Formation of Compound I in the Reaction of Native Myoglobins with Hydrogen Peroxide*

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Reaction of ferric native myoglobin (Mb) with hydrogen peroxide (H₂O₂) was studied by the aid of stopped-flow rapid-scan spectrophotometry. In contrast to the results in previous studies where compound I was reported to be undetectable, both sperm whale and horse heart metmyoglobins (metMbs) formed a significant quantity of compound I, an oxoferryl porphyrin (metMb) formed a significant quantity of compound I, an oxoferryl porphyrin (Por-Fe IV(O)), during their reactions with H₂O₂. With both kinds of Mbs, formation of compound I was more clearly observed in D₂O than in H₂O. The compound thus formed was capable of performing monooxygenation of thioanisole to methyl phenyl sulfoxide and a 2-electron oxidation of H₂O₉ giving O₂ and H₂O as products. It was also converted into ferryl myoglobin (Por-Fe IVV(O)-globin*) spontaneously. Rate constants for these reactions and that for a direct conversion of metMb to ferryl Mb through the homolysis of H₂O₂ were determined. These results established unambiguously that native metMb can form both compound I and ferryl Mb upon reaction with H₂O₂ and that these high valent iron compounds serve as essential intermediates in Mb-assisted peroxidative reactions. The observed deuterium effect on the apparent stability of compound I was attributable to that effect on the hydrogen abstraction step in the 2-electron oxidation of H₂O₂ by compound I.

A variety of organic compounds such as lipids, styrenes, and sulfides undergo various oxidative reactions with H₂O₂ in the presence of metmyoglobin (metMb)¹ (1–9). It has been shown that ferryl myoglobin (ferryl Mb), a high valent form of Mb with a structure of Por-Fe IV(V)(O)-globin*, is formed in these reactions as an intermediate of the reaction (1–4), where Por-Fe IVV(O) and globin* denote an oxoferryl neutral heme and an amino acid-centered organic radical in its globin moiety, respectively (10–19). The reaction sequence through which ferryl Mb is generated from metMb has been considered as follows (3–9):

Por-Fe III-globin + HOOH → Por-Fe IV(V)(O)-globin + HOH
REACTION 1
Por-Fe IV(V)(O)-globin + R → Por-Fe III-globin + RO
REACTION 2

where metMb is first oxidized by H₂O₂ to compound I (Por-Fe IV(V)(O)-globin) (Reaction 1), which is known to occur in the reactions of various other heme proteins such as peroxidases and possibly in the catalytic cycle of cytochromes P₄₅₀. The oxidizing equivalent on the porphyrin π-cation radical is then transferred to the globin moiety of Mb to produce ferryl Mb (Reaction 2), which oxidizes organic compounds such as lipids and styrenes. On the other hand, oxidation of certain other organic compounds such as sulfides by metMb has been postulated to proceed via a direct oxygen transfer from compound I in a similar way to those found in peroxidase-catalyzed monooxygenation reactions and probably in the catalytic cycle of cytochromes P₄₅₀ (Reaction 3) (6–9).

Por-Fe IV(V)(O)-globin + R → Por-Fe III-globin + RO
REACTION 3

Despite such formulations, however, the mechanisms for the peroxidative reactions catalyzed by metMb remained ambiguous in that the key intermediate in the above formula, compound I (Por-Fe IV(V)(O)-globin), has never been isolated nor observed as a discernible entity. In this connection, Watanabe and co-workers (7, 8) recently prepared a series of site-directed mutants of sperm whale metMb, in which the distal histidine (His-64) was replaced by a variety of different amino acid residues such as alanine, leucine, and serine, and they observed the formation of compound I of these mutant metMbs during the reaction with m-chloroperbenzoic acid. With native metMb, however, it was not possible to observe the formation of compound I under the same experimental conditions; only the formation of ferryl Mb was detected. They interpreted these findings to indicate that compound I of native metMbs was formed but was immediately converted to a ferryl state by accepting a reducing equivalent from the globin moiety through the distal histidine, His-64, and therefore did not accumulate as a discernible entity. On the other hand, the transfer of a reducing equivalent from the globin moiety to the heme could be slower in mutants due to the absence of His-64.

By employing stopped-flow rapid-scan spectrophotometry, we examined here the possible involvement of compound I and other high valent iron compounds in the H₂O₂-assisted oxidative reactions of organic compounds catalyzed by native metMb. The results have shown that compound I of native Mb is formed during its reaction with H₂O₂ and serves as an obligatory intermediate of peroxidative reactions. Further kinetic analyses have indicated that 1) compound I abstracts a

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¹ The abbreviations used are: metMb, metmyoglobin; Mb, myoglobin; ferryl Mb, ferryl myoglobin; mCPBA, meta-chloroperbenzoic acid.
hydrogen from H$_2$O$_2$ during the 2-electron oxidation of H$_2$O$_2$, 2) apparent stability of compound I is therefore mainly determined by its reaction with H$_2$O$_2$, and 3) native metMb carries out both homolysis and heterolysis of H$_2$O$_2$.

