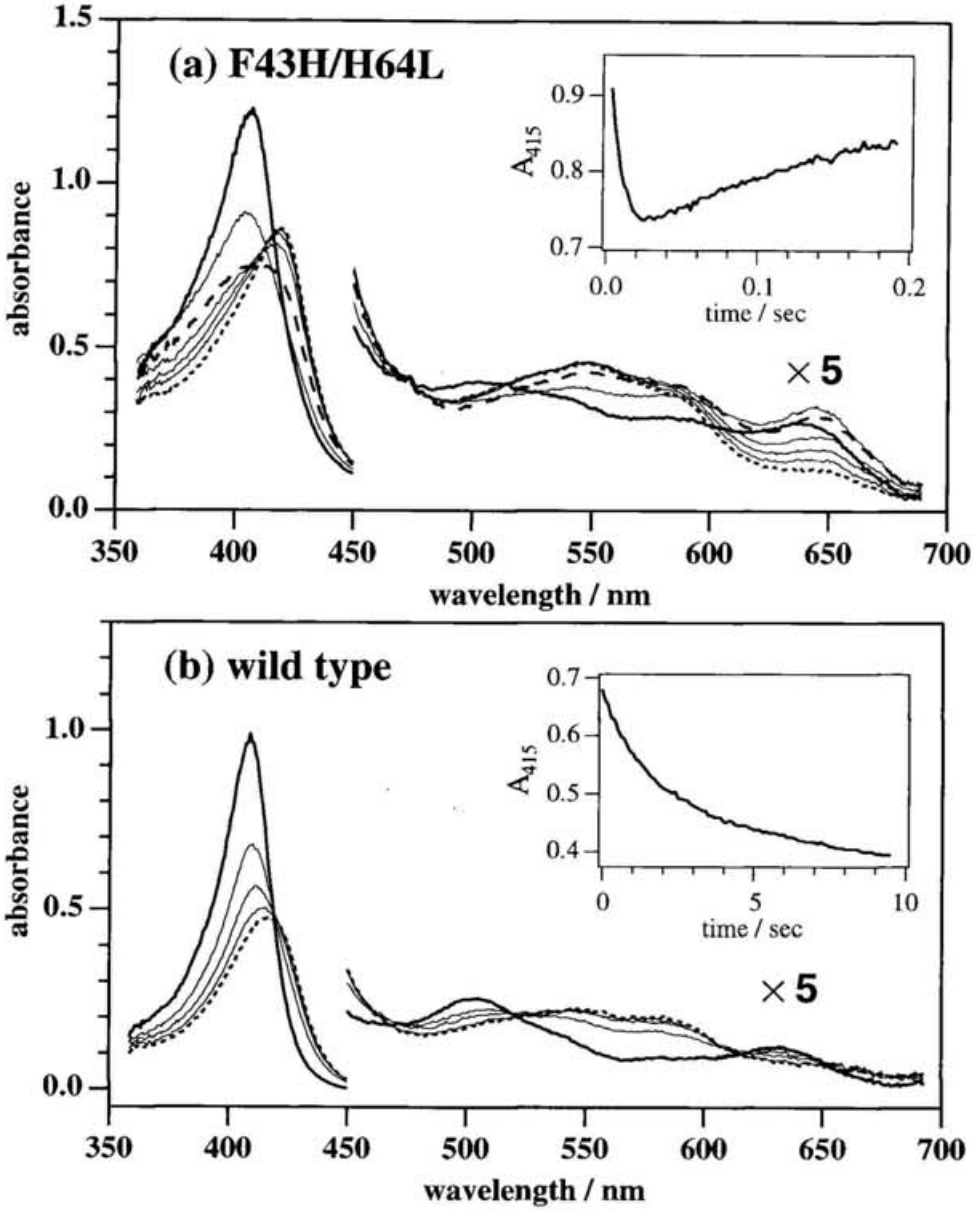


Figure 2. Absorption spectra of (a) ferric F43H/H64L (7 μM) or (b) wild type Mb (6 μM) with *m*CPBA (0.5 mM) in 50 mM sodium acetate buffer, pH 5.3. Insets are changes in absorbance at 415 nm. Thick line spectra represent ferric. Dashed and dotted line spectra are compound I and II, respectively.

addition of styrene or thioanisole to the compound I-like intermediate, absorbance of the Soret band for the intermediate is recovered back to the level of ferric state. Thus, it is now convincing that the observed species bears two electron oxidation equivalents. On the contrary, wild type and F43H Mb react with *m*CPBA more slowly than the

F43H/H64L mutant to generate compound II, and there is no accumulation of compound I (Figure 2b) presumably due to the rapid electron transfer from compound I to His64, which lies closer to the heme iron than the residue at the position 43 (Figure 1).

In summary, we have engineered the distal pocket of Mb to mimic the active site of peroxidase, and the F43H/H64L mutant is found to be a much better peroxygenase than wild type Mb and even HRP. Furthermore, the utilization of *m*CPBA enables us to identify a compound I-like species of F43H/H64L Mb. Since the percentage of enantiomeric excess for styrene epoxidation by F43H/H64L Mb with *m*CPBA is almost identical to the value with H_2O_2, the same catalytic species seems likely to be involved in the catalytic cycle as a reactive intermediate responsible for the peroxygenation. The close examination of a crystal structure for sperm whale Mb suggests that the orientation of the distal histidine in F43H/H64L Mb could be somewhat similar to that of bovine liver catalase,[21,22] and thus, compound I of the mutant appears to be readily reduced to the ferric state in the presence of hydrogen peroxide as suggested in chapter 1 of this part. The results clearly indicate that the alignment of the distal histidine is important for the reactivity as well as the direct observation of the transient catalytic species for Mb.

3.4 REFERENCES AND NOTES

(1) Ortiz de Montellano, P. R.; Catalano, C. E. *J. Biol. Chem.* **1985**, *260*, 9265-9271.

(2) Catalano, C. E.; Ortiz de Montellano, P. R. *Biochemistry* **1987**, *26*, 8373-8380.

(3) Adachi, S.; Nagano, S.; Ishimori, K.; Watanabe, Y.; Morishima, I.; Egawa, T.; Kitagawa, T.; Makino, R. *Biochemistry* **1993**, *32*, 241-252.

(4) Matsui, T.; Nagano, S.; Ishimori, K.; Watanabe, Y.; Morishima, I. *Biochemistry* **1996**, *35*, 13118-13124.

(5) King, N. K.; Winfield, M. E. *J. Biol. Chem.* **1963**, *238*, 1520-1528.

(6) Yonetani, T.; Schleyer, H. *J. Biol. Chem.* **1967**, *242*, 1974-1979.

(7) Dunford, H. B.; Stillman, J. S. *Coord. Chem. Rev.* **1987**, *19*, 187-251.

(8) Peroxygenase activity of HRP was previously reported in Ozaki, S.; Ortiz de Montellano, P. R. *J. Am. Chem. Soc.* **1995**, *117*, 7056-7064. The rate of sulfoxidation for F43H Mb is equal to that for native HRP, and the F43H/H64L mutant is found to oxidize thioanisole approximately 13 times faster than HRP. Styrene is oxidized by the Phe43 → His mutants at least 100-fold faster than HRP and its mutants.

(9) Phillips, S. E. *J. Mol. Biol.* **1980**, *142*, 531-534.

(10) Miller, M. A.; Shaw, A.; Kraut, J. *Nature Struct. Biology* **1994**, *1*, 524-531.

(11) Although X-ray crystal structure of HRP has not been published yet, the modeling studies in Zhao, D.; Gilfoyle, D. J.; Smith, A. T.; Loew, G. H. *Proteins: Struct. Funct. Genet.* **1996**, *26*, 204-216 suggest that the alignment of distal histidine in HRP is similar to that of CcP.

(12) Ozaki, S.; Matsui, T.; Watanabe, Y. *J. Am. Chem. Soc.* **1996**, *118*, 9784-9785.

(13) Personal communication from Phillips, G. N., Jr. (Rice University). The R and R-free values are 16.7% and 21.6%, respectively. The RMS deviation of bond length and bond angle are 0.010 Å and 1.825 degrees, respectively. The crystal structure of L29H/H64L Mb is essentially the same with that of wild type Mb except for residues at the position 29 and 64.

(14) Tschirret-Guth, R. A.; Ortiz de Montellano, P. R. *Arch. Biochem. Biophys.* **1996**, *335*, 93-101.

(15) Fenwick, C. W.; English, A. M. *J. Am. Chem. Soc.* **1996**, *118*, 12236-12237.

(16) Kelman, D. J.; DeGray, J. A.; Mason, R. P. *J. Biol. Chem.* **1994**, *269*, 7458-7463.

(17) The absorption maxima of compound II in the visible region are located at 548 and 581 nm in wild type, 546 and 584 nm in F43H/H64L, but 524 and 551 nm in F43H Mb.

(18) Stillman, J. S.; Stillman, M. J.; Dunford, H. B. *Biochemistry* **1975**, *14*, 3183-3187.

(19) Stillman, J. S.; Stillman, M. J.; Dunford, H. B. *Biochem. Biophys. Res. Commun.* **1975**, *63*, 32-35.

(20) Smith, A. T.; Sanders, S. A.; Thorneley, R. N.; Burke, J. F.; Bray, R. R. *Eur. J. Biochem.* **1992**, *207*, 507-519.

(21) Fita, I.; Rossmann, M. G. *J. Mol. Biol.* **1985**, *185*, 21-37.

(22) Fita, I.; Rossmann, M. G. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 1604-1608.

PART III

FORMATION AND REACTIONS OF COMPOUND I OF MYOGLOBIN

CHAPTER 1.

Formation and Reactivity of Compound I of His64 Myoglobin Mutants*

* published in *J. Biol. Chem.* **1997**, *272*, 32735-32738.

On the Formation and Reactivity of Compound I of the His64 Myoglobin Mutants.

Toshitaka Matsui, Shin-ichi Ozaki, and Yoshihito Watanabe

ABSTRACT: Myoglobin (Mb) catalyzes various two-electron oxidations; however, a ferryl porphyrin cation radical equivalent to peroxidase compound I has not been identified yet. Distal histidine mutants of sperm whale Mb (His64→Ala, Ser and Leu) react with *m*-chloroperbenzoic acid (*m*CPBA) to afford a novel intermediate followed by the formation of a ferryl heme (Mb-II). Since the intermediate exhibits characteristic absorption spectrum of compound I and is oxidized by two electrons above the ferric state, we have assigned the species as compound I of myoglobin (Mb-I). Although we have recently observed compound I of the F43H/H64L Mb mutant, F43H and wild type Mb react with *m*CPBA to give Mb-II without any accumulation of Mb-I. The results unambiguously indicate that His64 plays a key role in destabilizing wild type Mb-I. Furthermore, Mb-I is found to be capable of performing two-electron oxidation of styrene, thioanisole and H_2O_2.

