Kinetic and Spectroscopic Characterization of a Hydroperoxy Compound in the Reaction of Native Myoglobin with Hydrogen Peroxide*

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The reaction of metmyoglobin with H₂O₂ was investigated in a pH range between 8.5 and 6.0 with the aid of stopped flow-rapid scan and rapid freezing-EPR techniques. Singular value decomposition analyses of the stopped flow data at pH 8.5 revealed that a spectral species previously unknown accumulated during the reaction and exhibited a Soret absorption maximum at ≥423 nm. In the EPR experiments, the new species exhibited a set of g values at 2.32, 2.19, and 1.94, indicating that the species was assignable to a ferric hydroperoxy (Fe(III)[O–O–H]) intermediate species, which oxidizes the substrates (11, 15–19). In this context, the formation of compound I, which was derived from the oxygen-oxygen bond cleavage of the hydroperoxy compound relative to compound I showed a pH dependence with an apparent pKₙ of 7.27 depending on the metmyoglobins examined. This variation in pKₙ paralleled that in pKₘ of metmyoglobins, suggesting that the accumulation of hydroperoxy compound is controlled by the distal histidine. We propose that the H₂O₂ activation by metmyoglobin is promoted at the acidic condition due to the imidazolium form of the distal histidine, and we further propose that the controlled protonation state of the distal histidine is important for the facile O–O bond cleavage in heme peroxidases.

A large number of heme enzymes catalyze heterolysis of hydrogen peroxide (H₂O₂)¹ and utilize H₂O₂ as a source of oxidizing equivalents for biological oxidative reactions. The enzymes include a family of heme peroxidases from plants, yeasts, fungi, and mammals (1–6), soybean peroxygenase (7), and bacterial and fungal catalase peroxidases (8–10). High valent intermediates such as compounds I and II, which possess an oxoferryl porphyrin π-cation radical (Por’-[Fe(IV)O]²⁺), where Por’ represents porphyrin π-cation radical and an oxoferryl heme (Por-[Fe(IV)O]²⁺), respectively, are known to be created upon the reaction of the heme enzymes with H₂O₂ and to carry out various oxidation reactions (1–4). Compound I is the first detectable intermediate in the reactions of the heme enzymes previously studied, and the subsequent one-electron reduction of compound I by the substrate generates compound II (1–4). In some enzymes such as cytochrome c peroxidase (CcP), the oxidizing equivalent on the π-cation heme of compound I is rapidly transferred to an amino acid residue in the protein moiety, creating another type of compound I (CcP-type compound I), which oxidizes the substrates (2). The chemical and enzymatic properties of the reaction between ferric heme and H₂O₂ have attracted considerable research interests to understand the reaction mechanisms of the heme enzymes.

As an approach to the above-mentioned research interest, investigations on the reaction between myoglobin (Mb) and H₂O₂ have long been made by several groups (11–19). Mb is a single domain heme protein that primarily serves as an oxygen storage compound using the ferrous state of the heme. The ferric form of Mb (metMb) is known to be capable of reacting with H₂O₂, forms an analog of the porphyrin π-cation-type compound I (11), and also forms that of CcP-type compound I (12–14). Both analogs can oxidize organic compounds (11, 15–19). The latter analog is termed ferryl Mb, whereas the former one, which was found recently (11), is named myoglobin compound I. All the basic reactions catalyzed by heme enzymes, i.e. formation of compound I, intramolecular electron transfer from an aromatic amino acid residue to the heme of compound I, and oxidation of organic or inorganic compounds by the high valent species, can be observed in Mb (11, 15–19). In this context, the reaction between H₂O₂ and metMb has been considered as a model system to understand the activation mechanisms of H₂O₂ by heme enzymes (11, 15, 18, 19).

Chemical properties of the high valent species and mechanisms of reaction steps after formation of compound I have...
been studied extensively for peroxidases (1–6) and also for Mb (11, 15–19). On the other hand, mechanisms for compound I formation from ferric heme and H$_2$O$_2$ in heme proteins are still under debate (20–25). For compound I formation, Poulos and Kraut (26) proposed essential roles of a histidine residue at the distal vicinity of the heme, the distal histidine, which is highly conserved in peroxidases (26) and Mb (27). Based on their proposal, and also on results of recent quantum chemical studies (20–22), the catalysis of H$_2$O$_2$ by peroxidase has been supposed to proceed as shown in Fig. 1: a, the protonated form (imidazolium form) of the distal histidine donates its proton back to the terminal oxygen of the hydroperoxy group (acid catalysis); b, to the protonated form (imidazolium form) of the distal histidine donates its proton back to the terminal oxygen of the hydroperoxy group (acid catalysis); c, the distal histidine then abstracts a proton from H$_2$O$_2$ (base catalysis), producing a hydroperoxy anion species (Fe(III)[O–O–H$^-$]); d and e, the protonated form (imidazolium form) of the distal histidine donates its proton back to the terminal oxygen of the hydroperoxy group (acid catalysis) to assist the heterolytic oxygen-oxygen (O–O) bond cleavage, leading to the formation of compound I.