EXPERIMENTAL PROCEDURES

Materials—Sperm whale and horse heart metMbs were purchased from Sigma. The metMb dissolved in 10 mM potassium phosphate buffer, pH 7.4, was treated with a small amount of potassium ferricyanide to ensure the oxidation of ferrous Mb remaining in the commercial preparations. MetMb was extensively dialyzed against the same buffer and was purified by CM-cellulose column chromatography.

D$_2$O (99.9 atom %) was purchased from Aldrich and used without further purification. The pH value of the D$_2$O buffer was determined according to the relation: pH$_{obs}$ = pH$_{cal}$ + 0.39, where pH$_{cal}$ is a reading of an ordinary pH meter which employs a glass electrode (20). All other reagents used in this study were of highest grade commercially available.

Stopped-flow Rapid-scan Spectrophotometry—Changes in optical absorption were measured by using an RSP-601 stopped-flow rapid-scan system (Unisoku Co., Ltd., Osaka, Japan) (21). The dead time of the system with the system was determined to be 5 ms by employing a pseudo first-order reaction of 2,6-dichlorophenol-indophenol with ascorbic acid (21).

Determination of Rate Constants—As described under “Results,” absorption changes observed during the reaction of metMb with H$_2$O$_2$ in the presence and absence of thioanisole were found to proceed in a stopped-flow rapid-scan system. Spectra at 0 ms (broken lines in A and B) were obtained for metMb in the absence of H$_2$O$_2$, and others were recorded during the reaction at 10, 30, 60, 100, and 200 ms after the initiation of the reaction. The directions of the absorption changes are indicated by arrows. Inset, time profiles of absorption changes of Mb at 417, 616, and 667 nm. For the 616 and the 667 nm time profiles, the vertical scales are the same, but their origins were shifted for a clear separation.

Detection of a New Spectral Species—Fig. 1 shows changes in absorption spectra of sperm whale metMb in both Soret and visible regions during the reaction of metMb with 50 mM H$_2$O$_2$ at pH 6.0, 25°C. The spectrum of metMb (λ$_{max}$; 409, 505, and 634 nm) changed to that of ferryl Mb (λ$_{max}$; 421, 551, and 586 nm) (15) in 200 ms without giving any spectral intermediate distinct from the two species (Fig. 1, A and B). However, we noticed that the isosbestic points between the spectra of metMb and ferryl Mb around 417, 616, and 667 nm were dubious, suggesting that one or more other spectral species could be involved in the reaction. Such an interpretation was further supported by the biphasic curves obtained for the time-dependent changes in absorption at 417, 616, and 667 nm in the insets of Fig. 1, A and B. Hence, we carried out the next series of experiments in a D$_2$O medium, hoping that the use of D$_2$O would make the other spectral species prominent since D$_2$O frequently affects the rates of chemical reactions through solvent isotope effects (24) and sometimes helps to detect unstable reaction intermediates.

![Fig. 1. Soret (A) and visible (B) absorption changes for the reaction of sperm whale metmyoglobin with 50 mM H$_2$O$_2$ at pH 6.0, 25°C.](image)

FIG. 1. Soret (A) and visible (B) absorption changes for the reaction of sperm whale metmyoglobin with 50 mM H$_2$O$_2$ at pH 6.0, 25°C. 5.0 (A) or 25 (B) mM of Mb were used for the measurements on a stopped-flow rapid-scan system. Spectra at 0 ms (broken lines in A and B) were obtained for metMb in the absence of H$_2$O$_2$, and others were recorded during the reaction at 10, 30, 60, 100, and 200 ms after the initiation of the reaction. The directions of the absorption changes are indicated by arrows. Inset, time profiles of absorption changes of Mb at 417, 616, and 667 nm. For the 616 and the 667 nm time profiles, the vertical scales are the same, but their origins were shifted for a clear separation.