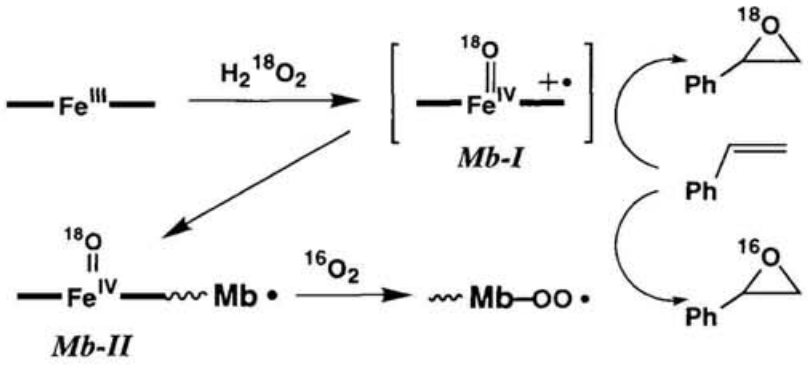
ABBREVIATIONS

Mb	myoglobin
compound I	a ferryl porphyrin cation radical
compound II	a ferryl heme
Mb-I	compound I of myoglobin
Mb-II	compound II of myoglobin
*m*CPBA	*m*-chloroperbenzoic acid
HRP	horseradish peroxidase

1.1 INTRODUCTION

The key intermediate in the catalytic cycles of heme-containing peroxidase and catalase is ferryl porphyrin cation radicals (Fe^{IV}=O $Por^{+\bullet}$) so called compound I formed by the reaction of the resting ferric enzymes with peroxide.[1,2] By the two sequential one-electron transfers from substrates, compound I is reduced back to the ferric state via a ferryl heme (Fe^{IV}=O Por) known as compound II. The two-electron oxidation which is often associated with the ferryl oxygen transfer to substrates also takes place by compound I. The reactive species responsible for the oxygenation by cytochrome P450, heme-containing monooxygenase, is assumed to be compound I.[3,4]

Myoglobin (Mb), a carrier of molecular oxygen, can catalyze H_2O_2-dependent two-electron oxidations including styrene epoxidation;[5-8] however, the reactions of Mb with peroxides are known to yield a ferryl heme (Mb-II) coupled to a transient protein radical in stead of a ferryl porphyrin cation radical (Mb-I).[9] The epoxidation by wild type Mb in the presence of $H_2{}^{18}O_2$ resulted in only 20 % ^{18}O-incorporation into the epoxide, and oxygen in the epoxide derived primarily from molecular oxygen.[5,6] The incorporation of the molecular oxygen has been attributed to the co-oxidation by the protein-peroxy radical which is formed by the reaction of molecular oxygen and the protein radical (Scheme I). Thus, Mb-I appears to be a branch to co-oxidation *versus* ferryl oxygen transfer mechanism.



Scheme I. Ferryl oxygen transfer *versus* co-oxidation mechanisms

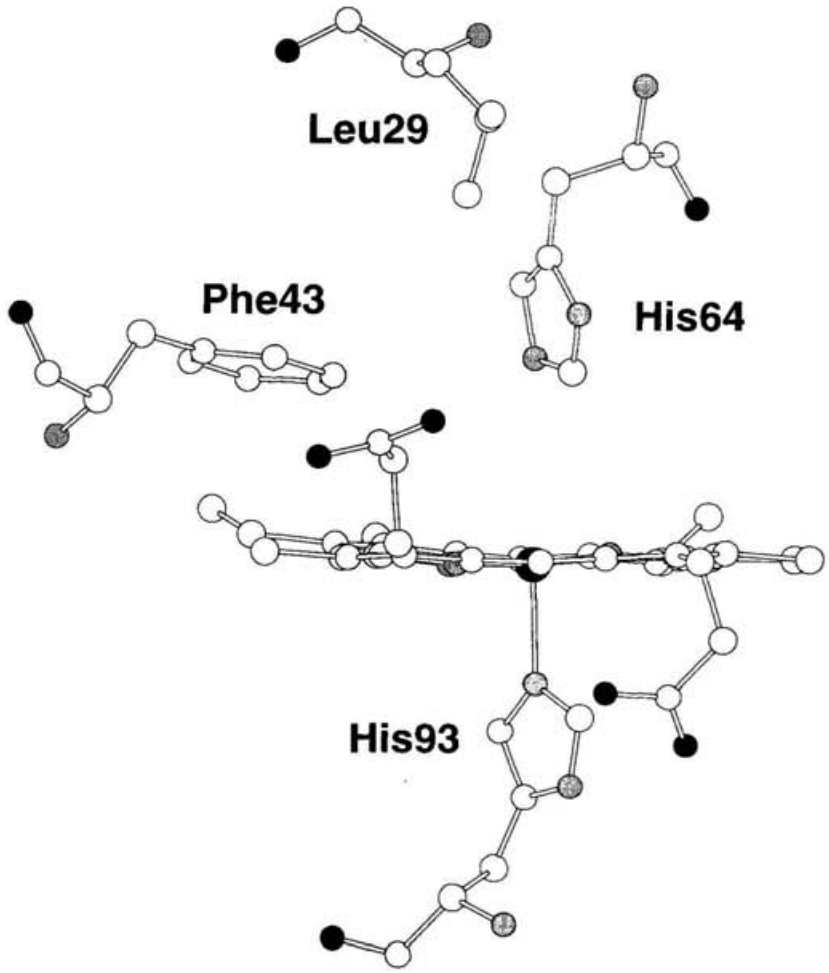


Figure 1. Heme environmental structure of sperm whale myoglobin. Heme and some selected residues including His64 (E7) are presented.

The Mb mutants in which distal histidine (His64, E7) is replaced by Leu or Val (Figure 1) showed drastic increase in the ratio of ferryl oxygen transfer even though the Mb-I as well as Mb-II of those mutants has not been observed when H_2O_2 is used as an oxidant.[6,10,11] We previously designed a F43H/H64L mutant to mimic the active site of peroxidases because the distal histidine of peroxidases functions as a general acid-base catalyst in the formation of compound I.[12] Compound I of the F43H/H64L mutant was not detected in the presence of H_2O_2 due to the enhanced catalase activity of the mutant, while the reaction of F43H/H64L Mb with *m*-chloroperbenzoic acid (*m*CPBA) afforded a compound I (Mb-I). We attributed the stabilization of Mb-I for the F43H/H64L double mutant to the replacement of His64 with an unoxidizable amino acid like Leu, but not to

Phe43→His mutation.[12] In order to examine the hypothesis, this chapter focuses on the reactions of *m*CPBA with H64A, H64S, H64L and F43H mutants of sperm whale Mb. Since these single mutants except for F43H Mb afford Mb-I, His64 is unambiguously identified as a critical residue for destabilizing wild type Mb-I. Furthermore, the reaction rates of H64A and H64S Mb-I with styrene, thioanisole and H_2O_2 have been determined by single turnover kinetics.

1.2 EXPERIMENTAL PROCEDURES

Materials *m*CPBA and thioanisole was obtained from Nakalai Tesque. Styrene and H_2O_2 (30%) was purchased from Wako. H64A, H64S and H64L Mb mutants were constructed by cassette mutagenesis. The cassette including the desired His64 substitution and a new silent *Hpa*I restriction site was inserted between the *Bgl*II and *Hpa*I sites. The expression and purification of the mutants were described in chapter 1 of part II.[13]

Reaction of Myoglobin Mutants with *m*-Chloroperbenzoic Acid The reactions between ferric Mb mutants and *m*CPBA were performed at 5.0°C in 50 mM sodium acetate buffer (pH 5.3) or 50 mM sodium phosphate buffer (pH 7.0). Whole spectral changes during the reactions were recorded on a Hi-Tech SF-43 stopped-flow apparatus equipped with a MG 6000 diode array spectrophotometer (5 μM of Mb, 100 μM of *m*CPBA with or without 4 mM of styrene). The formation and decay rates of Mb-I were determined at pH 7.0 using single wavelength mode of the stopped-flow apparatus (H64A and H64S, 406 nm; H64L, 411 nm). In order to assure the pseudo-first-order condition, the ferric Mb mutants (5 μM) were mixed with more than 10 mol equivalents of *m*CPBA (0.050–1.5 mM). The absorbance change recorded with a logarithmic time interval was fitted by a double-exponential function assuming sequential two-step reactions (eq 1,2). As expected from the reaction scheme, observed formation

rates of Mb-I were proportional to *m*CPBA concentration (eq 3) whereas its decay rates to Mb-II were essentially constant.

$$Fe^{III}\ Por + m\text{CPBA} \xrightarrow{k_1} Fe^{IV}{=}O\ Por^{+\bullet}\ (\text{Mb-I}) \quad (1)$$

$$Fe^{IV}{=}O\ Por^{+\bullet} \xrightarrow{k_{dec}} Fe^{IV}{=}O\ Por\ (\text{Mb-II}) + \text{protein radical} \quad (2)$$

$$k_{1,obs} = k_1 \times [m\text{CPBA}] \quad (3)$$

Two-electron Oxidation Rates by Myoglobin Compound I The reactivities of Mb-I with styrene, thioanisole and H_2O_2 were determined by means of a double-mixing rapid scan technique at 5.0°C in 50 mM sodium acetate buffer, pH 5.3. Ferric H64A or H64S mutant was mixed with 1.3 mol equivalent of *m*CPBA into an age loop to prepare compound I (aging time: 0.80 and 2.0 s, respectively), then, the aged solution was mixed with a substrate solution to monitor whole spectral changes. The final reaction mixture contained 2.2 μM of Mb and varied concentrations of styrene (0.10–1.0 mM), thioanisole (25–100 μM) or H_2O_2 (0.25–2.0 mM). The reaction rates were obtained by fitting the increase in absorbance at 406 nm to a single exponential function.

1.3 RESULTS AND DISCUSSION

Formation of Myoglobin Compound I

The reaction of ferric Mb with *m*CPBA was performed at pH 5.3 on a stopped-flow apparatus, and Figure 2A shows absorption spectra of H64A Mb during the reaction. Within 80 msec after the mixing, Soret absorption of H64A decreased to less than half and a broad visible band having a peak at 648 nm appeared. Then, the novel intermediate was slowly converted to a species with Soret shift to 419 nm which is readily assigned to Mb-II (Fe^{IV}=O Por). The formation of the first intermediate proceeded with distinct isosbestic points at 430, 462 and 540 nm (k_{obs} = 99 s^{-1} at [*m*CPBA] = 100 μM), and its spectrum is characteristic of a ferryl porphyrin cation

radical (Fe^{IV}=O $Por^{+\bullet}$), equivalent to peroxidase compound I.[1] On the contrary, subsequent Mb-II formation seems to be accompanied by partial degradation of heme chromophore since the latter reaction did not give isosbestic points, and the Soret absorption of Mb-II was less intense than that of wild type Mb-II (Figure 2A).

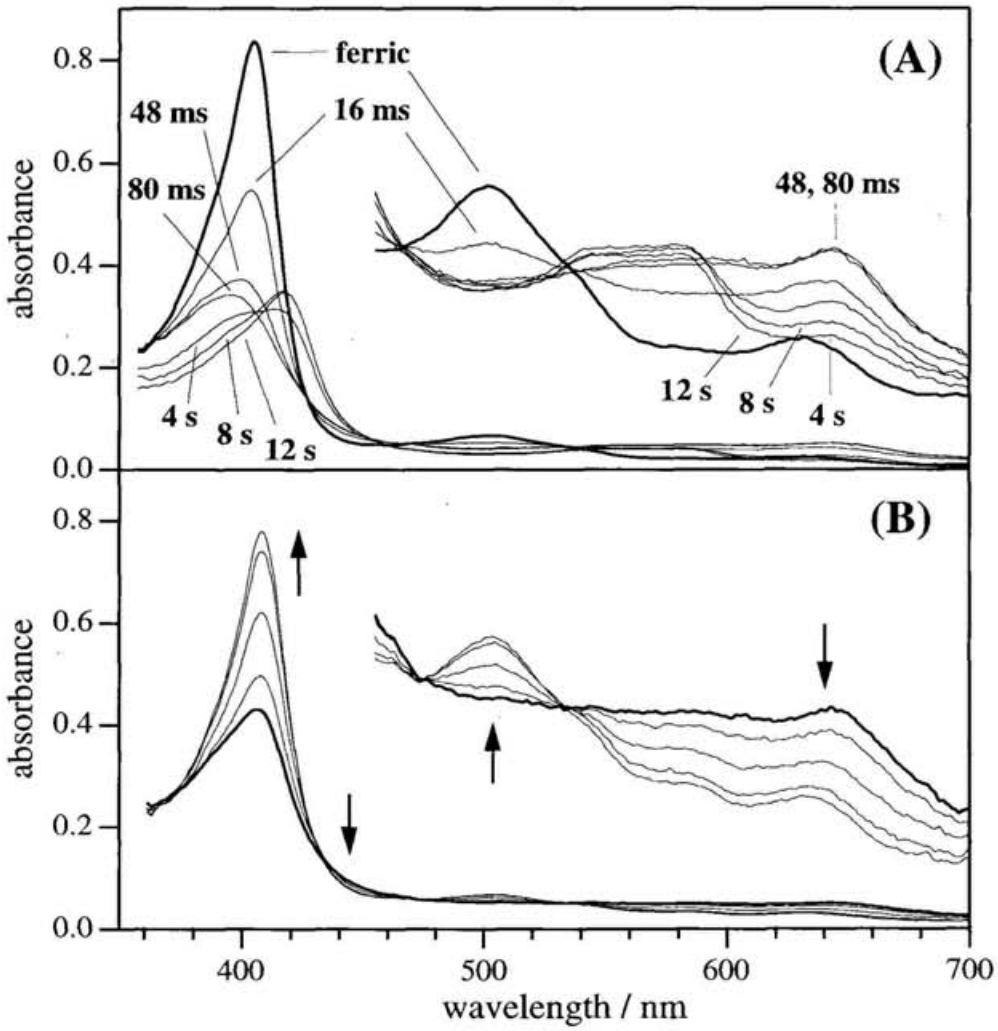


Figure 2. Absorption spectral changes of H64A Mb upon mixing with *m*CPBA at 5.0°C in 50 mM sodium acetate buffer, pH 5.3. (A) Final concentrations: 5.0 μM H64A and 100 μM *m*CPBA. Spectra were recorded before addition of *m*CPBA (*thick line*) and indicated time after mixing. (B) Final concentrations: 4.8 μM H64A, 100 μM *m*CPBA and 4.3 mM styrene. Spectra were recorded at 0.10 (*thick line*) and 0.40–1.3 sec after mixing with a 0.30 sec time interval. The directions of absorbance changes are indicated by arrows.