Site-directed mutagenesis has demonstrated the importance of the distal histidine in peroxidases; an aliphatic amino acid substitution leads to a significant decrease in the rate of formation of compound I (28–31). However, identification and characterization of the postulated intermediates as discernible entities were not successful. Up to now, stopped flow observations about intermediates, which form prior to compound I, upon adding H$_2$O$_2$ to peroxidases have only been reported for horseradish peroxidase (HRP). These studies were done on reactions of HRP with H$_2$O$_2$ in either 50% methanol or 100% chlorobenzene at subzero temperature (32, 33). It is, however, unknown whether the reactions in organic solvents are identical to those under physiological conditions. The properties of the above precursors are also unclear, because characterization by physicochemical techniques such as EPR and electron nuclear double resonance spectroscopies were not applied.

On the other hand, studies employing cryoradiolytic reductions of oxy hemes have succeeded in EPR and/or ENDOR characterizations of Fe(III)[O–O–H$^-$] hemes formed in heme proteins including hemoglobin (34–36), myoglobin (34, 35), cytochrome P450 (37), heme oxygenase (36), and HRP (35, 38). These studies have indicated that the Fe(III)[O–O–H$^-$] species are low spin.

Indeed, optical absorption spectra of Fe(III)-[O–O–H$^-$] species ($\lambda_{\text{max}} \sim 420$ nm) are typical of ferric low spin hemes (38, 39), and they are different from any reported spectra by the stopped flow studies described above. Therefore, in order to investigate the molecular mechanism of compound I formation and the functions of the distal histidine, it is necessary to examine whether the Fe(III)[O–O–H$^-$] species characterized by the oxy heme reduction studies are also involved in the reactions of H$_2$O$_2$ and ferric heme proteins having the histidine. For HRP, however, a recent study indicated that compound I formation is complete within a very short time (~200 µs) at ambient temperature in aqueous buffers, and identification of any precursor of compound I was difficult (40).

In order to address the above problem, and also to give insights into the roles of amino acid residues that form the heme pockets of heme enzymes, we followed the reaction between H$_2$O$_2$ and metMb in detail. By using stopped flow-rapid scan, singular value decomposition (SVD), and rapid freezing-EPR techniques, we succeeded in detecting a hydroperoxy anion compound (Fe(III)[O–O–H$^-$]) that is closely related to one of the postulated intermediates in the peroxidase reactions. Analyses of population changes of the hydroperoxy compound and compound I as a function of pH clearly demonstrated the involvement of the distal histidine in the reaction. Based on the present findings, we postulated mechanisms for the reaction between metMb and H$_2$O$_2$, and we also discussed the reaction mechanisms of the heme peroxidases.

**EXPERIMENTAL PROCEDURES**

**Materials**—Sperm whale and horse heart metMbs were purchased from Sigma. Human metMb and its T67N mutant were expressed in *Escherichia coli* and were purified as described elsewhere (18, 41). Each metMb dissolved in a potassium phosphate buffer was treated with a small amount of potassium ferricyanide as described previously (11) to eliminate the reduced form of Mb remaining in the commercial products or in the preparations extracted from *E. coli*. The oxidized proteins were extensively dia lyzed and further purified by CM-cellulose column chromatography (11).

**Stopped Flow-Rapid Scan Spectrophotometry**—Changes in the optical absorption were measured by using an RSP-601 stopped flow-rapid scan spectrophotometer (UNISOKU Co., Ltd., Osaka, Japan) (11, 42). The instrumental dead time was estimated to be ~5 ms by employing a pseudo first-order reaction of 2,6-dichlorophenolindophenol with ascorbic acid (43).

**Singular Value Decomposition**—Time-resolved absorption spectra, which were obtained by using the stopped flow-rapid scan spectrophotometer, were analyzed by the technique of SVD (11, 42, 44–48). In the present investigation, the rapid scan spectroscopic system allowed us to record absorption data at up to 512 points for both wavelength and time, and hence 512 × 512 data points were obtained upon each run of the stopped flow experiments. We collected data from 19 experimental runs and averaged them. Among the data thus obtained, those at every 1.62 nm from 322 to 525 nm (126 data points) and at every 3 ms from 6 to 195 ms (64 spectral scans) were selected. This time range was selected in order to cover most of the reaction period under the present experimental conditions. The data set of 126 × 64 points thus obtained was subjected to the SVD calculation. The computer program used for the calculation was described elsewhere (11, 42).