Compound I of Myoglobin

RESULTS

Detection of a New Spectral Species—Fig. 1 shows changes in absorption spectra of sperm whale metMb in both Soret and visible regions during the reaction of metMb with 50 mM H$_2$O$_2$ at pH 6.0, 25°C. The spectrum of metMb (λ$_{max}$; 409, 505, and 634 nm) changed to that of ferryl Mb (λ$_{max}$; 421, 551, and 586 nm) (15) in 200 ms without giving any spectral intermediate distinct from the two species (Fig. 1, A and B). However, we noticed that the isosbestic points between the spectra of metMb and ferryl Mb around 417, 616, and 667 nm were dubious, suggesting that one or more other spectral species could be involved in the reaction. Such an interpretation was further supported by the biphasic curves obtained for the time-dependent changes in absorption at 417, 616, and 667 nm in the insets of Fig. 1, A and B. Hence, we carried out the next series of experiments in a D$_2$O medium, hoping that the use of D$_2$O would make the other spectral species prominent since D$_2$O frequently affects the rates of chemical reactions through solvent isotope effects (24) and sometimes helps to detect unstable reaction intermediates.

Fig. 2 shows Soret and visible absorption changes in the reaction of sperm whale metMb with 50 mM H$_2$O$_2$ in a D$_2$O buffer containing 200 mM potassium phosphate, pH 6.0, at 25°C. Spectra at 0 ms (broken lines in Fig. 2, A and B) are those of metMbs, and those at 0.5 s (thin lines) were attributable to the ferryl form. At 50 s, the Soret absorption at 409 nm had decreased to almost a half (solid thick line in Fig. 2A) and accompanying increases in absorption in the visible region with prominent peaks at ~550, ~590, and 648 nm (solid thick line in Fig. 2B). Among the peaks in the visible region, one at 648 nm (marked by an asterisk) was unique to this intermediate spectrum not being found in the spectrum of metMb nor of ferryl Mb, whereas the other two were attributable to those of ferryl Mb at 551 and 586 nm. The Soret absorption maximum with a reduced intensity and a visible band around 650 nm are characteristic to compound I of other protoheme proteins such as...
Table I

Monooxygenation of thioanisole by compound I of myoglobin

The amount of methyl phenyl sulfoxide, which is the product of the monooxygenation of thioanisole by compound I, was determined for 1-min incubation of 1 ml of reaction mixture, at 25 °C, pH(pD) 6.0. The reaction mixture contained 5 μM metMb, 50 mM H$_2$O$_2$, 200 μM thioanisole, 0.4% methanol, and 200 mM potassium phosphate. The values are the mean ± S.D. of four experiments. The bimolecular rate constant ($k_s$) for the monooxygenation of thioanisole by compound I was determined as described in the text.

| Species | Myoglobin | Methyl phenyl sulfoxide formed | $k_s$ |
|---------|-----------|------------------------------|-------|
|         | in H$_2$O | in D$_2$O                    | in H$_2$O | in D$_2$O |
| Sperm whale | 19 ± 1 | 44 ± 4 | ND | 2.7 × 10$^4$ |
| Horse heart | 19 ± 1 | 42 ± 1 | ND* | 3.0 × 10$^4$ |

* ND, not determined, because compound I in the present reaction system accumulated so scarcely in H$_2$O, regardless the presence of thioanisole, that accurate values of $k_s$ could not be determined by the present method.

Compound I of Myoglobin

Fig. 2. Soret (A) and visible (B) absorption changes for the reaction of sperm whale metmyoglobin with 50 mM H$_2$O$_2$ in D$_2$O at pH 6.0, 25 °C. 5.0 (A) or 25 μM (B) of Mb were used for the measurements. Spectra at 0 ms (broken lines in A and B) were of metMb recorded in the absence of H$_2$O$_2$. An arrow in A indicates an apparent isosbestic point shared by the Soret spectra at 0, 20, and 50 ms. An asterisk in B shows the 648 nm band in the visible spectrum at 50 ms. B, spectra obtained at 20 and 150 ms were omitted to merely avoid complexities in the figure.

Reaction of the New Spectral Species with Thioanisole—In an attempt to characterize further the properties of the new spectral species, we examined its reaction with thioanisole (CH$_3$—S—C$_6$H$_5$); it has been shown that compound I of heme proteins (5–9) transfers an oxygen atom to thioanisole yielding methyl phenyl sulfoxide (CH$_3$—SO—C$_6$H$_5$) was formed both in H$_2$O and D$_2$O systems in significant quantities (Table I). As seen, the amount of the product formed in D$_2$O (44 nmol) was about twice as that in H$_2$O (19 nmol). Formation of other products was negligible, if present. A similar result was also obtained with horse heart metMb (see also Table I). These results, together with the spectral evidence described above, indicate that the new spectral species is compound I of native myoglobin.

We then carried out stopped-flow rapid-scan experiments under varying concentrations of thioanisole in the D$_2$O buffer system. When formation and decomposition of the spectral species were followed at 418 nm, the absorption first decreased and then increased, indicating that the spectral species had accumulated transiently and then decreased (inset of Fig. 3, broken line). As seen, the decrease in absorption became more shallow as thioanisole concentration was raised, suggesting that the new spectral species decomposed more rapidly as thioanisole concentration increased. This finding has further supported the above interpretation that the new spectral species is compound I; it decomposes by donating an oxygen atom to thioanisole giving the sulfoxide as product. In the following experiments, therefore, we determined the rate constants for the reaction between compound I and thioanisole.