When the ferric H64A Mb was mixed with *m*CPBA in the presence of styrene, the compound I-like species partially formed (approximately 70 % based on the Soret intensity) and directly went back to the ferric form (Figure 2B). The appearance of isosbestic points and the complete recovery of Soret absorbance after the completion of the reaction ruled out the Mb-II formation and heme degradation. During the reaction, styrene was oxidized by two-electron equivalents to produce styrene oxide and phenylacetoaldehyde, and total amount of these products was approximately equal to that of *m*CPBA added. These results clearly indicate that the compound I-like intermediate bears two electron oxidizing equivalents above the ferric state and oxidizes an equimolar amount of styrene. Thus, it is now convincing that the novel intermediate observed for H64A mutant is myoglobin compound I (Mb-I).

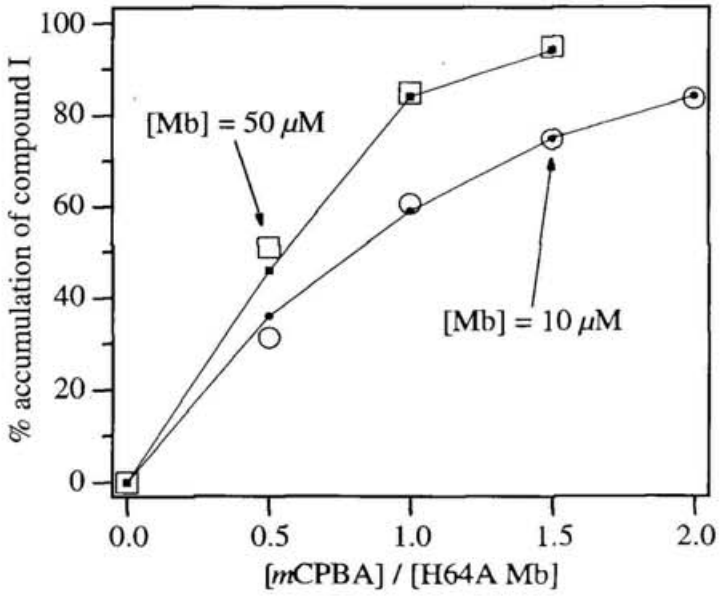


Figure 3. Yields of Mb-I during the reactions of the H64A mutant with varied amount of *m*CPBA at 5.0°C in 50 mM sodium acetate buffer, pH 5.3. The yields were based on absorbance changes at 416 or 646 nm for [Mb]=10 μM (E) and 50 μM (G), respectively. The close symbols with lines were the calculated based on eq 1 and 2. The values of k_1 (1×10^6 $M^{-1}s^{-1}$) and k_{dec} (0.4 s^{-1}) were used for the simulations.

Yields of Mb-I for the H64A mutant with varied amount of *m*CPBA at pH 5.3 were shown in Figure 3. An equimolar amount of *m*CPBA could convert 85 % of H64A Mb to Mb-I at [Mb] = 50 μM. Thus, the ferric Mb is revealed to need one mole equivalent of *m*CPBA to afford Mb-I. In contrast, when the concentration of Mb was 10 μM, approximately two and seven mol equivalents of *m*CPBA were required to accumulate 80 and 100 % of Mb-I, respectively. As simulations based on equation 1 and 2 roughly reproduced the curves (Figure 3), the excess peracid is necessary for the complete accumulation of Mb-I under the condition probably due to the decay of Mb-I to II.

H64S and H64L mutants exhibited similar spectral changes upon mixing with *m*CPBA, and sequentially afforded Mb-I and II in the absence of substrates (eq 1,2). While we have reported the first observation of Mb-I in F43H/H64L double mutant,[12] F43H Mb as well as the wild type was directly oxidized to Mb-II without any observable intermediate. On the basis of these findings, His64 so called distal histidine in Mb is unambiguously identified as a critical residue for destabilizing Mb-I. It has been proposed that transiently formed wild type Mb-I is immediately reduced to Mb-II by neighboring amino acid residues, and the resulted protein radical has been shown to be centered at least on Tyr103, Trp14, Lys42 and His64.[14-16] Our present result strongly suggests that the diffusion of one oxidizing equivalent from wild type Mb-I is mainly initiated by the oxidation of His64, which lies close vicinity of the heme (4.3 Å above heme iron, Figure 1). Therefore, the substitutions of His64 with unoxidizable amino acids drastically prolong the life-time of Mb-I even though there may be other leak pathways of the oxidizing equivalent. On the other hand, the histidine found in the active site of peroxidases is located 5.6–5.9 Å above heme iron and free from the oxidation by compound I.

Formation and Decay Rates of Myoglobin Compound I

At pH 5.3, the H64A and H64S mutants were almost completely oxidized to Mb–I at similar rates (99 and 104 s^{-1} at [*m*CPBA] = 100 μM, respectively). Only the H64L

mutant did not show 100 % accumulation of Mb-I due to at least 10-fold lower reactivity with *m*CPBA. Even though the stability of Mb-I appears to be essentially the same among the His64 mutants, the concomitant heme degradation and instability of Mb-II disabled us to measure decay rates of Mb-I to II at pH 5.3.[17] Thus, we have examined optimal pH levels for the reactions.

As increasing pH, the reaction rates of the ferric H64A with *m*CPBA showed roughly linear decrease (Figure 4), which implies the presence of plural ionizable groups affecting the reactivities with *m*CPBA. At pH 9 or higher, H64A Mb was directly oxidized to Mb-II without the accumulation of Mb-I (Figure 4) because the decay rates of Mb-I to II (k_{dec}) appeared to be greater at higher pH. The k_{dec} values were measurable between pH 7 and 8 (1.2 and 2.7 s^{-1}, respectively) where the heme degradation was negligible as judged from isosbestic conversion of Mb-I to II (Figure 5). Thus, the formation rates of Mb-I (k_1) and its decay rates to Mb-II (k_{dec}) in the Mb mutants were determined at pH 7.0 (Table 1).

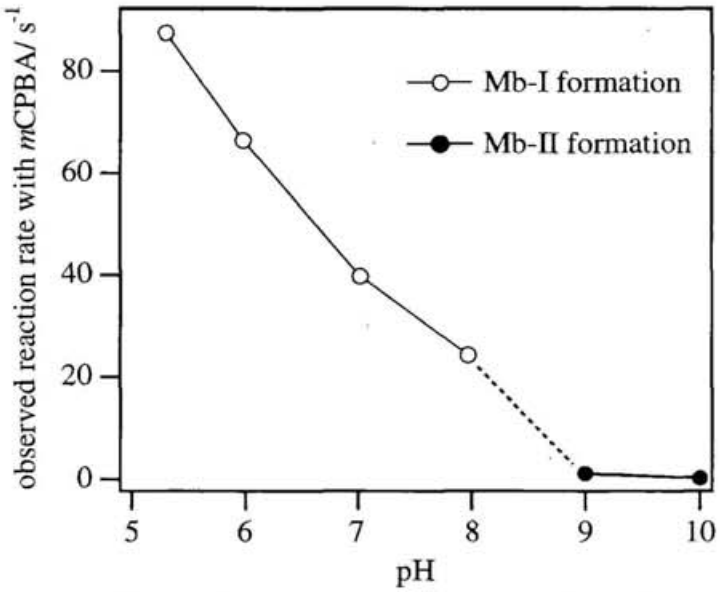


Figure 4. pH profiles of the reaction rates of H64A mutant with *m*CPBA at 5.0°C in 50 mM buffers. The open and close circles correspond to the formation rates of Mb-I and Mb-II, respectively.

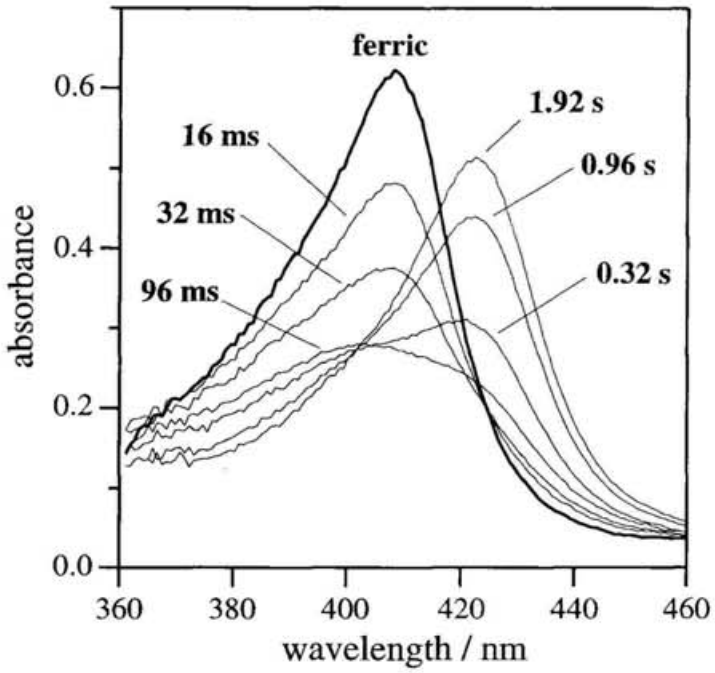


Figure 5. Soret spectral change of H64A Mb upon mixing with *m*CPBA at 5.0°C in 50 mM sodium phosphate buffer, pH 7.0. Final concentrations: 3.4 μM H64A and 100 μM *m*CPBA. Spectra were recorded before addition of *m*CPBA (*thick line*) and indicated times after mixing.

Table 1. Formation and decay rates of Mb compound I in the reaction with *m*CPBA [a]

	k_1 ($M^{-1}s^{-1}$)	k_{dec} (s^{-1})
H64A	4.2×10^5	1.2
H64S	3.0×10^5	1.2
H64L	1.1×10^4	0.9
HRP	1.9×10^7	–

[a] Rate constants were measured in 50 mM sodium phosphate buffer (pH 7.0) at 5.0 °C.

The H64A and H64S mutants exhibited similar k_1 values which are about 50-fold lower than that of horseradish peroxidase (HRP). In comparison with the H64A and H64S, 30-fold decrease in the k_1 was observed for H64L. Although reasons for the

poor reactivity of the H64L are unclear, it is not due to the steric bulkiness of Leu because the reaction rate with peracetic acid was also 45-fold lower in the H64L than the H64A (~7×10^3 and 3.2×10^5 $M^{-1}s^{-1}$, respectively). The decay rates of Mb-I to II (k_{dec}) are essentially identical (0.9–1.2 s^{-1} at pH 7.0) for the three His64 mutants (Table 1). As compared to these single mutants, F43H/H64L exhibited about 10–fold higher k_{dec} value even at pH 5.3 (9.2 s^{-1}), and the Mb-I formation in the double mutant was not apparent at pH 7.0.[12] Therefore, His43 in F43H/H64L might be also oxidized by Mb-I (Figure 1) possibly due to the close location to the β-*meso* heme edge, while the distance of His43 from the iron is expected to be at least 1 Å longer than that of His64 in wild type Mb (Figure 1) and similar to that of distal histidine in peroxidase.