**Kinetic Parameters in Three Component Reactions**—Concentrations of chemical species in three component sequential reaction represented by Reaction 1 are by Equations 1 and 2,

$$R \rightarrow I \rightarrow P$$

**Reaction 1**

$$[R] = [I]_0 \exp(-k_1 t)$$  \hspace{1cm} (Eq. 1)

$$[I] = [R]_0 k_1$$

$$[P] = [R]_0 \frac{k_1}{k_2 - k_1} \exp(-k_2 t) - \exp(-k_1 t)$$ \hspace{1cm} (Eq. 2)

where $k_1$ and $k_2$ are rate constants of the reaction, and $[R]_0$ is the initial concentration of the reactant (R) (49). Integrating Equations 1 and 2 with respect to time (t) from 0 to $\infty$, we obtained equations for total accumulated amounts of R and I in Reaction 1, and their ratio is given by Equation 3,

$$[I]_{\text{total}}/[R]_{\text{total}} = k_1/k_2$$  \hspace{1cm} (Eq. 3)

On the other hand, the equation used for determining maximum accumulation time ($t_{\text{max}}$; time at which concentration reaches the maximum
Fig. 2. Absorption changes of the reaction of 5 μM sperm whale metMb with 50 mM H$_2$O$_2$ at pH 6.0 (A) and 8.5 (B). Absorption spectra in both A and B were recorded at 6, 15, 30, 60, 100, and 200 ms after starting the reaction by the stopped flow-rapid scan system. Arrows indicate the directions of the absorption changes. Time profiles of absorption changes were followed at 418 nm (insets a and b). Buffer systems used were 200 mM potassium phosphate and 200 mM glycylglycine-NaOH at pH 6.0 and 8.5, respectively. All measurements were carried out at 25 °C.

The absorption changes of the reaction depends on the pH. Fig. 2B shows the absorption changes of the reaction at pH 8.5 in 200 mM glycylglycine-NaOH (11).

Rapid Freezing-EPR Spectroscopy—Samples for EPR measurements were prepared by using a recently developed rapid freezing apparatus that was composed of a rapid mixing chamber and a freeze-quench device. The details of this apparatus are described elsewhere (40). We mixed metMb (1 mM) and H$_2$O$_2$ (200 mM) solutions in a 1:1 ratio and rapidly froze the mixed solution at 77 K to obtain the frozen flakes. The frozen flakes were collected and transferred into an EPR tube, and the EPR spectra of the samples were recorded at 15 K. The freezing dead time (time period from mixing to complete quenching) of our apparatus is 200 μs at minimum and is changeable by adjusting conditions such as mixing flow rate and distance between the mixing and freeze-quench devices (40). Methods to calibrate the dead time were described already (40). Methods to calibrate the dead time were described elsewhere (40). We used an Oxford ESR-900 liquid helium cryostat. Detailed descriptions of this apparatus and the cryostat are described elsewhere (40). We equipped with an Oxford ESR-900 liquid helium cryostat. Measurements of EPR spectra were measured by a Varian E-12 spectrometer (40). In the present experiments, the freezing dead time was fixed at 3 ms.

RESULTS

Changes in Absorption Spectra during the Reaction between metMb and H$_2$O$_2$—Fig. 2A shows spectral changes observed during the reaction of 5 μM sperm whale metMb with 50 mM H$_2$O$_2$ in 200 mM potassium phosphate buffer, pH 6.0. The spectral changes shown here well reproduce those we reported previously for the same reaction (11). As we already indicated, formation of myoglobin compound I is detectable under the above experimental conditions (11). Although these spectral changes might be viewed as an apparently single process of conversion from metMb (λ$_{max}$ 409 nm) to ferryl Mb (λ$_{max}$ 422 nm), the time profile of the absorption at an isosbestic point (418 nm) between the spectra of metMb and ferryl Mb shows formation of an intermediate (Fig. 2, inset a). The temporary decrease in the time profile is caused by the accumulation of compound I in the reaction system (11). This accumulation of compound I can be observed more clearly in D$_2$O (at pH 6.0) (11).

In the present study, we show that the kinetics of the above reaction depends on the pH. Fig. 2B shows the absorption changes of the reaction at pH 8.5 in 200 mM glycylglycine-NaOH buffer. The reaction conditions other than pH and the buffer were identical to those used for Fig. 2A at pH 6.0. Although the gross features of the spectral changes at pH 8.5 were quite similar to those at pH 6.0, the absorption at 418 nm was almost unchanged during the entire reaction period (Fig. 2B). This result indicates that compound I scarcely accumulates at pH 8.5.

