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

To clarify how the location of distal histidine affects the activation process of H₂O₂ by heme proteins, we have characterized reactions with H₂O₂ for the L29H/H64L and F43H/H64L mutants of sperm whale myoglobin (Mb), designed to locate the histidine farther from the heme iron. Whereas the L29H/H64L double substitution retarded the reaction with H₂O₂, an 11-fold rate increase versus wild-type Mb was observed for the F43H/H64L mutant. The V₅₀ₐₓ values for 1-electron oxidations by the myoglobins correlate well with the varied reactivities with H₂O₂. The functions of the distal histidine as a general acid-base catalyst were examined based on the reactions with cumene hydroperoxide and cyanide, and only the histidine in F43H/H64L Mb was suggested to facilitate heterolysis of the peroxide bond. The x-ray crystal structures of the mutants confirmed that the distal histidines in F43H/H64L Mb and peroxidase are similar in distance from the heme iron, whereas the distal histidine in L29H/H64L Mb is located too far to enhance heterolysis. Our results indicate that the proper positioning of the distal histidine is essential for the activation of H₂O₂ by heme enzymes.

Peroxidase is a heme enzyme that catalyzes 1-electron oxidations of a variety of substrates (1, 2). The ferric enzyme is oxidized by H₂O₂ to yield a ferryl porphyrin cation radical (FeV=O Por⁺) known as compound I in the first step of the catalytic cycle (3). Compound I is reduced to the ferric state through a ferryl species (FeV=O Por), so-called compound II, by two sequential 1-electron oxidations of substrates. The invariant histidine in the distal heme pocket (1–6) is a critical residue for peroxidases, and its replacement by aliphatic residues retards compound I formation by 5–6 orders of magnitude (7–9). As shown in Scheme I, the distal histidine is believed to function (i) as a general base to accelerate binding of H₂O₂ to the ferric heme iron by deprotonating the peroxide and (ii) as a general acid to facilitate the heterolytic cleavage of the O–O bond of a plausible FeIV-OOH complex by protonating the terminal oxygen atom (10). The charge separation in heterolysis is suggested to be also enhanced by a positively charged distal arginine (Scheme I), whose substitution results in 2 orders of magnitude slower formation of compound I (10–12).

Myoglobin (Mb),¹ a carrier of molecular oxygen, similarly possesses a distal histidine (His-64) in the heme pocket (Fig. 1) and can catalyze various oxidation reactions using H₂O₂ (13–19). However, Mb reacts with H₂O₂ much slower (~10² m⁻¹ s⁻¹) than peroxidases (~10⁻² m⁻¹ s⁻¹) to afford ferryl Mb (Mb-II) paired with a transient protein radical (Scheme II). Compound I of wild-type Mb (Mb-I) has not been observed and is considered to decay to Mb-II immediately (Scheme II). The poor reactivity of Mb with H₂O₂ might be partly due to the malfunction of the distal histidine as a general acid-base catalyst and the absence of the distal arginine. Whereas the distal histidine in peroxidase is suggested to raise the basicity of imidazole by a hydrogen bond with the adjacent asparagine (20, 21), the absence of the hydrogen bond in Mb (13, 14) is indicative of less basicity of its distal histidine. Furthermore, wild-type Mb cleaves the O–O bond of the heme-bound peroxide not only heterolytically, but also homolytically to give Mb-II and a hydroxy radical as shown in Scheme II (22–24). It has been suggested that the distal histidine in Mb is unable to enhance heterolysis as a general acid (25); however, the structural factor for this inability is still obscure.

Comparison of crystal structures of sperm whale Mb and horseradish peroxidase led us to propose that the distal histidine in Mb is too close to the heme center to enhance heterolysis as a general acid catalyst (Fig. 1) (14, 26). The distances between N° of the distal histidine and the ferric heme iron are normally 4.1–4.6 Å for globins (4.3 Å for sperm whale Mb) and 5.5–6.0 Å for peroxidases (6.0 and 5.6 Å for horseradish peroxidase and cytochrome c peroxidase, respectively) (6, 14, 26). To examine our hypothesis, we have studied the reactions of ferric wild-type, L29H/H64L, and F43H/H64L sperm whale Mb with H₂O₂ because the estimated iron-distal histidine distances for the L29H/H64L and F43H/H64L mutants are 6.6 and 5.4 Å, respectively (27, 28). The functions of distal histidines as general acids and general bases are examined based on the ratio of heterolysis versus homolysis of the O–O bond in cumene hydroperoxide and association rate constants of cyanide and azide, respectively. The F43H/H64L mutant shows the highest reactivity with H₂O₂, and its distal histidine functions as a general acid-base catalyst most efficiently. Furthermore, the x-ray crystal structures of the double mutants in this report confirm that the distal histidine in F43H/H64L Mb is at a