Employing the rapid-scan spectrophotometric system and methods described under “Experimental Procedures,” we performed kinetic least-squares fits of the time profiles of the reaction to a double-exponential function, and we obtained $k_1^{obs}$ and $k_2^{obs}$ where $k_1^{obs}$ and $k_2^{obs}$ denote apparent rate constants for describing the absorption changes. Then theoretical time profiles of absorption at 418 nm which were drawn by using $k_1^{obs}$ and $k_2^{obs}$ values thus obtained were overlaid on the experimental curves in the inset of Fig. 3 (solid line). As seen, theoretical curves agreed well with experimental data. Experimental time profiles at every other wavelengths examined (322–525 nm) were all well reproduced when the same $k_1^{obs}$ and $k_2^{obs}$ values as above were applied (data not shown), ensuring the validity of our kinetic analyses. When we plotted the parameter $k_1^{obs} + k_2^{obs}$ against thioanisole concentrations, the plot showed a linear increase with increasing concentration of thioanisole (Fig. 3), where the slope corresponds to the bimolecular rate constant ($k_S$) between compound I and thioanisole (see “The General Formula for Reaction Systems Involving Three Spectral Species” under “Appendix”). Values of $k_S$ thus obtained were listed in Table I. Based on all these results so far described, we conclude that the new spectral species found in this study is compound I of native myoglobin.

Overall Reaction Scheme for the Reaction of MetMb with H$_2$O$_2$—Since it is now clear that the new spectral species is compound I, the scheme for the present reaction system can be given by Scheme I, where $k_{12}$ and $k_{13}$ are both pseudo first-order rate constant with respect to H$_2$O$_2$ for the formation of compound I and ferryl Mb from metMb under the experimental conditions. The latter conversion of metMb to ferryl Mb was suggested to proceed through a homolytic cleavage of H$_2$O$_2$ by metMb (Reactions 4 and 5) (5, 28).

$$\text{Por-Fe}^{III}\text{-globin} + \text{HOOH} \rightarrow \text{Por-Fe}^{IV(O)}\text{-globin} + \text{H}^+ + \cdot \text{OH}$$

Reaction 4
It should be noted that H2O2 that converts metMb to compound thioanisole is present in the reaction system (Reaction 3) (6–9). 

The above reaction, which resembles those found in catalytic cycles of catalase and certain peroxidases (25–27), was already observed for compound I of the His-64 mutant Mbs (8, 9). It is a catalase-like reaction that involves a 2-electron oxidation of H2O2 to dioxygen (O2) and water (H2O). By using an oxygen electrode, we were able to find that a detectable amount of O2 was evolved during the reaction of metMbs with H2O2, indicating that the reaction of compound I with H2O2 actually proceeded in the present reaction system (data not shown).

As described previously, the least squares fits of absorption time profiles observed for the present reaction system have given two apparent rate constants, k1obs and k2obs. When specific oxygen acceptors (sulfides etc.) are absent and H2O2 is excess as compared with Mb, these rate constants can be related to rate constants involved in Scheme I according to Equations 2 and 3 below,

\[ k_{1\text{obs}} = \frac{(k_{12} + k_{13} + k_{21})([\text{H}_2\text{O}_2] + k_{23})}{2} \]

\[ \sqrt{\frac{[(k_{12} + k_{13} - k_{21})^2([\text{H}_2\text{O}_2] - k_{23})^2 + k_{12}k_{21}[\text{H}_2\text{O}_2]^2]}{4}} \] (Eq. 2)

\[ k_{2\text{obs}} = \frac{(k_{12} + k_{13} + k_{21})([\text{H}_2\text{O}_2] + k_{23})}{2} \]

\[ \sqrt{\frac{[(k_{12} + k_{13} - k_{21})^2([\text{H}_2\text{O}_2] - k_{23})^2 + k_{12}k_{21}[\text{H}_2\text{O}_2]^2]}{4}} \] (Eq. 3)