Reaction of Compound I with Styrene, Thioanisole and H_2O_2

Two-electron oxidation ability of Mb-I was examined by means of a double-mixing rapid-scan technique at pH 5.3. Although Mb-I was prepared by a slight excess amount of *m*CPBA for avoiding its catalytic regeneration, H64L Mb-I was not apparent due to its poor reactivity with *m*CPBA. Thus, reactions of Mb-I with styrene, thioanisole and H_2O_2 were examined for H64A and H64S mutants (approximately 80 % yield of Mb-I). Upon mixing with the substrates, both H64A and H64S Mb-I were directly reduced to the ferric states with complete recovery of the Soret intensity. These spectral changes clearly reveal that Mb-I is capable of performing two-electron oxidation of styrene, thioanisole and H_2O_2. The time course of Mb-I reduction using the absorbance increase at 406 nm obeyed pseudo-first-order kinetics. The reduction rates were proportional to the substrate concentrations, and the second-order rate constants are listed in Table 2. There was no significant difference in the reactivities of H64A and H64S Mb-I.

The H_2O_2 oxidation by Mb-I is about 3000-fold slower than that reported for catalase compound I.[18] Nevertheless, the complete reduction of Mb-I by H_2O_2 indicates that the resulting ferric Mb does not react with H_2O_2 in the time scale of the reduction; i.e., Mb-I appears to be reduced by H_2O_2 much faster than its formation by H_2O_2. Due

to the catalase-like reaction, the direct observation of Mb-I in these mutants was failed when H_2O_2 was employed as the oxidant.

Table 2. Reaction rate constants for the oxidation of styrene, thioanisole and H_2O_2 by Mb compound I / $M^{-1}s^{-1}$ [a]

myoglobin	styrene	thioanisole	H_2O_2
H64A	2.2×10^4	1.5×10^6	5.9×10^3
H64S	2.6×10^4	1.5×10^6	6.2×10^3

[a] Rate constants were determined in 50 mM sodium acetate buffer (pH 5.3) at 5.0 °C.

Styrene oxidation by Mb-I was about 4-fold faster than the H_2O_2 oxidation (Table 2). Thioanisole was oxidized to methyl phenyl sulfoxide at further 60-fold higher rates (Table 2) which are comparable to guaiacol and iodide oxidation rates by compound I of HRP.[19,20] There are few reports on the oxygenation rates of styrene and thioanisole by compound I of hemoproteins even though chloroperoxidase compound I is shown to immediately sulfoxidize thioanisole derivatives.[21] We have examined the reactions of HRP compound I with styrene and thioanisole, but only photoreduction of compound I to II was observed ($k_{obs} = 0.035\ s^{-1}$) under the exposure to a xenon lamp used for the rapid scanning.[22] Since the oxygen transfer requires direct interaction of substrates with the ferryl oxygen, the low oxygenation ability of HRP compound I is normally ascribed to the poor accessibility of substrates to the ferryl species.[23] Although the distal side of Mb is considered to be more spacious than those of peroxidases,[24] the replacements of His64 to the smaller Ala and Ser are expected to make the ferryl oxygen further accessible. Thus, the high peroxygenation ability of Mb-I could be partially due to the preferable accessibility of aromatic substrates.

In summary, the replacement of His64 by unoxidizable amino acids enables us to observe compound I of Mb (Mb-I) in the reaction with *m*CPBA, and Mb-I is capable of performing two-electron oxidation of styrene, thioanisole, and H_2O_2. The results clearly indicate that His64 is a critical residue for destabilizing Mb-I, and the location of distal histidine is suggested to be important to control the stability of compound I in heme enzymes.

1.4 REFERENCES AND NOTES

(1) Dunford, H. B.; Stillman, J. S. *Coord. Chem. Rev.* **1987**, *19*, 187-251.

(2) Schonbaum, G. R.; Chance, B. In *The Enzymes*; 3rd ed.; P. D. Boyer, Ed.; Academic Press: New York, 1976; Vol. 13; pp 363-408.

(3) White, R. E.; Coon, M. J. *Annu. Rev. Biochem.* **1980**, *49*, 315-350.

(4) Egawa, T.; Shimada, H.; Ishimura, Y. *Biochem. Biophys. Res. Commun.* **1994**, *201*, 1464-1469.

(5) Ortiz de Montellano, P. R.; Catalano, C. E. *J. Biol. Chem.* **1985**, *260*, 9265-9271.

(6) Rao, S. I.; Wilks, A.; Ortiz de Montellano, P. R. *J. Biol. Chem.* **1993**, *268*, 803-809.

(7) Adachi, S.; Nagano, S.; Ishimori, K.; Watanabe, Y.; Morishima, I.; Egawa, T.; Kitagawa, T.; Makino, R. *Biochemistry* **1993**, *32*, 241-252.

(8) Matsui, T.; Nagano, S.; Ishimori, K.; Watanabe, Y.; Morishima, I. *Biochemistry* **1996**, *35*, 13118-13124.

(9) King, N. K.; Winfield, M. E. *J. Biol. Chem.* **1963**, *238*, 1520-1528.

(10) Phillips, G. N., Jr.; Arduini, R. M.; Springer, B. A.; Sligar, S. G. *Proteins: Struct. Funct. Genet.* **1990**, *7*, 358-365.

(11) Ozaki, S.; Matsui, T.; Watanabe, Y. *J. Am. Chem. Soc.* **1996**, *118*, 9784-9785.

(12) Ozaki, S.; Matsui, T.; Watanabe, Y. *J. Am. Chem. Soc.* **1997**, *119*, 6666-6667.

(13) Springer, B. A.; Egeberg, K. D.; Sligar, S. G.; Rohlfs, R. J.; Mathews, A. J.; Olson, J. S. *J. Biol. Chem.* **1989**, *264*, 3057-3060.

(14) Tew, D.; Ortiz de Montellano, P. R. *J. Biol. Chem.* **1988**, *263*, 17880-17886.

(15) Fenwick, C. W.; English, A. M. *J. Am. Chem. Soc.* **1996**, *118*, 12236-12237.

(16) DeGray, J. A.; Gunther, M. R.; Tscirret-Guth, R.; Ortiz de Montellano, P. R.; Mason, R. P. *J. Biol. Chem.* **1997**, *272*, 2359-2362.

(17) King, N. K.; Winfield, M. E. *J. Biol. Chem.* **1963**, *238*, 1520-1528.

(18) Chance, B.; Greenstein, D. S.; Roughton, F. J. W. *Arch. Biochem. Biophys.* **1952**, *37*, 301-321.

(19) Nagano, S.; Tanaka, M.; Ishimori, K.; Watanabe, Y.; Morishima, I. *Biochemistry* **1996**, *35*, 14251-14258.

(20) Roman, R.; Dunford, H. B. *Biochemistry* **1972**, *11*, 2076-2082.

(21) Casella, L.; Gullotti, M.; Ghezzi, R.; Poli, S.; Beringhelli, T.; Colonna, S.; Carrea, G. *Biochemistry* **1992**, *31*, 9451-9459.

(22) Stillman, J. S.; Stillman, M. J.; Dunford, H. B. *Biochemistry* **1975**, *14*, 3183-3187.

(23) Ozaki, S.; Ortiz de Montellano, P. R. *J. Am. Chem. Soc.* **1995**, *117*, 7056-7064.

(24) Ortiz de Montellano, P. R. *Acc. Chem. Res.* **1987**, *20*, 289-294.

CHAPTER 2.

Formation and Catalytic Roles of Compound I in the Hydrogen Peroxide-Dependent Oxidations by His64 Myoglobin Mutants

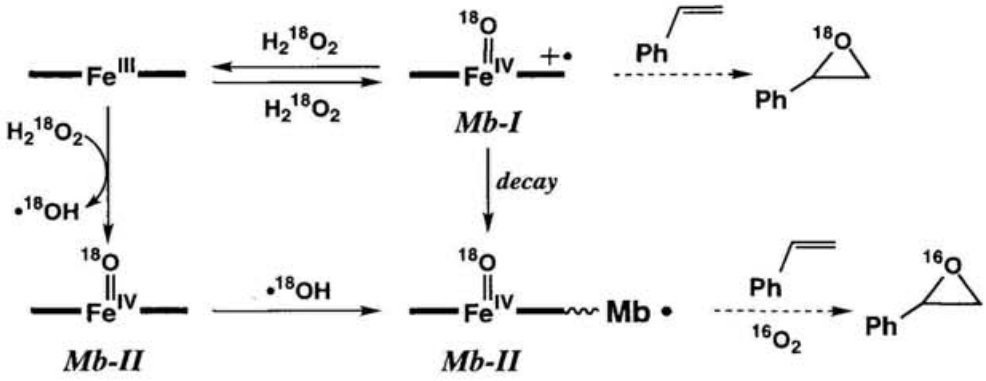
ABSTRACT: Recently, we have shown that replacements of distal histidine (His64) by unoxidizable amino acids enable us to observe compound I of Mb (Mb-I) in the reaction with *m*-chloroperbenzoic acid. However, the His64 Mb mutants react with H_2O_2 without any considerable accumulation of Mb-I. In this chapter, a novel His64 mutant of sperm whale Mb, H64D Mb, has been prepared to mimic the active site of chloroperoxidase from the marine fungus *Caldariomyces fumago* in which distal glutamic acid is suggested to enhance the compound I formation by H_2O_2. The His64 $\rightarrow$ Asp replacement substantially improves the reactivity of ferric Mb with H_2O_2 and allows us to see the accumulation of Mb-I in the reaction with H_2O_2 for the first time. The examinations on the H_2O_2-dependent oxidations catalyzed by wild type Mb and a series of His64 Mb mutants elucidate the effects of the reactivity with H_2O_2 and stability of Mb-I on their oxidation activities and mechanisms.

ABBREVIATIONS

Mb	myoglobin
compound I	a ferryl porphyrin cation radical
Mb-I	compound I of Mb
Mb-II	a ferryl heme of Mb
*m*CPBA	*m*-chloroperbenzoic acid
ABTS	2,2'-azino-*bis*(3-ethylbenzothiazoline-6-sulfonic acid)

2.1 INTRODUCTION

Myoglobin (Mb) is an oxygen transport hemoprotein that catalyzes a variety of oxidation reactions including oxygenation of styrene and thioanisole in the presence of peroxides.[1-6] Ferric Mb reacts with H_2O_2 to form a ferryl heme (Fe^{IV}=O Por), termed Mb-II in this text, and a transient protein radical.[7-9] The styrene epoxidation by wild type Mb has been shown to be mainly mediated by the protein radical with incorporation of an oxygen atom of molecular oxygen (Scheme I).[1,2] A ferryl porphyrin cation radical (Fe^{IV}=O $Por^{+\bullet}$), equivalent of compound I of peroxidase, has not been identified in the wild type yet.



Scheme I. Proposed mechanisms for the reaction of Mb with H_2O_2 and styrene epoxidation catalyzed by Mb

Replacements of distal histidine (His64, Figure 1A) by unoxidizable amino acids are known to enhance the incorporation of an oxygen atom of peroxides, which has been attributed to ferryl oxygen transfer from transiently formed Mb-I (Scheme I), in the H_2O_2-dependent oxygenations (peroxygenation).[2,10,11] Recently, we have shown that His64 Mb mutants react with *m*-chloroperbenzoic acid (*m*CPBA) to form compound I (Mb-I).[11,12] The absence of wild type Mb-I even in the reaction with *m*CPBA suggests rapid oxidation of His64 by Mb-I.[11] The high reactivities of Mb-I in the His64 mutants with styrene and thioanisole[12] strongly suggest that the peroxygenation activity of Mb is controlled both by stability and formation rate of Mb-I. However, the His64 Mb mutants react with H_2O_2 without detectable accumulation of Mb-I.[2,10-12] The rapid reduction of

Mb-I by H_2O_2[12] can rationalize the absence of Mb-I (Scheme I), but there is no direct evidence for the formation of Mb-I when H_2O_2 is used as an oxidant.