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1 The abbreviations used are: Mb, myoglobin; Mb-I, compound I of Mb; Mb-II, compound II of Mb; HPLC, high pressure liquid chromatography; mCPBA, m-chloroperoxybenzoic acid; CHP, cumene hydroperoxide.
similar distance from the heme iron compared with those in peroxidases, whereas the distal histidine in L29H/H64L Mb is located too far to activate H$_2$O$_2$.

**EXPERIMENTAL PROCEDURES**

**Preparation of Myoglobin Mutants**—The expression vectors for wild-type and H64L sperm whale Mb were gifts from John Olson (Rice University) (29, 30). L29H and F43H mutations with new silent HindIII and PvuI sites, respectively, were introduced by use of a polymerase chain reaction-based technique. Expression in Escherichia coli strain and some selected residues including distal histidines (His-64 in Mb and His-42 in horseradish peroxidase) in crystal structures of sperm whale Mb (ball and stick) and horseradish peroxidase (thin lines). A, side view; B, top view. HRP, horseradish peroxidase.

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

**Reactions with Hydrogen Peroxide**—All reactions of ferric Mb with H$_2$O$_2$ were carried out in 50 mM sodium phosphate buffer (pH 7.0). Formation rates of a ferryl heme in wild-type and F43H/H64L Mb (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 observed rate versus [H$_2$O$_2$] concentration. The amounts of H$_2$O$_2$ were kept >10 molar eq over Mb for ensuring the pseudo first-order condition ([Mb] = 1.0 and 0.5 $\mu$M for wild-type and F43H/H64L Mb, respectively). Whole spectral changes of rapid reactions were monitored by using a Hi-Tech SF-43 stopped-flow apparatus equipped with an MG 6000 diode array spectrophotometer.

The catalase activity of Mb was measured at 25 °C from amounts of molecular oxygen formed with a Hansatech DW1 oxygen electrode. The reaction mixture contained 10 $\mu$M Mb and 1 mM H$_2$O$_2$.

**Activations of Oxidation Activities**—1-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 oxidations of guaiacol and ABTS (2,2’-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)) were obtained by measuring the initial rates while 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 absorbance at 470 nm using a molar extinction coefficient of 3.8 × 10$^3$ M$^{-1}$ cm$^{-1}$. The 1-mL final assay volume contained 1 $\mu$M Mb, 0.2 mM H$_2$O$_2$, and variable amounts of guaiacol (0.08–2.5 mM). The formation of an ABTS cation radical was monitored at 730 nm, where the absorption of Mb was negligible (19). The absorption coefficient of the ABTS cation radical at 730 nm ($\varepsilon$$_{353}$ = 1.4 × 10$^4$ M$^{-1}$ cm$^{-1}$) was calculated from that at 415 nm (3.6 × 10$^3$ M$^{-1}$ cm$^{-1}$) (33). The reaction mixture contained 0.5 $\mu$M Mb, 0.2 mM H$_2$O$_2$, and 20–300 $\mu$M ABTS.

**Reaction with Cumene Hydroperoxide**—A reaction mixture containing 10 $\mu$M Mb and 270 $\mu$M 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
Activation of Hydrogen Peroxide by Myoglobin Mutants

RESULTS

Spectroscopic Features of L29H/H64L and F43H/H64L Mb Mutants—The ferric F43H/H64L and L29H/H64L Mb mutants exhibited absorption spectra similar to that of wild-type Mb (Fig. 2). The Soret maxima is around 408 nm, which indicates a typical hexa-coordinated ferric high-spin heme. The sixth ligand in wild-type Mb is a water molecule stabilized by His-64 through hydrogen bonding (13, 14). Since the loss of water ligation in H64L causes a Soret shift to 393 nm (Fig. 2) (36), the novel histidines in the double mutants appear to stabilize the heme-bound water. The absorption maxima of the ferric CN, ferrous, and ferrous CO forms of the L29H/H64L and F43H/H64L Mb mutants are essentially identical to the corresponding states of wild-type and H64L Mb (data not shown).