Detection of a New Spectral Species in the Reaction at pH 8.5—To uncover the difference in the reactions at pH 6.0 and 8.5, we analyzed the above spectral data by SVD. In general, SVD calculations transform an absorption data matrix (i.e., a set of time-resolved absorption spectra) into a product of three matrices, U, S, and V, where the ith column of the matrix U is termed ith basis spectrum (44). Among the basis spectra, only a few columns from 1st to nth of the matrix U carry information about absorption spectra of the chemical species. Each of these basis spectra, which are called the significant basis spectra, can be expressed as an appropriate linear combination of the absorption spectra of the chemical species involved in the reaction (11, 42, 45–48). The total number, n, of the significant basis spectra corresponds to the minimal number of chemical species that contributes to the absorption data matrix (11, 42, 45–48). Other basis spectra (>n) consist of optical noises and have no significant spectral features (11, 42, 45–48).

SVD calculations for the present data found three significant basis spectra for the reactions at pH 6.0 and 8.5. In Fig. 3, the first, second, and third basis spectra thus obtained at pH 6.0 are shown in A–C, whereas those at pH 8.5 are in D–F, respectively (solid lines). The presence of three significant basis spectra at both pH 6.0 and 8.5 indicates that the reaction involves at least three spectral species. We have assigned these to metMb, ferryl Mb, and another state of Mb, which is characterized below. The results at pH 6.0 are more easily interpreted because compound I can be detected due to its distinctive absorption changes (11). On the other hand, at pH 8.5 it is less clear that either compound I or any other well known species contributes to the absorption changes. We therefore simulated the above basis spectra in terms of linear combinations of optical absorption spectra of metMb, ferryl Mb, and compound I that were obtained independently.

The standard absorption spectra of metMb (thin solid line), ferryl Mb (broken line), and compound I (thick solid line) in Fig. 3G are used for the analyses. Among them, the spectrum of compound I is that reported for H64A mutant of sperm whale Mb by Matsui et al. (19). According to Matsui et al., compound

$^2$ Numerical data of the absorption spectrum of the mutant compound I was kindly provided by Drs. Toshitaka Matsui, Shinichi Ozaki, and Yoshihito Watanabe (19).
I was detectable as an almost pure product when m-chloroperoxybenzoic acid was added to ferric H64A Mb (19). It is also possible to obtain the absorption spectrum of compound I for the reaction of native Mb with H$_2$O$_2$ using a D$_2$O buffer system; however, contributions of metMb and ferryl Mb to the observed spectrum of compound I cannot be removed completely (11). As the standard absorption spectrum of metMb, a spectrum at pH 6.0 was utilized to analyze the basis spectra at both pH 6.0 and 8.5. Although the absorption spectrum of metMb is known to depend on pH and shows an acid-alkaline transition of the standard absorption spectrum of metMb, a spectrum at pH 8.5 is given by

\[ \frac{0.0995 \times \text{metMb spectrum} + 0.343 \times \text{ferryl Mb spectrum}}{1} \]

for the H64A mutant of Mb (19). All the basis spectra except for F could be reproduced by combining the standard absorption spectra of metMb, ferryl Mb, and compound I in G. The resultant linear combination spectra are shown in A–E (broken lines) with their linear combination factors. All the basis spectra in A–F were calculated from the sets of time-resolved absorption spectra represented in Fig. 2.

Because more than one chemical species contributes to each of the significant basis spectra, the negative peak position at 434 nm of the third basis spectrum (Fig. 3F) does not necessarily mean that the unidentified species exhibits an absorption peak at exactly 434 nm. The presence of the negative peak at 434 nm, which is red-shifted from the Soret absorption peak of ferryl Mb at 423 nm, rather indicates that the species exhibits a Soret band at a wavelength longer than 423 nm. Therefore, it is obviously impossible to reproduce the negative peak at 434 nm of the third basis spectrum at pH 8.5 by any combinations of the standard absorption spectra.

The presence of the above unidentified species was not specific to the buffer system we employed. When the reaction was carried out in potassium phosphate buffer at pH 8.0, the shapes of the third basis spectrum was essentially unchanged (see below).

**Time Profile of the Concentration of the New Spectral Species**—According to the principle of SVD, ith row of the matrix $V^T$ represents time progression of ith column of the matrix $U$ (i.e., ith basis spectrum), and it can be expressed as an appropriate
linear combination of the actual time profiles of concentrations of the chemical species in the reaction (11, 42, 45–48). Thus, least squares analyses on \( V \) rows enable us to compute concentration changes of chemical species, if appropriate equations for the concentration changes are given (11, 42, 45–48). In the present study, however, this method did not work well; the computed value of the decay constant of the new intermediate at pH 8.5, which corresponds to \( k_2 \) in Reaction 1, was accompanied by a large error. Because the accumulated amount of the intermediate was very small, the signal-to-noise ratio of the third \( V \) row, which mostly carries information about the intermediate, was poor. The reaction was approximately a single exponential process, and only one apparent rate constant, which corresponds to \( k_1 \), was determined to be \( 2.4 \times 10^{-1} \) s\(^{-1} \) at 50 mM \( \text{H}_2\text{O}_2 \). However, we could comprehend a rough trend of the concentration changes of the intermediate when we investigated singular values of the reaction.