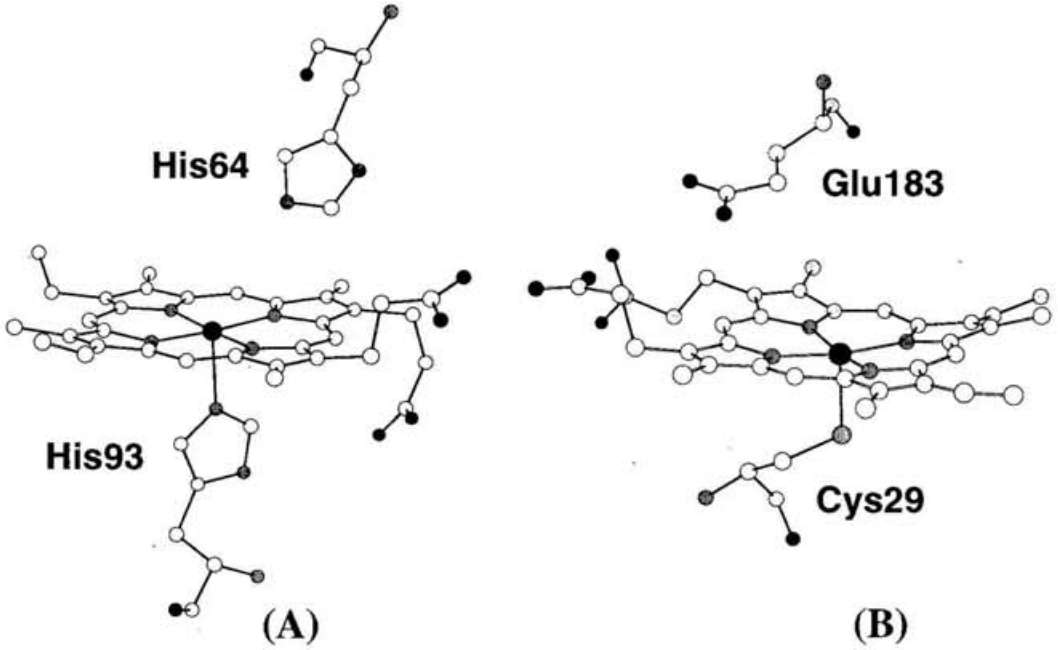
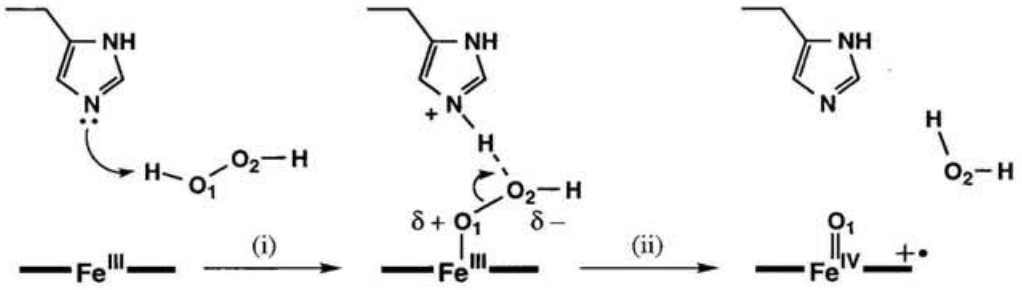


Figure 1. Active site structure of (A) sperm whale Mb and (B) chloroperoxidase.



Scheme II. Proposed mechanism for the reaction of peroxidase with H_2O_2

In this chapter, a novel His64 mutant of sperm whale Mb, H64D Mb, has been prepared to mimic the active site of chloroperoxidase from the fungus *Caldariomyces fumago* (Figure 1B). While classical peroxidases and catalases utilize distal histidine to improve the reactivity with H_2O_2 possibly as a general acid-base catalyst (Scheme II), distal glutamate (Glu183) in chloroperoxidase is suggested to enhance the compound I

formation.[13] The His64 → Asp replacement substantially improves the reactivity of ferric Mb with H_2O_2 and allows us to see the accumulation of Mb-I in the reaction with H_2O_2 for the first time. The H_2O_2-dependent oxidations catalyzed by wild type Mb and a series of His64 Mb mutants are also examined to elucidate the effects of the reactivity with H_2O_2 and stability of Mb-I on their oxidation activities and mechanisms.

2.2 EXPERIMENTAL PROCEDURES

Materials H64D, H64L, H64A, and H64S Mb mutants were constructed by cassette mutagenesis as described in chapter 1 of this part. Procedures for expression and purification of myoglobin mutants were mentioned in chapter 1 of part II.

Reaction with *m*CPBA and Hydrogen Peroxide Measurements of the decay rate of Mb-I to Mb-II and reaction rates of Mb-I with substrates for H64D Mb mutant were carried out on a Hi-Tech SF-43 stopped-flow apparatus equipped with a MG 6000 diode array spectrophotometer at 5.0°C in 50 mM sodium acetate buffer (pH 5.3). Details for the measurements were described in chapter 1 of this part. The reaction with H_2O_2 was also performed on the stopped-flow apparatus at 20°C in 50 mM sodium phosphate buffer (pH 7.0) with monitoring whole spectra.

Assay of the One-Electron Oxidations One-electron oxidation activities of guaiacol and ABTS were measured at 20°C in 50 mM sodium phosphate buffer (pH 7.0) on a Shimadzu UV-2400 spectrophotometer. The formation rate of the guaiacol oxidation product was determined from the increase in the absorbance at 470 nm ($\varepsilon = 2.6 \times 10^4\ M^{-1}\ cm^{-1}$).[14] The 1 ml final assay volume contained 2 mM guaiacol, variable amounts of H_2O_2 (0.2–5 mM), and 1 μM Mb except for the H64D mutant (0.5 μM). The formation of ABTS cation radical was monitored at 730 nm ($\varepsilon = 3.6 \times 10^4\ M^{-1}cm^{-1}$) as noted in chapter 1 of part II. The reaction mixture contained 1 mM ABTS and variable amounts of H_2O_2 (0.1–2 mM). Final concentrations of Mb were 0.5 μM for wild type, H64A, and H64S Mb, 1 μM for H64L Mb, and 10 nM for H64D Mb.

Assay of the Peroxygenation Reactions Peroxygenations of thioanisole and styrene were performed at 20°C in 50 mM sodium phosphate buffer (pH 7.0) or 50 mM sodium acetate buffer (pH 5.3). The reaction mixture contained 10 μM Mb, 1 mM H_2O_2, and either 1 mM thioanisole or 8.7 mM styrene. Incubation and work up for determining oxidation rates were carried out as described in chapter 2 of part II. In order to determine total yields of the *m*CPBA-supported styrene oxidation, 100 μM *m*CPBA was added to a solution containing 10 μM Mb and 8.7 mM styrene. Incubation time was 5 min for wild type Mb and 0.5–1 min for the His64 mutants.

Kinetic measurements for association of cyanide The association rate of ferric Mb with cyanide was measured at 20°C on a Hi-Tech stopped-flow apparatus in 50 mM sodium phosphate buffer (pH 7.0). The kinetic traces at 408 nm were used for determining pseudo-first-order rates. The association rate constants were given by the slope of a plot of the observed rates *versus* cyanide concentration.

Reaction with cumene hydroperoxide The reaction mixture containing 10 μM Mb and 270 μM cumene hydroperoxide was incubated at 20°C in 50 mM sodium phosphate buffer (pH 7.0). The time-dependent formation of cumylalcohol and acetophenone was analyzed by a Shimadzu HPLC system on a reverse phase column (Tosoh inertsil ODS) as described in chapter 1 of part II.

2.3 RESULTS

Stability and Reactivity of Mb-I in H64D Mb

As shown in Figure 2, H64D Mb as well as H64A, H64S, and H64L Mb[12] reacted with *m*-chloroperbenzoic acid (*m*CPBA) to afford a ferryl porphyrin cation radical (Fe^{IV}=O $Por^{+\bullet}$, Mb-I). Because wild type Mb was oxidized by *m*CPBA directly to a ferryl heme (Mb-II),[11] the His64 → Asp replacement was found to significantly prolong the life-time of Mb-I. The H64D Mb-I slowly decayed to Mb-II at the rate of 1.2 s^{-1} at pH 7.0 and 5.0°C, which is essentially the same with corresponding rates in other His64

mutants (0.9–1.2 s^{-1}).[12] Thus, the stabilization of Mb-I upon the His64 → Asp replacement is due to the elimination of the oxidizable histidine in close vicinity of the heme, but not to the anionic nature of Asp64.

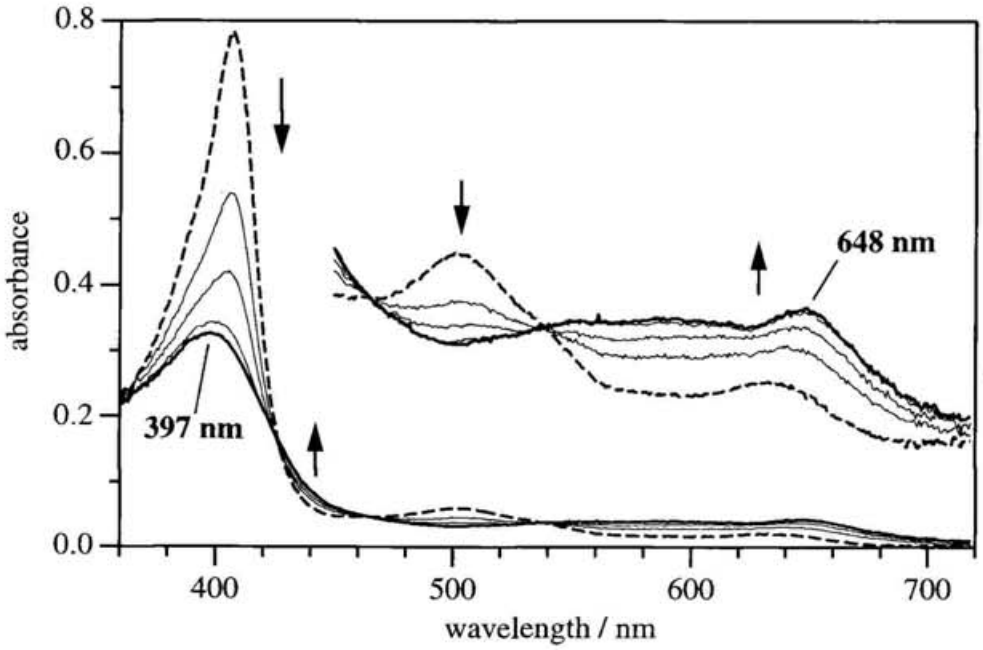


Figure 2. Rapid-scan absorption spectra of H64D Mb in the reaction with *m*CPBA in 50 mM sodium acetate buffer (pH 5.3) at 5.0°C. Broken line represents the spectrum of the ferric mutant and solid lines are spectra recorded at 50, 100, 200, and 300 ms after mixing. Directions of the spectral change were indicated in the figure by arrows.

Table 1 Reduction rate constants of compound I in 50 mM sodium acetate buffer (pH 5.3) at 5.0°C / $mM^{-1}s^{-1}$

	thioanisole	styrene	H_2O_2
H64A	1500	22	5.9
H64S	1500	26	6.2
H64D	220	21	18

The reactions of Mb-I with thioanisole, styrene, and H_2O_2 were performed at pH 5.3 and 5.0°C (Table 1). Upon mixing with the substrates, H64D Mb-I was reduced to the ferric state without appearance of Mb-II as reported for the H64A and H64S

mutants.[12] The spectral changes clearly indicate that H64D Mb-I can oxidize these substrates by two-electron. While the reactivities of Mb-I with styrene do not seem to differ greatly among the His64 mutants, H64D Mb-I exhibited 7-fold lower and 3-fold higher rates in the oxidation of thioanisole and H_2O_2, respectively, than the others (Table 1).