Fig. 3 presents hyperfine-shifted $^1$H NMR spectra of the 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. Although the heme methyl signals of the mutants were slightly downfield-shifted compared with those of wild-type Mb (mean methyl shift: 74.6, 77.3, and 78.3 ppm for wild-type, L29H/H64L, and F43H/H64L Mb, respectively), the double mutations did not greatly alter the whole spectra. A similar shift of the heme methyl protons has been also reported for various His-64 Mb mutants (37, 38). Therefore, the introduction of histidines at positions 29 and 43 does not appear to greatly alter the electronic structures of the ferric hemes.

Reactions of Ferric Myoglobins with $H_2O_2$—Ferric wild-type Mb reacted with $H_2O_2$ to yield a ferryl heme (Fe$^{IV}=O$ Por), equivalent to compound II of peroxidase, paired with a protein radical as reported previously (22–24). The ferryl heme (Mb-II) formation in wild-type Mb showed isosbestic points and obeyed pseudo first-order kinetics. A ferryl porphyrin cation radical (Fe$^{IV}=O$ Por$^+$) for wild-type Mb similar to compound I in horse-radish peroxidase (39) has not been observed, probably due to its rapid decay to Mb-II and a protein radical (Scheme II). We have recently shown that the distal histidine (His-64) in wild-type Mb plays a crucial role in destabilizing compound I (40).

The reaction between ferric F43H/H64L and $H_2O_2$ also occurred in nearly isooisoelectric conversion to Mb-II (Fig. 4A). The kinetic trace obeyed pseudo first-order kinetics in the incubation with 25 $\mu$M $H_2O_2$, but not with 500 $\mu$M $H_2O_2$ (Fig. 4B). In the presence of a large amount of $H_2O_2$, the apparent deviation of the trace from single exponential curvature reveals a slight accumulation of an intermediate species, which was not clearly observed by varying $H_2O_2$ concentration, pH, and temperature. The most probable candidate for the intermediate is compound I (Mb-I) because F43H/H64L Mb-I is stable enough for its direct observation when the oxidant is $m$-chloroperbenzoic acid (mCPBA) (28). The lesser accumulation of Mb-I with $H_2O_2$ than mCPBA could be attributed to the rapid reduction of Mb-I by $H_2O_2$ to the ferric state ($2 \times 10^6$ $s^{-1}$, $M^{-1}$) for F43H/H64L Mb at 5.0 $\mathrm{pH}$ and p5.3 (28, 40). In fact, F43H/H64L Mb dismutated $H_2O_2$ to molecular oxygen and water at a 50-fold higher rate than wild-type Mb (Table I). Therefore, F43H/H64L Mb-I is formed in the reaction with $H_2O_2$, and conceivable reactions are summarized in Scheme III.

The reaction rates of ferric wild-type and F43H/H64L Mb with $H_2O_2$ were determined under the condition where the Mb-I accumulation was negligible. As noted above, there is no kinetic evidence for the formation of wild-type Mb-I. When incubated with a low concentration of $H_2O_2$ (5.0–20 $\mu$M), F43H/H64L Mb-I did not appear to be formed. Plots of the observed...
formation rates of Mb-II versus H₂O₂ concentrations showed good linearity (Fig. 4B, inset). The rate constant for the F43H/H64L mutant is $5.6 \times 10^3$ M⁻¹ s⁻¹, which is 11-fold higher than that for wild-type Mb (Table I). The slight accumulation of F43H/H64L Mb-I with H₂O₂ (Fig. 4A) is consistent with the fact that F43H/H64L Mb-I is more reactive with H₂O₂ than its ferric form. In the incubation with 20 mM H₂O₂, F43H/H64L Mb-I decayed much faster (9.2 s⁻¹ at 5.0 °C and pH 5.3) than its formation or reduction by H₂O₂ (0.1 and 0.4 s⁻¹, respectively).