where k12, k13, and k21 are bimolecular rate constants corresponding to k12, k13, and k21, i.e., k12 = k12 [H2O2], k13 = k13 [H2O2], and k21 = k21 [H2O2] (see “Rate Constant for the Decay of Compound I to Ferryl Mb” under “Appendix”). We then mathematically examined properties of the functions of Equations 2 and 3, and we derived the following relationships I–III as follows. Relationship I, parameter k1obs and k2obs linearly increases as increasing [H2O2], and the slope of the plot of k1obs + k2obs versus [H2O2] corresponds to k12 + k13 + k21, whereas the intercept of it on the k1obs + k2obs axis corresponds to k23 (see “Rate Constant for the Decay of Compound I to Ferryl Mb” under “Appendix”). Relationship II, if k21 is negligible as compared with k12, k13, and k23, then k1obs linearly increases as increasing [H2O2], whereas k2obs is independent of [H2O2] (see “Dependences of k1obs and k2obs on [H2O2] under Condition That k23 Is Negligible” under “Appendix”). Relationship III, if k’21 is not negligible, then k1obs and k2obs, respectively, approach linear functions of [H2O2], whereas parameter k1obs × k2obs approaches to k’21 [H2O2] as increasing [H2O2] (see “Relationships between k1obs and k2obs and k1’21, k’13, and k’21 at a Higher Concentration of H2O2” under “Appendix”).

Based on the above findings, e.g., the relationships I–III, we carried out stopped-flow rapid-scan experiments under the conditions essentially the same as those for Figs. 1A or 2A except that the concentration of H2O2 was changed in a range of 25–100 mM. Values of k1obs and k2obs were then obtained by the least squares fits as described above. In H2O, as well as in D2O, both k1obs and k2obs were nearly proportional to the concentration of H2O2 (Fig. 4, A and B). These linear dependences were reproducible with horse heart metMb (Fig. 4, C and D). These findings indicate that k’21 is not negligible (see relationships I–III described above). In other words, the reaction between compound I and H2O2 (Reaction 6) proceeded with a significant rate in the reaction system under the present conditions. From the plots of k1obs + k2obs versus [H2O2] (Fig. 4), we also estimated values for k23; the intercept of the plots on the k1obs + k2obs axis corresponds to k23. The rate constants thus obtained are listed in Table II.

\[^{2}\text{T. Matsui, S. Ozaki, and Y. Watanabe, personal communication.}\]
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have expressed relationships between these fitting parameters and the rate constants by using another set of parameters, \( l_1 \) and \( l_2 \), as follows in Equations 4 and 5,

\[
\text{[metMb]}_0 \cdot d \cdot e_\text{opt} - c_\text{chl} = c_\text{chl} \cdot l_1 + c_\text{chl} \cdot l_2 \quad (\text{Eq. 4})
\]

\[
k'_2([\text{H}_2\text{O}_2]) = (l_1(k'^{\text{obs}}_{12} - k_{23}) - l_1(k'^{\text{obs}}_{12} - k_{23}))/l_2 - l_1 \quad (\text{Eq. 5})
\]

where \([\text{metMb}]_0\) is the initial concentration of metMb used for the stopped-flow experiments; \( d \) is the length of the light path adopted for the optical measurements, and \( e_\text{opt} \) is the molar extinction coefficient of compound I at \( \lambda \) wavelength. These equations indicate that the value of \( k_2' \) is obtainable according to Equation 5, whereas those of \( l_1 \) and \( l_2 \) are given by resolving Equation 4. Among the values of other parameters in Equations 4 and 5, however, those of \( e_\text{opt} \) at any \( \lambda \) were not determined exactly in the present study because either metMb or ferryl Mb contributed to the observed absorption changes in the spectrophotometric detection of compound I (see Fig. 2B, for example). In the case of the H64A mutant of sperm whale Mb, on the other hand, the values of \( e_\text{opt} \) were determined in a wavelength region from 360 to 710 nm.\(^2\) We therefore applied the \( e_\text{opt} \) values of H64A mutant to the present kinetic analyses for native sperm whale Mb under the assumption that the molar extinction coefficients of compound I of native Mb were very similar to those of the H64A mutant, if not entirely the same. Substituting the \( e_\text{opt} \) values at every 10 nm from 360 to 460 nm into Equation 4, we obtained a set of simultaneous equations for \( l_1 \) and \( l_2 \), and then a numerical solution of \( l_1 \) and \( l_2 \) was derived. The value of \( k_2' \) was then obtained by substituting the \( l_1 \) and \( l_2 \) values, together with that of \( k_{23} \), into Equation 5 (Table II).