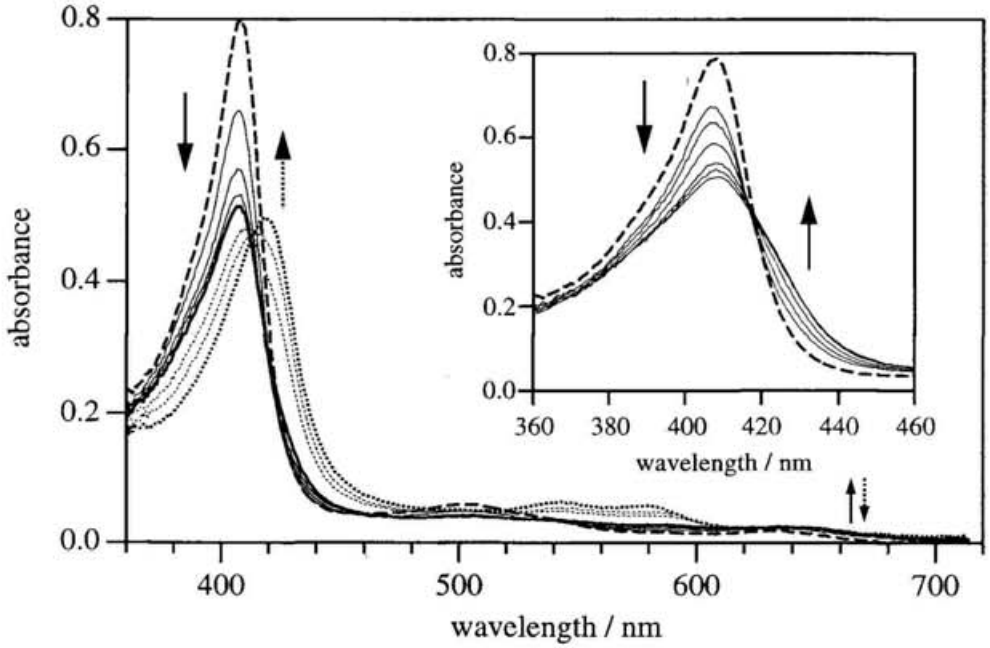


Figure 3. Spectral changes of H64D Mb in the reaction with 1 mM H_2O_2. The spectra were recorded before mixing (*broken line*) and at 10, 30, 60, and 150 ms (*solid line*) and 1, 3, and 6 sec (*dotted line*) after mixing. Directions of absorbance changes are indicated by arrows. (*inset*) The same reaction in the presence of 0.87 mM styrene. The spectra recorded before mixing (*broken line*) and 0.5, 1, 2, 4, 6, and 8 sec (*solid line*) after mixing.

Reaction with H_2O_2

When H_2O_2 was used as an oxidant, wild type Mb showed isosbestic conversion to Mb-II,[7] and the H64A, H64S, and H64L mutants did not show any considerable accumulation of Mb-I.[12] The formation rate of Mb-II in the wild type, that is to say, the reaction rate of the ferric wild type with H_2O_2 ($k_{H_2O_2}$), was determined as 5.1×10^2 $M^{-1}s^{-1}$ at pH 7.0 and 20°C. On the contrary, the H64D mutant reacted with H_2O_2 to

sequentially afford Mb-I and Mb-II (Figure 3). The initial Soret decrease and simultaneous growth of the band around 650 nm are typical of the conversion of ferric Mb to Mb-I (Figure 2). In the presence of styrene (Figure 3 *inset*), only the partial formation of Mb-II was observed presumably due to the rapid reduction of Mb-I by styrene. These results provide direct evidences for the formation of Mb-I in the reaction with H_2O_2 and its involvement in the H_2O_2-dependent styrene oxidation catalyzed by H64D Mb. The approximately 40 % accumulation of Mb-I based on the Soret absorbance means that the formation rate of H64D Mb-I by H_2O_2 is comparable with its reduction rate by H_2O_2 (1.8×10^4 $M^{-1}s^{-1}$ at pH 5.3 and 5.0°C, Table 1). Therefore, the His64 → Asp replacement appears to substantially improve the reactivity of the ferric mutant with H_2O_2. However, the complex reaction mechanism (Scheme 1) disables us to determine the exact rates for the His64 Mb mutants including H64D Mb.

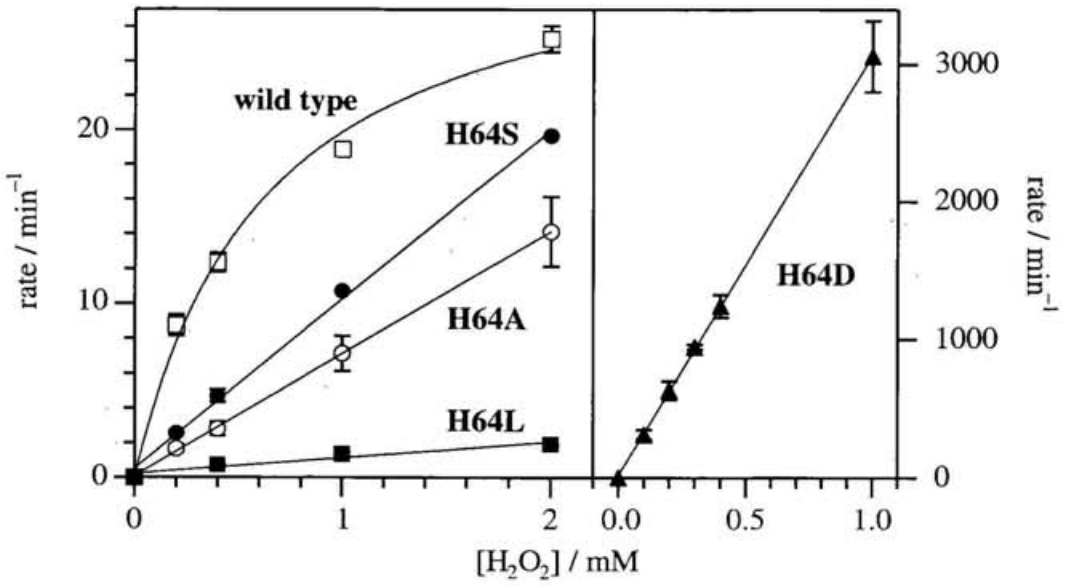


Figure 4. Initial oxidation rates of ABTS as a function of the concentration of H_2O_2 at 20°C in 50 mM sodium phosphate buffer (pH 7.0).

One-Electron Oxidations by Wild Type Mb and Its Mutants

In order to estimate the reaction rate between the ferric Mb and H_2O_2, one-electron oxidations of ABTS and guaiacol supported by H_2O_2 (peroxidations) were examined at 20°C and pH 7.0 (Figure 4). As discussed in chapter 1 of part II, the peroxidation activity of Mb is mainly dependent on the reactivity with H_2O_2 but not on the stability of Mb-I. Also in the case of the His64 Mb mutants, the linear increase of the oxidation rates of ABTS *versus* concentration of H_2O_2 clearly indicates that the reaction of ferric Mb with H_2O_2 is a major rate-determining step for the peroxidase reaction. Although wild type Mb showed a hyperbolic dependency (Figure 4), in the presence of small amount of H_2O_2, the slowest step in the catalytic cycle of the wild type is also the Mb-II formation. Very similar results were observed for the peroxidation of guaiacol by wild type Mb and the His64 mutants. Table 2 summarizes the slopes of the *[H_2O_2]–rate* plots for the mutants, and those determined from the oxidation rates at 0.2 mM H_2O_2 for the wild type.

Table 2. One-electron oxidations catalyzed by wild type Mb and its mutants at 20°C in 50 mM sodium phosphate buffer (pH 7.0) [a]

	guaiacol	ABTS
wild type	5.6	44
H64L	0.017	0.73
H64A	0.99	7.0
H64S	1.3	9.5
H64D	280	3050

[a] The unit is $min^{-1} \cdot mM^{-1}(H_2O_2)$

The His64 → Asp replacement raises the peroxidation activities by 50–70-fold while the other His64 substitutions especially by Leu drop the activities (Table 2). Thus, the ferric H64D Mb is strongly suggested to be highly reactive with H_2O_2 as compared to the wild type. The reaction rate of ferric H64D Mb with H_2O_2 ($k_{H_2O_2}$) can be calculated

from its ABTS oxidation activity (Table 2) to be greater than 2.5×10^4 $M^{-1}s^{-1}$, which is 50-fold higher than that of wild type Mb (5.1×10^2 $M^{-1}s^{-1}$) determined from the formation rate of Mb-II. Since a similar calculation based on the ABTS oxidation activity for the wild type gives 3.7×10^2 $M^{-1}s^{-1}$ as the $k_{H_2O_2}$ value, the ABTS oxidation activity appears to be a good measure of the reactivity of ferric Mb with H_2O_2.

Oxygenation Reactions Catalyzed by Wild Type Mb and Its Mutants

The H_2O_2-dependent oxygenations (peroxygenations) were examined at 20°C and pH 7.0. The H64D mutant showed 600-fold and 200-fold rate increase *versus* wild type Mb for the thioanisole sulfoxidation and styrene epoxidation, respectively (Table 3). Although the His64 mutants gave almost racemic sulfoxide, the enantiomeric excess of the epoxide are over 75 % in H64A, H64S, and H64D Mb (Table 3). The styrene oxidation by the Mb/H_2O_2 systems afforded not only styrene oxide but also phenylacetoaldehyde and benzaldehyde as reported earlier.[6] Plots of the sulfoxidation, epoxidation, and total styrene oxidation activities (epoxide plus aldehydes) *versus* peroxidation activities show roughly linear correlation (Figure 5). The result clearly indicates that the peroxygenation activities of the His64 mutants also strongly depend on the reactivity with H_2O_2 ($k_{H_2O_2}$). As described above, the stability of Mb-I is almost identical among the mutants.

Table 3. H_2O_2-dependent oxygenations catalyzed by Mb at 20°C in 50 mM sodium phosphate (pH 7.0)

	thioanisole sulfoxidation			styrene epoxidation		
	rate / min^{-1}	ee / *R*%	^{18}O from H_2O_2 / %	rate / min^{-1}	ee / *R*%	^{18}O from H_2O_2 / %
wild type	0.25	25	92	0.015	9	20
H64L	0.072	27	89	0.020	34	73
H64A	0.74	6	98	0.074	75	87
H64S	1.7	9	99	0.12	75	86
H64D	145	6	99	3.4	88	95

The peroxygenation activities of wild type Mb are apparently lower than those expected from its peroxidation activity (Figure 5). Only 20 % incorporation of an oxygen atom of H_2O_2 into styrene oxide by the wild type (Table 3) has been attributed to the incorporation of molecular oxygen mediated by a protein radical (co-oxidation, Scheme I).[1,15] Thus, the rapid decay of Mb-I to Mb-II and a protein radical seems to drop the peroxygenation activity of the wild type. It has been shown that Mb-II is unable to epoxidize styrene[16] and the co-oxidation is apparently slower than the oxidation by Mb-I. On the contrary, the His64 mutants mainly incorporate the peroxide oxygen to the epoxide, suggesting Mb-I being responsible for the oxygenations (Table 3, Scheme I). As shown above (Figure 3 *inset*, Table 3), the reactive intermediate of the peroxygenations is Mb-I in the case of H64D Mb.