The ferric L29H/H64L and H64L mutants showed few absorption spectra changes upon the addition of H₂O₂. Nevertheless, L29H/H64L and H64L Mb slowly consumed H₂O₂ (0.11 and 0.003 min⁻¹, respectively, at [H₂O₂] = 50 μM) and produced a protein radical as reported for H64V Mb (16). It is likely that the ferric mutants react with H₂O₂, but much slower than the decay of the oxidized heme to the ferric state. L29H/H64L Mb-I prepared by mCPBA, as well as F43H/H64L Mb-I, was immediately reduced by H₂O₂ to the ferric state, although a requirement for a large excess of mCPBA for the Mb-I preparation prevented us from determining the exact reaction rate. The catalase activity of the L29H/H64L mutant was 5-fold higher than that of wild-type Mb (Table I). Because of the mutants being in the ferric form during incubation with H₂O₂, the reactivity of ferric L29H/H64L and H64L Mb with H₂O₂ can be estimated from the consumption rates of H₂O₂ to be ~3–6- and ~100-fold lower than that of wild-type Mb, respectively. The results reveal that the enhancement of H₂O₂ activation by His-64 is more effective than that by His-29.

**Oxidation Activities of Wild-type Mb and Its Mutants—**

Electron oxidations of guaiacol and ABTS were examined at pH 7 using H₂O₂ as an oxidant. The initial rates of oxidation by Mb showed hyperbolic dependence on the concentration of the substrates under the conditions employed. Table II summarizes V_max and K_m values for the oxidations. F43H/H64L Mb exhibited ~6-fold higher V_max values than wild-type Mb (Table I). The changes in the V_max values correlate well with those observed for the reactivities of ferric Mb with H₂O₂, i.e. 11-fold higher versus wild-type Mb in F43H/H64L Mb (Table I) and 3–6-fold lower in L29H/H64L Mb. Steady-state absorption spectra of wild-type Mb during the guaiacol oxidation (Fig. 5) indicated that most of wild-type Mb existed as Mb-II in the presence of 40 μM guaiacol, but as the ferric form with 4 mM guaiacol. Therefore, the rate-determining step in the presence of a large excess amount of guaiacol is the reaction of ferric Mb with H₂O₂.

**Reaction with Cumene Hydroperoxide—**

To examine the capability of the distal histidines as general acids (Scheme I), the reaction of ferric Mb with cumene hydroperoxide (CHP) was performed. The heterolytic O–O bond cleavage of CHP is
Roles of the Distal Histidine in the Reaction with Hydrogen Peroxide—We have prepared L29H/H64L and F43H/H64L mutants—

As described above, the L29H/H64L and F43H/H64L mutants showed similar $k_{CN}$ values (Table IV), the accessibility to the heme center of the mutants does not seem to greatly differ from each other. Thus, it is likely that His-43, as well as His-64, facilitates cyanide binding as a general base. The $k_{CN}$ value for the L29H/H64L mutant was 40-fold lower than that of wild-type Mb, whereas wild-type and L29H/H64L Mb reacted with azide anion at similar rates (Table IV). Thus, His-29 in the L29H/H64L mutant appears to be a less effective base than His-64 and His-43 in supporting cyanide binding.

Crystal Structures of Ferric L29H/H64L and F43H/H64L Mb Mutants—As described above, the L29H/H64L and F43H/H64L mutations to rearrange the distal histidine strongly affected the reactions of Mb with peroxides and anions. To verify the location of the distal histidine, the crystal structures of L29H/H64L and F43H/H64L Mb were determined at 1.8-Å resolution (Fig. 6). The data collection and refinement statistics are listed in Table V. Both Mb mutants exhibited few structural changes upon the double mutations outside the immediate vicinity of the substituted residues (Fig. 6). The distal histidines in the mutants (His-29 in L29H/H64L Mb and His-43 in F43H/H64L Mb) are directed to the heme center. The coordination structures of the ferric heme iron (aquohexacoordinations) are consistent with their absorption spectra (Fig. 2). The coordinated water molecules appear to be stabilized by a hydrogen bond with the distal histidine through another water molecule in the active site (Fig. 6). The distance between N$^\text{e}$ of the distal histidine and the ferric heme iron is 5.7 Å in F43H/H64L Mb, which is similar to distances in structurally known peroxidases (5.5–6.0 Å). In contrast, the distal histidine in L29H/H64L Mb is located farther (6.6 Å) from the heme iron than in peroxidases.