In general, the \( i \)th singular value (i.e. \( i \)th diagonal elements of the matrix \( S \)) is a measure for contribution of \( i \)th basis spectrum to the absorption data matrix (45–48). Therefore, if extinction coefficients of a chemical species, which is represented by the \( i \)th basis spectrum, are not largely different from those of other chemical species in the reaction, the relative magnitude of \( i \)th singular value roughly corresponds to relative total concentration of that chemical species. For the reaction of metMb and \( \text{H}_2\text{O}_2 \) at pH 8.5, singular values of the first, second, and third basis spectra were \( 1.8 \times 10^3 \), \( 2.6 \times 10^3 \), and \( 1.0 \times 10^2 \), respectively. Among them, the first two represent both metMb and ferryl Mb; the first and second basis spectra are linear combinations of met and ferryl spectra (see Fig. 3, D and E). Considering the weights (i.e. absolute values of the linear combination factors) of the met spectrum (0.1 and 0.6 in the first and second basis spectra), the total contribution of the met spectrum to the absorption data matrix was determined to be \( 0.1 (1.8 \times 10^3) + 0.6 (2.6 \times 10^3) \approx 4 \times 10^3 \). On the other hand, the intermediate is represented by the third basis spectrum (Fig. 3F) giving the third singular value (1.0 \( \times 10^2 \)), and its weight in the third basis spectrum is at most 1. Based on these values, we determined that the total concentration of the intermediate was more than 2 orders of magnitude smaller than that of metMb. Thus, \( k_2/k_1 \) is less than \( 1 \times 10^{-2} \) (see Equation 3). Based on this, together with the experimental value of \( k_1 \), we estimated the maximum accumulation time, \( t_{\text{max}} \) of the intermediate to be \(< 2 \) ms according to Equation 4.

On the other hand, when \( k_1 \ll k_2 \), Equation 2 can be reduced to Equation 5,

\[
[\text{I}] = [\text{R}]_0 \frac{k_1}{k_2} \exp (-k_1 t) \quad \text{(Eq. 5)}
\]

Equation 5 indicates that concentration of intermediate species in such cases decreases at a rate \( (k_1) \) the same as decay rate of reactant. Accordingly, we determined that the concentration of the unidentified intermediate of Mb reached a maximum value within a very short time (<2 ms), which is shorter than the dead time (6 ms) of the stopped flow experiments, and decreased with decreasing the concentration of metMb. Based on these estimations, we planned rapid-freezing EPR experiments to identify the intermediate (see below).

Identification of the New Spectral Species by Rapid Freezing-EPR—To identify the new spectral species found at pH 8.5, a series of EPR experiments was carried out with the aid of the rapid freezing technique. The reaction was initiated by mixing 1 mM metMb and 200 mM \( \text{H}_2\text{O}_2 \) in a 1:1 ratio at pH 8.5 or 6.0; the concentrations of metMb and \( \text{H}_2\text{O}_2 \) (500 mM and 100 mM after the mixing) were larger by factors of 100 and 2, respectively, than those in the stopped flow-rapid scan experiments. The condition of the concentrations (\( [\text{H}_2\text{O}_2] \gg [\text{metMb}] \)) allows the reaction to be pseudo first-order with respect to \( \text{H}_2\text{O}_2 \); thus, the reaction is expected to proceed at a rate twice that in the stopped flow-rapid scan experiments. Since \( t_{\text{max}} \) of the intermediate is expected to be very small, we quenched the reaction within a short time (<3 ms), which was comparable to the dead time of the stopped flow experiments.

Fig. 4 illustrates the EPR spectra of the quenched samples measured at 15 K. Although the sample prepared at pH 8.5 (spectrum \( a \)) showed several peaks (asterisks), the signal-to-noise ratio was rather poor. We therefore raised the temperature of the sample to 220 K for 150 s (annealing), expecting that the intermediate species, if any, would decompose without drastic alterations of the sample conditions. After the annealing, the sample was cooled to 15 K, and the EPR spectrum was recorded again. The spectrum thus obtained (spectrum \( b \)) indicates that several signals presented before the annealing disappeared or were weakened. The changes are more clearly shown in the difference spectrum (spectrum \( c \): spectrum \( a \) minus spectrum \( b \)), which clearly indicates the presence of the signals marked with asterisks in spectrum \( a \). On the other hand, the EPR spectra collected before and after the annealing (spectra \( c \) and \( d \), respectively) at pH 6.0 were essentially the same, and their difference spectrum (spectrum \( f \) did not give any of the notable EPR signals observed at pH 8.5.