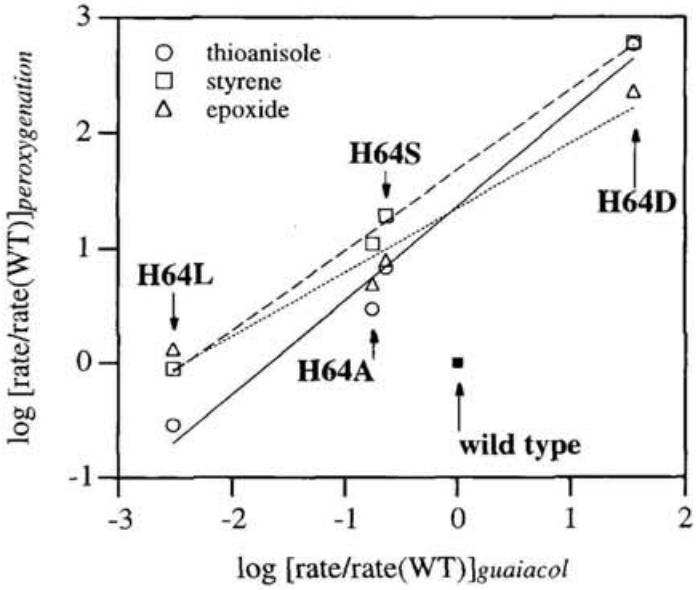


Figure 5. Relationships between guaiacol oxidation activities and peroxygenation activities of wild type Mb and the His64 mutants. All the values were normalized by corresponding rates of the wild type.

General Acid-Base Functions of the Residues at the Position 64

Finally, we have examined the roles of Asp64 in H64D Mb in the activation of H_2O_2 especially as a general acid-base catalyst (Scheme II).[17] In order to evaluate the

capability of Asp64 as a general base, association rates of cyanide (k_{CN}) to the ferric heme were measured at pH 7.0 (Table 4). At neutral pH, cyanide is dominantly protonated (HCN, pKa ~9) and the crucial step for cyanide association has been shown to be the deprotonation of HCN in the distal heme pocket, as suggested for the binding of H_2O_2 (pKa 11.6).[17] The His64 → Asp replacement, however, was found to retard the cyanide association by 10-fold, which is consistent with the lower basicity of the aspartate than histidine.

Table 4. Association rate constants of cyanide and product analysis of the reaction with cumene hydroperoxide [a]

	k_{CN} / $mM^{-1}s^{-1}$	$\frac{\text{cumylalcohol}}{\text{acetophenone}}$
wild type	0.32	3.3
H64L	0.002	3.3
H64A	0.075	3.7
H64S	0.41	4.7
H64D	0.034	4.0

[a] Both experiments were carried out at 20°C in 50 mM sodium phosphate buffer (pH 7.0).

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

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

$$PhC(CH_3)_2O\bullet \longrightarrow PhCOCH_3 + CH_3\bullet \quad (3)$$

The capability of Asp64 as a general acid (Scheme II) was estimated from product analysis in the reaction of the ferric Mb with cumene hydroperoxide. Heterolytic and homolytic cleavage of O–O bond of the peroxide affords cumylalcohol and acetophenone, respectively (eq. 1–3). The acid catalyst selectively enhances the heterolysis, leading to

the increase in the ratio of heterolysis over homolysis (cumylalcohol/acetophenone). As shown in Table 4, the His64 replacements do not seem to greatly improve the cumylalcohol/acetophenone ratios while H64S showed a slightly higher heterolysis ratio. The result indicates that neither His64 nor Asp64 significantly enhances the heterolytic cleavage of the O–O bond of cumene hydroperoxide as a general acid.

2.4 DISCUSSION

Roles of the Distal Aspartate in the Formation of Mb-I

The reaction of ferric Mb with H_2O_2 has been believed to afford compound I (Mb-I) as a transient intermediate. The ferric Mb cleaves the O-O bond of peroxides, at least in part, heterolytically to be oxidized by two-electron.[18] In the H_2O_2-supported oxygenation reactions, partial incorporation of an oxygen atom of the peroxide used has been attributed to a ferryl oxygen transfer from Mb-I.[1,2] One possible explanation for the absence of Mb-I is rapid oxidation of distal histidine (His64, Figure 1A) by Mb-I,[7,8] and its replacement by an unoxidizable amino acid suppresses the styrene co-oxidation mediated by a protein radical (Scheme I).[2] Recently, we have succeeded in direct observation of Mb-I in the reaction of His64 Mb mutants with *m*CPBA, and identified His64 as a critical residue to destabilize Mb-I.[11,12] Nevertheless, there has been no direct evidence for the formation of Mb-I in the presence of H_2O_2 though the absence of Mb-I might be attributable to the rapid reduction of Mb-I by H_2O_2.[12] In order to establish the formation of Mb-I in the reaction with H_2O_2, we have designed a novel H64D Mb mutant based on the active site of chloroperoxidase[13] to improve the reactivity of ferric Mb with H_2O_2 (Figure 1B).

The ferric H64D was almost completely oxidized by *m*CPBA to Mb-I (Figure 2), and about 40 % accumulation of Mb-I was observed in the reaction with H_2O_2 (Figure 3). The stability of H64D Mb-I is almost identical with those in the H64A, H64S, and H64L mutants, and the reaction rate with H_2O_2 is 3-fold higher with H64D Mb-I than the

others (Table 1). Thus, the successful observation of Mb-I in the presence of H_2O_2 is due to the substantial improvement of the reaction rates with H_2O_2 ($k_{H_2O_2}$) upon the His64 → Asp replacement. Although the multi-step reaction mechanism of Mb with H_2O_2 prevents us from determining the exact $k_{H_2O_2}$ values for the His64 mutants (Scheme 1), that of H64D Mb is greater than 2.5×10^4 $M^{-1}s^{-1}$ suggested by its high ABTS oxidation activity (Table 2). Because of the rapid decay of wild type Mb-I to Mb-II, the $k_{H_2O_2}$ value for the wild type can be determined from the formation rate of Mb-II as 5.1×10^2 $M^{-1}s^{-1}$. Therefore, the His64 → Asp replacement is found to enhance the reactivity of ferric Mb with H_2O_2 at least by 50-fold.

Distal glutamate in chloroperoxidase (Figure 1B) as well as distal histidine in classical peroxidases is suggested to enhance the formation of compound I as a general acid-base catalyst (Scheme II).[13,17] Tanaka et al. reported a horseradish peroxidase (HRP) mutant in which distal histidine (His42) was replaced by glutamic acid.[19] While the His42 → Glu substitution retarded the formation rate of compound I of HRP by 2900-fold, the reactivity of the H42E mutant with H_2O_2 was still 35–490-fold higher than other His42 HRP mutants reported.[20-22] They have suggested the distal glutamate to be capable of a general acid-base catalyst; however, their suggestion is based only on the greater reactivity of the H42E mutant than the other His42 mutants.

In order to examine the capability of Asp64 in H64D Mb as a general acid-base catalyst, we have measured the binding rates of cyanide to the ferric heme iron as a model reaction for the binding of H_2O_2 (Table 4). The 10-fold decrease in k_{CN} upon the His64 → Asp replacement indicates lower basicity of Asp64 than His64. Thus, Asp64 in H64D Mb seems inferior to His64 in wild type Mb as a general base to help the binding of H_2O_2. The reaction with cumene hydroperoxide was also carried out to obtain insight into the O–O bond cleavage mode of the heme-bound peroxide (eq. 1–3). The cumylalcohol/acetophenone ratio was not greatly altered by the His64 replacements (Table 4), implying neither His64 nor Asp64 significantly facilitates the heterolytic O–O bond cleavage of the heme-bound peroxide. On the basis of these results, Asp64 does not appear to function as a general acid-base catalyst in the activation of H_2O_2.

Recently, Brittain et al. examined the reactions of His64 Mb mutants with H_2O_2, and found a novel intermediate which was proposed to be a peroxy species (Fe^{3+}–O_2^{2-}).[23] On the basis of the finding, they have suggested that His64 in wild type Mb accelerate the cleavage of the peroxide bond since the accumulation of the peroxy intermediate requires relative impairment of the O–O bond cleavage. Their suggestion is contradict to the result obtained from the reactions with organic peroxides that His64 does not help the heterolysis of the O–O bond (Table 4 and ref. 17). However, Brittain et al. did not take the reduction of Mb-I by H_2O_2 into account although most of the reactions were performed in the presence of a huge excess H_2O_2 (90 mM). Increasing the amount of H_2O_2 facilitates the binding rates of H_2O_2 and the reduction rates of Mb-I but not the cleavage rate of the O–O bond. Under the condition, the accumulation of the peroxy intermediate is also possible even if the O–O bond cleavage is not altered by the His64 substitutions.

Although the Mb-I formation in the reaction with H_2O_2 is apparently facilitated by Asp64 in H64D Mb, its roles in the activation of H_2O_2 are unclear at this point. It may be possible that the highly polar distal heme pocket of the H64D mutant raises the affinity of H_2O_2 to the pocket and/or Asp64 fixes H_2O_2 through a hydrogen-bonding to a preferable position to bind to the heme iron.

Peroxygenation Activity

In the presence of styrene, the H64D mutant did not give detectable amount of Mb-I in the reaction with H_2O_2 (Figure 3 *inset*). The result provides a direct evidence for the involvement of Mb-I in the H_2O_2-dependent peroxygenations catalyzed by the H64D mutant. In addition, dominant incorporation of the peroxide oxygen and high enantioselectivity for the styrene epoxidation catalyzed by the other His64 mutants (Tables 3) indicate that a highly oxidized heme is responsible for the peroxygenations. When *m*CPBA was used as an oxidant, the reactive intermediate of the His64 mutants has been shown to be Mb-I.[12] The His64 mutants showed similar enantioselectivities in the

*m*CPBA- and H_2O_2-dependent epoxidation (data not shown), which also supports the involvement of Mb-I when H_2O_2 was used as an oxidant.

The peroxygenation activities of the His64 Mb mutants exhibit good linear correlation with their peroxidation activities (Figure 4). As described above, the peroxidation activities can be a good measure for the reaction rates of ferric Mb with H_2O_2 ($k_{H_2O_2}$). The stability of Mb-I and its reactivity with styrene and thioanisole are almost identical among the mutants. Thus, the rate-determining step of the peroxygenations catalyzed by the His64 mutants is revealed to be the Mb-I formation by H_2O_2. The $k_{H_2O_2}$ value among the mutants is the highest in H64D Mb (~2.5×10^4 $M^{-1}s^{-1}$), which is essentially the same with the reduction rate of Mb-I by styrene (Table 1). Therefore, under the assay condition (1 mM H_2O_2 and 8.7 mM styrene), the formation of Mb-I should be slower than its reduction by styrene even in the H64D mutant. The other His64 mutants are less reactive with H_2O_2, and thioanisole is more reactive with Mb-I than styrene (Table 1).

Based on the peroxygenation–peroxidation plots for the His64 mutants (Figure 4), the thioanisole and styrene oxidation activities of wild type Mb are respectively calculated to be 23-fold and 47-fold lower than those expected from its peroxidation activity; i.e., the reactivity with H_2O_2. The low peroxygenation efficiency of the wild type can be attributed to the instability of Mb-I since the wild type Mb-I has not been observed even in the reaction with *m*CPBA, and is likely to competitively oxidize His64 and the exogenous substrates. In the case of the wild type, most of the epoxide is produced through the co-oxidation mechanism mediated by a protein radical (Scheme II, Table 3).[1,2] Thus, the net efficiency for the styrene oxidation by Mb-I is much lower than that calculated from Figure 4 (47-fold lower than those in the His64 mutants). The decrease in the sulfoxidation efficiency is smaller than that in styrene oxidation because thioanisole, which is highly reactive with Mb-I than styrene (Table 1), is expected to react with the unstable wild type Mb-I more efficiently.

In summary, a H64D mutant of sperm whale myoglobin have been prepared to mimic the active site of chloroperoxidase. The His64 → Asp replacement considerably improved the reactivity of ferric Mb with H_2O_2 and allowed us to observe compound I of Mb for the first time in the reaction with H_2O_2. The result also provides a direct evidence of Mb-I being responsible for the H_2O_2-dependent peroxygenations. The peroxygenation activities of Mb are clearly shown to be strongly dependent on both the stability and formation rate of Mb-I.

2.5 REFERENCE AND NOTES

(1) Ortiz de Montellano, P. R.; Catalano, C. E. *J. Biol. Chem.* **1985**, *260*, 9265-9271.