The general acid catalyst is expected to selectively enhance heterolysis, which should raise the ratio of heterolysis over homolysis (cumyl alcohol/acetophenone). As shown in Table IV, the cumyl alcohol/acetophenone ratio was 2.8 for H64L Mb, whereas wild-type and L29H/H64L Mb reacted with azide anion at similar rates (Table IV). Thus, His-29 in the L29H/H64L mutant appears to be a less effective base than His-64 and His-43 in supporting cyanide binding.

\[ \text{Fe}^{III} \text{Por} + \text{PhC(CH}_3)_2\text{OOH} \xrightarrow{\text{heterolysis}} \text{Fe}^{IV}\text{O Por} + \text{PhC(CH}_3)_2\text{OH} \] (Eq. 1)

On the other hand, homolysis of the O–O bond gives compound II and the cumyloxy radical, which subsequently eliminates the methyl radical to afford acetophenone (Equations 2 and 3) (41).

\[ \text{Fe}^{III} \text{Por} + \text{PhC(CH}_3)_2\text{OOH} \xrightarrow{\text{homolysis}} \text{Fe}^{IV}\text{O Por} + \text{PhC(CH}_3)_2\text{O}^- + \text{H}^+ \] (Eq. 2)

\[ \text{PhC(CH}_3)_2\text{O}^- \rightarrow \text{PhCOCH}_3 + \text{CH}_3^- \] (Eq. 3)

The general acid catalyst is expected to selectively enhance heterolysis, which should raise the ratio of heterolysis over homolysis (cumyl alcohol/acetophenone).

As shown in Table IV, the cumyl alcohol/acetophenone ratio was not affected by the H64L substitution, indicating that His-64 in wild-type Mb does not facilitate the heterolysis of the peroxide bond as reported earlier (25). While L29H replacement did not alter the ratio greatly, the F43H/H64L mutant showed a higher cumyl alcohol/acetophenone ratio than the others (Table III). Thus, only His-43 in F43H/H64L Mb 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—Roles of the distal histidine in H$_2$O$_2$ binding (Scheme I) were proposed on the basis of rates of association of cyanide ($k_{CN}$) with the ferric heme iron at pH 7 (Table IV). Most of the cyanide is protonated at neutral pH ($pK_a \approx 9$), and the crucial step for the cyanide association with ferric Mb has been shown to be the deproto-
Possible interactions between the distal histidines and heme-bound peroxide are depicted in Scheme IV. Our results strongly suggest that the distal histidine in wild-type Mb (His-64) is too close to the heme center to support heterolysis of the peroxide bond to generate compound I. His-64 may interact with both oxygen atoms in the ferric-peroxide complex (Scheme IV). In ferrous oxy-Mb (with structural relevance to the ferric-peroxide complex), heme-bound and terminal oxygen atoms are similar distances from N\text{64} of His-64 (2.7 and 3.0 Å, respectively) (36). Therefore, although His-64 in oxy-Mb forms a hydrogen bond with the terminal oxygen (43), the iron-bound oxygen atom in the ferric-peroxide complex could also interact with the distal histidine. In fact, the heme-bound water in ferric wild-type Mb makes a direct hydrogen bond with His-64 (13, 14). The interaction of the distal histidine with both oxygen atoms of the iron-bound peroxide (Scheme IV) does not facilitate the charge separation, as suggested for the transition state of the heterolysis (Scheme I).
are rationalized in terms of roles of the distal histidine as a general acid-base catalyst. Whereas His-64 in wild-type Mb functions only as a general base, His-43 in the F43H/H64L mutant is suggested to work as a general acid-base catalyst. It appears that His-29 in the L29H/H64L mutant lacks complete acid-base functionality. Thus, the proper positioning of the distal histidine in heme enzymes is essential for the activation of H$_2$O$_2$ especially as a general acid. High catalytic activities are also observed for the F43H/H64L mutant because of the highest reactivity with H$_2$O$_2$ and the prolonged lifetime of Mb-I.

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