In order to improve the signal-to-noise ratio, we repeated the
same experiments at pH 8.5, and we averaged the difference spectra. The averaged spectrum (spectrum g) gave us a set of g values at 2.32, 2.19, and 1.94. Because the g values for ferric hydroperoxy hemes (Fe(III)–[O–O–H] ) were reported at 2.25–37, 2.16–9, and 1.91–6 (34–39, 51), the current data indicate the formation of a hydroperoxy compound in the reaction of metMb with H2O2 at pH 8.5. It should be noted that the g values of the ferric hydroperoxy hemes are unique and distinct from those of other ligand complexes of ferric and ferrous iron porphyrins, and therefore, they serve as an unmistakable signature of the Fe(II)[O–O–H] structure (34–39, 51).

By using rapid freezing-EPR techniques, Brittain et al. (51) detected EPR signals of hydroperoxy species in reactions of distal histidine mutants (H64V and H64Q) of sperm whale metMb with H2O2 at pH 7.5, although corresponding EPR signals were not detected for wild type metMb under their experimental conditions. In our EPR experiments, the hydroperoxy signals were detected together with an EPR signal giving positive and negative peaks around g ~ 2, which was assigned to the amino acid radical of ferryl Mb formed within the quenching dead time. We found that the hydroperoxy signals were 2 orders of magnitude weaker than the radical signal (data not shown). On the other hand, in the experiments by Brittain et al. (51) for H64Q mutant, the observed EPR intensities of hydroperoxy signals were comparable with that of the amino acid radical signal of ferryl Mb at a wide range (9–44 ms) of quenching dead time (see Fig. 4A of Ref. 51) that covered $t_{\text{max}}$ of the hydroperoxy compound (20 ms) under their conditions. Therefore, we estimate that the accumulated amount of the hydroperoxy intermediate relative to ferryl Mb was at least 2 orders of magnitude smaller in native Mb than in the mutant. This further means that the concentration of the hydroperoxy intermediate in our EPR experiments was at least 2 orders of magnitude smaller than the total concentration of Mb. By careful examinations of the pH effects on the reaction kinetics and the use of a higher concentration of Mb in the EPR experiments, we successfully detected the hydroperoxy compound in native metMb.

We assigned the new spectral species of Mb detected by the SVD analyses to the hydroperoxy compound observed by the EPR experiments on the following bases. 1) The SVD analysis indicated that the concentration of the new spectral species, which was the sole detectable intermediate at pH 8.5 and distinct from heme degradation products, reaches the maximum value within a very short time (<2 ms) and decreases relatively slowly as the reaction proceeds. Therefore, the hydroperoxy compound detected for the samples quenched within 3 ms should be assigned to the spectral species. Although we did not carry out rapid freezing-EPR experiments at a freezing dead time longer than 3 ms, the annealing experiments are reasonable alternatives to such experiments; when the reaction proceeded upon the annealing, the hydroperoxy signals quenched at 3 ms indeed disappeared. 2) The pH dependence of the new spectral species and the hydroperoxy EPR signals agree with each other; they were detected at pH 8.5 but were not detected at pH 6.0. 3) The low spin type Soret band ($\lambda_{\text{max}}$ $\approx$ 423 nm) of the spectral species is consistent with the assignment, because the hydroperoxy hemes are ferric low spin species (34–39, 51).

Although the estimations were rough, the agreement between the populations of the new spectral species and the hydroperoxy species in the EPR experiments may further support the assignment; they both were estimated to be less than 1% of total Mb. Accordingly, we conclude that the unidentified intermediate species in the reaction of metMb with H2O2 at pH 8.5 is the hydroperoxy compound of Mb. Effects of pH on the Kinetics of the Reaction—The present results indicate that the reaction of metMb with H2O2 is pH-dependent. Acidic conditions are favorable for the accumulation of compound I, and alkaline conditions favor accumulation of the hydroperoxy compound. Because the pH-dependent changes of the reaction strongly suggest the participation of the distal amino acid residues, we further analyzed the pH dependence based on extensive pH titration experiments. We measured and analyzed absorption changes of the reaction in a pH range from 6.25 to 8.0. The SVD analyses indicated that the number of the significant basis spectra was 3 at every pH condition and that the first and second basis spectra did not depend on pH (data not shown). The changes in the third basis spectrum at pH 6.25–8.0 were illustrated in Fig. 5A, where directions of the spectral changes upon rising pH were indicated by arrows. The spectral difference amplitudes between the positive peak at 422 nm and the negative peak at 434 nm are plotted versus pH in Fig. 5E (closed circles). The plot for sperm whale metMb agrees well with a theoretical titration curve (thick solid line) using a single pKapp value of 7.27. This result indicates that the protonation state of a certain chemical group regulates the conversion of the third basis spectrum and gives an apparent pKapp (hereafter termed as pKapp for the transition of the kinetics. The most plausible origin of the pKapp value at around pH 7 is the distal histidine (His-64) located in the close vicinity of the heme, because all other amino acid residues facing the distal heme pocket of Mb (Ile-28, Leu-29, Phe-43, and Val-68) (27) are not ionizable.