As already described, the parameter \( k'^{\text{obs}}_1 \times k'^{\text{obs}}_2 \) becomes roughly equivalent to \( k'^{\text{obs}}_{12} \times k'^{\text{obs}}_{23} \) \( [\text{H}_2\text{O}_2] \) \(^2\) (Equation 6) when \([\text{H}_2\text{O}_2] \) increases (see relationship III or “Relationships between \( k'^{\text{obs}}_1 \) and \( k'^{\text{obs}}_2 \) and \( k'_1 \), \( k'_2 \), \( k'_3 \), and \( k'_2' \) at a Higher Concentration of \( \text{H}_2\text{O}_2 \)” under “Appendix”).

\[
k'^{\text{obs}}_1 \times k'^{\text{obs}}_2 = k'_1 k'_2 [\text{H}_2\text{O}_2]^2
\]
  \( (\text{Eq. 6}) \)

As we noted under “Relationships between \( k'^{\text{obs}}_1 \) and \( k'^{\text{obs}}_2 \) and \( k'_1 \), \( k'_2 \), \( k'_3 \), and \( k'_2' \) at a Higher Concentration of \( \text{H}_2\text{O}_2 \)” under “Appendix”, it was a good approximation to assume the above equation when the \( k'^{\text{obs}}_1 \) and \( k'^{\text{obs}}_2 \) values at 100 mM \( \text{H}_2\text{O}_2 \) (Fig. 4, A and B) were applied. Thus we substituted these \( k'^{\text{obs}}_1 \) and \( k'^{\text{obs}}_2 \) values, together with that of \( k'_2' \) which was already obtained, into Equation 6, and we have calculated values of \( k'_1 \). On the other hand, \( k'_2' \) was calculated by using Equation 7, where the value of the parameter \( k'_1 + k'_3 + k'_2' \) was obtained as the slope of the plot of \( k'^{\text{obs}}_1 + k'^{\text{obs}}_2 \) versus \([\text{H}_2\text{O}_2] \) (see relationship I or “Rate Constant for the Decay of Compound I to Ferryl Mb” under “Appendix”).

\[
k'^{\text{obs}}_1 + k'^{\text{obs}}_2 = (k'_1 + k'_3 + k'_2')[\text{H}_2\text{O}_2] + k_{23}
\]
  \( (\text{Eq. 7}) \)

All these results are depicted in Table II. Corresponding data for horse heart Mb were also derived by the same procedure by adopting the \( e_\text{opt} \) values of the H64A mutant except for \( k'_1 \) and \( k'_3 \) values in \( \text{D}_2\text{O} \) since it could not be a good approximation to assume Equation 6 in this case (see “Relationships between \( k'^{\text{obs}}_1 \) and \( k'^{\text{obs}}_2 \) and \( k'_1 \), \( k'_2' \), \( k'_3 \), and \( k'_2' \) at a Higher Concentration of \( \text{H}_2\text{O}_2 \)” under “Appendix”).

Accordingly, we were able to describe all the rate constants in this reaction system, i.e. the reaction of native metMb with \( \text{H}_2\text{O}_2 \) in the presence and absence of an oxygen acceptor such as thiouanisole, indicating that Scheme I, which has long been suggested for \( \text{H}_2\text{O}_2 \)-assisted reactions by Mb, is indeed correct. Based on the rate constant values given for the Scheme I, chemical mechanisms of the individual reaction steps are discussed under “Discussion.”

**DISCUSSION**

It has long been postulated that oxidative reactions catalyzed by metMb using \( \text{H}_2\text{O}_2 \) as the oxidant proceed through the formation of compound I as an essential reaction intermediate (4–9). Up to now, however, formation of compound I has never been experimentally proven in the reaction system of native Mb with \( \text{H}_2\text{O}_2 \). Evidence for the formation of compound I during the reaction of metMb with \( \text{H}_2\text{O}_2 \) reported here comprises the following: 1) detection of the UV-visible absorption spectra typical to compound I of a protoheme protein; 2) decomposition of the spectral species upon reaction with thiouanisole, yielding methylphenyl sulfoxide; and 3) all the experimental findings observed here satisfactorily fit to Scheme I which assumes the formation and decomposition of compound I in the reaction. Rate constants for all the component reactions in the scheme have been successfully determined.

Among the rate constants for the reactions in Scheme I, those for \( k'_{21} \) showed the largest \( \text{D}_2\text{O} \) isotope effect as depicted in Table II, where the values in \( \text{D}_2\text{O} \) were 6 and 11 times smaller than those in \( \text{H}_2\text{O} \) for sperm whale and horse heart Mbs, respectively. Since \( k'_{21} \) is the rate constant for the degradation of compound I by \( \text{H}_2\text{O}_2 \), such a decrease in \( k'_{21} \) value in \( \text{D}_2\text{O} \) makes the degradation slower in the medium, explain-
ing why the accumulation of compound I was more easily seen in D2O rather than in H2O. No such a remarkable isotope effect was observed for the rate constant of other reactions in the scheme (Table II). Then, from a mechanistic point of view, the observed isotope effect on k_{21} suggests that compound 1 abstracts either a proton, hydrogen atom, or hydride ion from H2O2 when it performs the 2-electron oxidation of H2O2 according to Reaction 6.