(2) Rao, S. I.; Wilks, A.; Ortiz de Montellano, P. R. *J. Biol. Chem.* **1993**, *268*, 803-809.

(3) Tschirret-Guth, R. A.; Ortiz de Montellano, P. R. *Arch. Biochem. Biophys.* **1996**, *335*, 93-101.

(4) Adachi, S.; Nagano, S.; Ishimori, K.; Watanabe, Y.; Morishima, I.; Egawa, T.; Kitagawa, T.; Makino, R. *Biochemistry* **1993**, *32*, 241-252.

(5) Matsui, T.; Nagano, S.; Ishimori, K.; Watanabe, Y.; Morishima, I. *Biochemistry* **1996**, *35*, 13118-13124.

(6) Levinger, D. C.; Stevenson, J.-A.; Wong, L.-L. *J. Chem. Soc., Chem. Commun.* **1995**, 2305-2306.

(7) King, N. K.; Winfield, M. E. *J. Biol. Chem.* **1963**, *238*, 1520-1528.

(8) King, N. K.; Looney, F. D.; Winfield, M. E. *Biochim. Biophys. Acta* **1967**, *133*, 65-82.

(9) Yonetani, T.; Schleyer, H. *J. Biol. Chem.* **1967**, *242*, 1974-1979.

(10) Ozaki, S.; Matsui, T.; Watanabe, Y. *J. Am. Chem. Soc.* **1996**, *118*, 9784-9785.

(11) Ozaki, S.; Matsui, T.; Watanabe, Y. *J. Am. Chem. Soc.* **1997**, *119*, 6666-6667.

(12) Matsui, T.; Ozaki, S.; Watanabe, Y. *J. Biol. Chem.* **1997**, *272*, 32735-32738.

(13) Sundaramoorthy, M.; Terner, J.; Poulos, T. L. *Structure* **1995**, *3*, 1367-1377.

(14) Ozaki, S.; Ortiz de Montellano, P. R. *J. Am. Chem. Soc.* **1995**, *117*, 7056-7064.

(15) Catalano, C. E.; Ortiz de Montellano, P. R. *Biochemistry* **1987**, *26*, 8373-8380.

(16) Choe, Y. S.; Rao, S. I.; Ortiz de Montellano, P. R. *Arch. Biochem. Biophys.* **1994**, *314*, 126-131.

(17) Poulos, T. L.; Kraut, J. *J. Biol. Chem.* **1980**, *255*, 8199-8205.

(18) Allentoff, A. J.; Bolton, J. L.; Wilkis, A.; Thompson, J. A.; Ortiz de Montellano, P. R. *J. Am. Chem. Soc.* **1992**, *114*, 9744-9749.

(19) Tanaka, M.; Ishimori, K.; Mukai, M.; Kitagawa, T.; Morishima, I. *Biochemistry* **1997**, *36*, 9889-9898.

(20) Erman, J. E.; Vitello, L. B.; Miller, M. A.; Shaw, A.; Brown, K. A.; Kraut, J. *Biochemistry* **1993**, *32*, 9798-9806.

(21) Newmyer, S. L.; Ortiz de Montellano, P. R. *J. Biol. Chem.* **1995**, *270*, 19430-19438.

(22) Rodriguez-Lopez, J. N.; Smith, A. T.; Thorneley, R. N. F. *J. Bioinorg. Chem.* **1996**, *1*, 136-142.

(23) Brittain, T.; Baker, A. R.; Butler, C. S.; Little, R. H.; Lowe, D. J.; Greenwood, C.; Watmough, N. J. *Biochem J* **1997**, *326*, 109-115.

PART IV

SUMMARY AND CONCLUSIONS

In the present thesis, the author has aimed to endow hemoproteins with novel catalytic functions. The author has employed sperm whale myoglobin (Mb) has been employed as a model hemoprotein for his purpose, and some Mb mutants have been prepared by site-directed mutagenesis.

Part II described effects of the location of distal histidine (His64) on oxidation reactions catalyzed by Mb. In order to examine the effects, His64 was replaced by apolar leucine, and the novel distal histidine was introduced at the positions 29 and 43, yielding L29H/H64L and F43H/H64L Mb. The His43 in the F43H/H64L mutant can be expected to be located at a similar distance from the heme iron to the distal histidine in peroxidase. The His29 in the L29H/H64L mutant is 1.0 Å farther from the heme center than that in peroxidase. The effects of the L29H/H64L and F43H/H64L double-mutations on the reaction with H_2O_2 and one-electron oxidations were examined in chapter 1. Chapters 2 and 3 described the effects of the mutations on the peroxygenations and stability of catalytic intermediate.

The F43H/H64L mutant reacted with H_2O_2 11-fold faster than the wild type whereas the L29H/H64L mutation retarded the reaction. The enhanced reactivity of F43H/H64L Mb can be, at least partly, attributed to the capability of His43 to facilitate the heterolytic cleavage of O-O bond of peroxides as suggested by the product analysis in the reaction with cumene hydroperoxide. Neither His64 in the wild type nor His29 in the L29H/H64L mutant appeared to enhance the heterolysis. The varied reactivities with H_2O_2 were reflected on the one-electron oxidation activities, in which rate-determining step was revealed to be the formation of reactive intermediates by H_2O_2.

The peroxygenation activities of F43H/H64L and L29H/H64L Mb were much higher than those expected from the varied reactivity of the mutants with H_2O_2. The double substitution enhanced the incorporation of a peroxide oxygen into substrates, and allowed us to observe compound I of Mb (Mb-I) in the reaction with *m*-chloroperbenzoic acid (*m*CPBA). Thus, the drastic increase in the peroxygenation activity can be attributed to the efficient ferryl oxygen transfer from Mb-I. These results suggest that His64 is too close to the heme center to be free from the oxidation by Mb-I.

Interestingly, the distal histidines in the double mutants appeared to regulate the binding mode of the substrates drastically to enhance the enantioselectivities for the peroxygenations, especially in L29H/H64L Mb.

Part III dealt with the reactive intermediate, compound I of Mb (Mb-I), which has never been observed before. In order to establish its destabilization by His64, substrate oxidations, its formation and catalytic roles in the H_2O_2-dependent oxidations, a series of His64 Mb mutants, H64A, H64S, H64L (chapter 1), and H64D (chapter 2), were prepared.

All the His64 mutants reacted with *m*CPBA to afford Mb-I. The high oxidizing ability of Mb-I was elucidated by double-mixing experiments. In spite of the rapid reduction of Mb-I by H_2O_2, H64D Mb could accumulate Mb-I in the reaction with H_2O_2 due to at least 50-fold increase in the formation rate of Mb-I by H_2O_2 *versus* wild type Mb. These results demonstrate that the peroxygenation activity of Mb is strongly dependent both on the formation rate and stability of Mb-I, and that His64 in wild type Mb substantially decreases the two-electron oxidation activity.

Throughout the work in part II, the location of the distal histidine is clearly indicated to be an important factor for controlling the reactivity with H_2O_2, oxidation activity, and the stability of the catalytic intermediate. The studies in part III shed light on a long-standing "mystery of myoglobin compound I." Compound I of Mb is substantially destabilized by distal histidine (His64) while it is the catalytic intermediate in the H_2O_2-supported oxidation reactions in the absence of His64.

ACKNOWLEDGMENT

The present thesis is a summary of the author's studies from 1995 to 1997 at Department of Structural Molecular Science, the Graduate University for Advanced Studies. This work is generously supported by Institute for Molecular Science and carried out under the supervision of Prof. Yoshihito Watanabe. The author wishes to express his cordial gratitude to Prof. Yoshihito Watanabe for his continual direction, stimulating discussion, and hearty encouragement. The author also owes his accomplishment of the studies to the discerning advice and helpful discussion by Dr. Shin-ichi Ozaki.

The author is really grateful to Prof. John S. Olson and Prof. George N. Phillips, Jr. (Rice University) for providing cDNA of sperm whale myoglobin and X-ray structural analysis, Dr. Akio Murakami (National Institute for Basic Biology) for his help in measurement of catalase activity, Prof. Teizo Kitagawa and Dr. Masahiro Mukai (Institute for Molecular Science) for resonance Raman measurements.

This work would not have been possible without help of the members and co-workers in the Prof. Watanabe's laboratory. The author is deeply indebted to Drs. Senji Wada, Seiji Ogo, Mark P. Roach, Messrs. Yoshio Goto, Masakazu Iwase, Akira Wada, Shigeyuki Nakamura, Miss Miki Kanazawa, and Mrs. Yang Hui-Jun for valuable suggestion and unfailing encouragement. Acknowledgments are also due to Mrs. Misako Tanizawa and Mrs. Akiyo Ota for their office work and heartfelt kindness.

Finally, the author expresses his sincere gratitude to his parents for their supports, generous understanding, and affectionate encouragement.

Okazaki, November 1997

Toshitaka Matsui

LIST OF PUBLICATIONS

1. Conversion of Myoglobin into a Highly Stereospecific Peroxygenase by the L29H/H64L Mutation.

   Shin-ichi Ozaki, Toshitaka Matsui, and Yoshihito Watanabe

   J. Am. Chem. Soc. **1996**, *118*, 9784-9785.

2. Conversion of Myoglobin into a Peroxygenase: a Catalytic Intermediate of Sulfoxidation and Epoxidation by the F43H/H64L Mutant.

   Shin-ichi Ozaki, Toshitaka Matsui, and Yoshihito Watanabe

   J. Am. Chem. Soc. **1997**, *119*, 6666-6667.

3. On the Formation and Reactivity of Compound I of the His64 Myoglobin Mutants.

   Toshitaka Matsui, Shin-ichi Ozaki, and Yoshihito Watanabe

   J. Biol. Chem. **1997**, *272*, 32735-32738.

OTHER PUBLICATIONS

1. Studies on the Oxygen Activation Mechanisms by Heme Enzymes: Usage of Hemoprotein Mutants and Synthetic Heme Models.

   Yoshihito Watanabe and Toshitaka Matsui

   J. Syn. Org. Chem. Jpn., **1996**, *54*, 1045-1054.

2. Preparation and Reactions of Myoglobin Mutants Bearing both Proximal Cysteine Ligand and Hydrophobic Distal Cavity: Protein Models for the Active Site of P-450.

   Toshitaka Matsui, Shingo Nagano, Koichiro Ishimori, Yoshihito Watanabe, and Isao Morishima

   Biochemistry **1996**, *35*, 13118-13124.

3. Effects of the Location of Distal Histidine on the Reaction of Myoglobin with Hydrogen Peroxide.

   Toshitaka Matsui, Shin-ichi Ozaki, and Yoshihito Watanabe

   in preparation

4. Formation and Reactions of Compound I in the Hydrogen Peroxide-Dependent Oxidations by the His64 Myoglobin Mutants.

   Toshitaka Matsui, Shin-ichi Ozaki, and Yoshihito Watanabe

   in preparation

5. Effects of the Arrangement of a Distal Histidine on Regioselectivity of the Coupled Oxidation of Sperm Whale Myoglobin Mutants.

   Tatsuya Murakami, Isao Morishima, Toshitaka Matsui, Shin-ichi Ozaki, and Yoshihito Watanabe

   J. Chem. Soc., Chem. Commun. in press.

6. Preparation, Crystal Structure and Electrical Properties of Dimethyl Substituted Unsymmetrical 2,5-Bis(1',3'-dithiol-2'-ylidene)-1,3,4,6-tetrathia-pentalenes.

   Yohji Misaki, Kazuya Kawakami, Toshitaka Matsui, Tokio Yamabe, and Motoo Shiro

   J. Chem. Soc., Chem. Commun. **1994**, 459-460.

7. 2,5-Bis(1',3'-dithiol-2'-ylidene)-1,3,4,6,-tetrathia-pentalene and Its Related Unsymmetrical Donors.

   Yohji Misaki, Toshitaka Matsui, Kazuya Kawakami, Hiroyuki Nishikawa, Tokio Yamabe, and Motoo Shiro

   Chem. Lett. **1993**, 1337-1340.