As described earlier, the third basis spectrum at pH 6.0 could not be fully reproduced by any linear combinations of the standard spectra of metMb, ferryl Mb, and compound I due to the negative shoulder near 430 nm (see Fig. 3C). The titration plot (Fig. 5E, closed circles) indicates that the transition to the acid state is not complete at pH 6.0, and a small amount of the hydroperoxy compound is formed at pH 6.0. The negative shoulder in the spectrum of Fig. 3C may therefore be attributable to the hydroperoxy compound. The present reaction obviously involves four chemical species, i.e. metMb, the hydroperoxy compound, compound I, and ferryl Mb, at around pH 7, but only three significant basis spectra were obtained. This is possibly because the accumulated amounts of compound I and the hydroperoxy compound were very small. The absorption spectra of the hydroperoxy compound and compound I were combined and appeared on each of the third basis spectrum in Fig. 5A, and the relative population of these two compounds at the given pH reflected the shapes of each third basis spectrum. The changes in the third basis spectrum observed for sperm whale Mb were reproducible in horse heart (Fig. 5B) and human (Fig. 5C) Mbs. These results indicate that the accumulation observed here of compound I under acidic and the hydroperoxy compound under alkaline conditions are commonly encountered for reactions between H2O2 and Mb species. As shown in Fig. 5E, the pKapp values for the different species of metMb were quite similar. They were 7.18 and 7.00 for horse heart (Fig. 5E, open circles and thin solid line) and human (closed triangles and thick broken line) Mbs, respectively. Interestingly, the pKapp of Mbs (sperm whale, 7.27 > horse heart, 7.18 > human, 7.00) roughly parallels the acid-alkaline transition, which reflects ability of the distal histidine to abstract a proton from a ligand water, at the Met state of these Mbs. Reported values of pKapp of the acid-alkaline transition, pKapp, are as follows: sperm whale, 8.99 > horse heart, 8.93 > human, 8.72 (41, 50). In order to examine further this parallel relationship, we carried out experiments with a mutant of human Mb (T87N), which shows a pKapp value (8.49) lower than
that of wild type human metMb (41). The mutant also showed significant changes in the SVD third basis spectrum (Fig. 5D), and the pK$_a$ of the spectral changes was indeed shifted to a lower value (6.95) from that of the wild type protein (Fig. 5E, open triangles and thin broken line). This confirms the parallel relationship. Meanings of this relationship are discussed later.

**Association Rate between metMb and H$_2$O$_2$**

The apparent decay of metMb could be approximated to a single pseudo first-order exponential process. The rate of the process corresponds to association rate between metMb and H$_2$O$_2$. The estimated association rate constant for sperm whale metMb at pH 7.0 was 4.8 M$^{-1}$s$^{-1}$, which is in reasonable agreement with the reported values (14, 52). The association rate constant was decreased slightly under both of the acidic and alkaline conditions (data not shown), but no obvious pK$_a$ value related to pK$^{app}$ was observed. Similar results were also obtained for other Mbs examined here. Therefore, the chemical step(s) that are subjected to the pH effect and give pK$^{app}$ are not involved in the association processes of H$_2$O$_2$ to the ferric heme of metMb.

**DISCUSSION**

In this study, we investigated the reaction between metMb and H$_2$O$_2$, and we obtained evidence for the formation of the hydroperoxy compound in the reaction. It was also demonstrated that the distal histidine plays key roles in accumulation of the hydroperoxy compound. These results give insights into the mechanism of the O–O bond activation in metMb and in heme peroxidases.

**Reaction Mechanisms**—The entire reaction between H$_2$O$_2$ and ferric heme proteins consists of the association of H$_2$O$_2$ to the ferric heme and the subsequent chemical steps including the cleavage of the O–O bond. The association process involves a diffusion of H$_2$O$_2$ into the heme pocket and the coordination of H$_2$O$_2$ to the ferric heme, which leads to the formation of the ferric heme-H$_2$O$_2$ complex (Fig. 1, a $\rightarrow$ b) (20–22, 25). For the reaction between metMb and H$_2$O$_2$, the pH dependence of the association process does not correlate with the apparent pK$_a$ (pK$^{app}$) of the accumulation kinetics for the hydroperoxy compound. Therefore, the step(s) controlled by the distal histidine most likely occur after the formation of the Mb-H$_2$O$_2$ complex.
Thus, the conversion from the Mb-H2O2 complex to the hydroperoxy compound and/or the decomposition of the hydroperoxy compound are affected by the distal histidine. This conclusion is consistent with the mechanism proposed by Poulos and Kraut (26) in which the distal histidine catalyzes both of the formation and decomposition of the hydroperoxy intermediate in peroxidases.