In peroxidase-catalyzed reactions, the O–O bond of H2O2 is known to be cleaved in a heterolytic fashion (29). For the cleavage, a positive charge on an arginine residue present near the heme in the distal side (Arg-38 in horseradish peroxidase and Arg-48 in cytochrome c peroxidase) has been suggested to assist the heterolysis (29–31). On the other hand, hydrophobic amino acid residues such as Phe-43 and Val-68 occupy the distal space in Mb (32) resulting in a lack of amino acid residue(s) analogous to the arginine residue of peroxidases. Such a deficit in a charged residue(s) in metMb is in accordance with the view that the homolytic cleavage of H2O2 occurs at least in part in Mb-assisted peroxidative reactions (3–9, 28). However, the ratio of the homolysis to heterolysis (homo/hetero) has not been hitherto reported, because there was no way to measure the ratio. In this regard, we were able to obtain the values for k_{13} and k_{12} which correspond to the rate constants for the homolytic and heterolytic cleavages of H2O2, respectively. Hence the homo/hetero ratio corresponds to k_{13}/k_{12}. When the k_{13} and k_{12} values in Table II were applied, the ratio was found to be 2.7 and 3.5 in H2O for sperm whale and horse heart Mb, respectively. We were not able to exclude, however, the possibility that the homolytic cleavage also contributed to the k_{12} values in part; when the hydroxyl radical formed via the homolysis (Reaction 4) oxidizes not only the globin moiety but also the heme, it may also generate compound 1. If such an oxidation reaction was partly involved, the homo/hetero ratio determined here has been underestimated since k_{13} and k_{12} were determined in this study as the rate constants for the formations of ferryl Mb and compound I, respectively. It should be also noted that the data of k_{12} in Table II had somewhat larger uncertainties. Nevertheless, the present results in Table II (k_{13} 3.9–4.9 ± 0.5–0.7 × 10^6 M^{-1} s^{-1}; k_{12} 1.1–1.8 ± 1.5 × 10^6 M^{-1} s^{-1}) clearly indicate that the homolytic cleavages indeed proceeded at significant rates which were in a comparable order of magnitude as compared with that of the heterolysis.

The results of previous studies (4, 7, 8, 10) have suggested that His-64 plays a crucial role in the conversion of compound 1 to ferryl Mb (Reaction 2). As mentioned earlier, Watanabe and co-workers (7, 8) prepared several mutants of sperm whale Mb in which His-64 was replaced by an aliphatic amino acid such as alanine, serine, and leucine. When the reaction of such mutant Mb's with mCPBA was studied, they observed an accumulation of compound I that was not detected with native metMb. They interpreted the findings to indicate that the role of His-64 in the reaction is to assist a quick conversion of compound 1 to ferryl Mb, and hence its replacement with other kinds of amino acid made k_{23} smaller resulting in a slower conversion. No evidence was available, however, on whether or not the amino acid substitution indeed affected the rate of conversion; the rate constants (k_{23}) for the mutant Mbs were reported to be all about 1 s^{-1} at 5 °C (8), whereas no data were available for the native metMb until this study. As seen in Table II, we obtained k_{23} values of 4–6 and 3 ± 7 s^{-1} at 25 °C for sperm whale and horse heart metMbs, respectively. Although the S.D. values were large, we have assumed from these data the upper limit of the value to be 10 s^{-1}, which corresponds to about 2–3 s^{-1} at 5 °C if one takes the effects of temperature into account in an ordinary way. Thus it is difficult to judge whether such a difference in k_{23} values, 1:2–3, is significant enough to explain the accumulation of compound I. As will be discussed below, the substitution of His-64 may affect other factors such as the bimolecular rate constant for the reaction between mCPBA and mutant Mbs.

The values of k_{S1}, the bimolecular rate constant for the monooxygenation of thioanisole by compound I, were 2.7 × 10^4 and 3.0 × 10^4 M^{-1} s^{-1} for sperm whale and horse heart Mbs, respectively (Table I). These values were smaller by 2 orders of magnitude than 1.5 × 10^6 M^{-1} s^{-1} for H64A and H645 mutant Mbs (8). One of the possible causes for this difference is the steric hindrance offered by the side chain of His-64 which is greater in volume than the small side chains of Ala and Ser. If this is true, a similar difference in the bimolecular reaction rate of mCPBA with native and mutant Mbs can be expected to occur. The structure and the size of mCPBA (Cl-C6H5-C(O)OOH) are not grossly different from those of thioanisole (C6H5–S–CH3), and hence it is expected that His-64 can also hinder the access of mCPBA to the heme. Therefore, the replacement of His-64 by Ala or Ser possibly accelerates the reaction rate of mCPBA with metMb and in turn the formation of compound I of the mutant Mbs.