In order to discuss further details on possible mechanisms of the reaction of metMb with H2O2, we first consider the origin of the reaction of metMb with H2O2, we first consider the origin of the reaction of metMb with H2O2. It should be noted that the left equilibrium of the distal histidine forms a hydrogen bond with a proton of the ligand water of the heme, whereas Nδ is protonated (Fig. 6a) (50). Under alkaline conditions, Nδ releases its proton, and Ne accepts a proton from the water, converting the ligand water to hydroxide anion (Fig. 6c) (50). The transient state between the acid and alkaline forms is consistent with the mechanism proposed by Poulos and Kraut (26) (Fig. 6b) (50). In addition, pKa of the acid-alkaline transition under given pH should be determined essentially by pKs of the equilibrium between the HP* and HP states.

Because the ferric heme and the distal histidine concertedly facilitate the ionization of the ligand water, pKsAB is significantly down-shifted to −9 from the intrinsic pKs value of water at −16 (50). In addition, pKsAB varies slightly with different species of Mb and depends on the slight conformational variations of the distal histidine and the chemical environment of the heme pockets. Thus, sperm whale, horse heart, human, and human T67N mutant Mbs exhibit different pKsAB values (41, 50). Similarly, it is expected that the pKs value of the equilibrium between the HP* state and HP* state is lower than the intrinsic pKs value of H2O2 (−12), which is remarkably lower than that of water (−16). Therefore, the pKs value is also expected to be lower than pKsAB and to show a variation that parallels pKsAB. When the pH is below this pKs value, heterolysis and the formation of compound I is favored (Fig. 6, b’ → d → e), whereas above the pKs value, stabilization of the HP* state is favored.

The above mechanism for the reaction of metMb with H2O2 was proposed on the basis of the established knowledge on the acid-alkaline transition of Mb, the acid characteristics of imidazole and imidazolium, and the chemical characteristics of H2O and H2O2. It should be emphasized that the present observations are fully consistent with the mechanism thus proposed; the stable hydroperoxy intermediate, that is HP*, was detect-

![Fig. 6](http://www.jbc.org/Downloaded from)
deprotonation equilibrium at the Nδ nitrogen. Such a function of the conserved asparagine (distal asparagine) has been clearly demonstrated in the ferric cyanide complexes of HRP (56), CcP (57), and LiP (58) by proton NMR techniques. When cyanide binds to the ferric heme of these peroxidases, the proton of HCN is transferred to the Nδ nitrogen of the distal histidine, whereas Nδ remains protonated, allowing the histidine to be in the imidazolium form (56–58). On the other hand, in the corresponding cyanide complex of sperm whale metMb, the distal histidine can accept the proton from HCN at the Nδ nitrogen and releases the Nδ proton to remain in the imidazolide form (59). These observations strongly suggest that the Nδ nitrogen of the distal histidine in peroxidases is also resistant to deprotonation during the H₂O₂ catalysis. The formation of a stable hydroperoxy species corresponding to the HP* state (Fig. 6c) should be suppressed in the peroxidases, because the formation is coupled with the deprotonation at the Nδ nitrogen (Fig. 6, b→c). Thus, the reaction of the peroxidases with H₂O₂ should proceed according to the Poulos and Kraut mechanism without accumulating the stable hydroperoxy state. This proposal is consistent with the observations that the detection of the hydroperoxy species was difficult for the reactions of the peroxidases with H₂O₂ but was successful for the corresponding reaction of metMb as demonstrated in this study.

Nagano et al. (60) prepared the distal asparagine mutants (N70V and N70D) of HRP, and showed that the rate of the compound I formation by H₂O₂ was decreased by an order of magnitude in the mutants. One of the mutants, N70D, was subjected to detailed proton NMR experiments in the ferricyanide state (61). The NMR experiments indicated that the distal histidine of the cyanide complex of the mutant possesses the Ne and Nδ protons, but the Nδ proton signal showed significant broadening, which was not found for the wild type cyanide complex. This was attributed to a rapid exchange of the Nδ proton with protons of bulk water (61). These results indicate that the distal asparagine of HRP indeed hinders the release of the Nδ proton effectively. Although it was not examined whether the hydroperoxy compound was detectable in the mutants, their decreased rates of compound I formation are consistent with the suggestion that the distal asparagine suppresses the formation of the HP* state and accelerates the compound I formation.

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Hydroperoxy Compound of Myoglobin

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