Finally a few words may be necessary for an additional reaction intermediate that had been expected to occur but was not detected in this study. Prior to the formation of compound I of heme proteins such as peroxidases, it has been considered that H2O2 coordinates to a ferric heme to form a hydroperoxy (Fe(OH)-O–H) intermediate termed compound 0 which undergoes a subsequent cleavage of the O–O bond in a heterolytic manner to give compound I (29, 33). Furthermore, several lines of evidence have indicated that the distal histidine of peroxidases (His-42 of horseradish peroxidase and His-52 of cytochrome c peroxidase) acts as a base catalyst to abstract one proton from H2O2, accelerating the formation of compound 0 (29, 34, 35). Also for Mb, the formation of compound I has been indicated to require a precursor compound corresponding to compound 0 (Scheme II) (3–9),

### Table II

| Compounds | $k_{13}$ | $k_{23}$ | $k_{12}$ | $k_{13}$ | $k_{23}$ | $k_{12}$ |
|-----------|---------|---------|---------|---------|---------|---------|
| Sperm whale | 1.8 ± 1.5 | 3.9 ± 0.5 | 12 ± 1 | 12 ± 1 | 12 ± 1 | 12 ± 1 |
| Horse heart | 3.9 ± 0.7 | 4.9 ± 0.7 | 12 ± 1 | 12 ± 1 | 12 ± 1 | 12 ± 1 |

The symbol $k_{12}/k_{13}$ denotes the slope of the plot of $k_{12}/k_{13}$ versus [H2O2].

Data of $k_{12} + k_{13}$ were obtained according to $\Delta(k_{12} + k_{13}) = k_{23} = k_{12} + k_{13}$.

Experimental data were not available for calculating $k_{12}$ and $k_{13}$ values by assuming Equation 6 (see text).

**Mean values ± S.D. of at least four experiments were obtained for $k_{12} + k_{13}$.** Based on the $k_{12} + k_{13}$ values thus obtained, data in the table were calculated according to the methods described in the text. The data of $k_{12} + k_{13}$ are those at 100 mM H2O2. $k_{12}$, $k_{13},$ and $k_{23}$ denote those in the absence of specific oxygen. Note that H2O2 acts as an oxygen acceptor in this case.
although such a compound has not been detected yet for the reaction of native metMb with H$_2$O$_2$.

Under the present experimental conditions, metMb showed a direct conversion to either compound I or ferryl Mb with rate constants $k_{12}$ or $k_{13}$, and no accumulation of compound 0 was detected. This finding indicates that the formation of compound 0 is the rate-limiting step in the pathway in Scheme II. In other words, $k_0$ in the above pathway is much smaller than $k'$ and $k''$. In such a case, $k_{14}$ and $k_{15}$ respectively correspond to $k_k k'/(k'' + k')$ and $k_k k''/(k' + k'')$, and therefore $k'_{12} + k''_{13}$ corresponds to $k_0' (= k_k[H_2O_2])$ which is the bimolecular rate constant for the formation of compound 0. The value of $k'_{12} + k''_{13}$ in D$_2$O (6.3 × $10^2$ M$^{-1}$ s$^{-1}$) was essentially the same as that in H$_2$O (6.7 × $10^2$ M$^{-1}$ s$^{-1}$) for sperm whale Mb (Table II). The corresponding data for horse heart Mb were 3.6 × $10^2$ M$^{-1}$ s$^{-1}$ in D$_2$O and 5.0 × $10^2$ M$^{-1}$ s$^{-1}$ in H$_2$O, which were only slightly different from each other. These results indicate that, if the coordination reaction of H$_2$O$_2$ to the heme involves a chemical step where a proton is detached from His-64 or the other amino acid residues in Mb act as a base catalyst in the coordination of H$_2$O$_2$ to the heme, at least under the present experimental conditions. Such a different feature of the distal histidine of Mb from that of peroxidases had already been suggested on the basis of the findings that the overall rate of the conversion from metMb to ferryl Mb upon the reaction with H$_2$O$_2$ is 5 orders of magnitude smaller than the bimolecular reaction rate between peroxidases and H$_2$O$_2$ (34). Further efforts to detect compound 0, if present, in the reaction of native metMb with H$_2$O$_2$ are underway in this laboratory.

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APPENDIX

The General Formula for Reaction Systems Involving Three Spectral Species—For the kinetic analyses, we first calculated singular value decomposition (36) of the absorption changes observed for the present reaction system. Upon analyses of the calculated results of singular value decomposition (22), we made a conclusion that only one spectral species other than metMb and ferryl Mb contributed to the absorption changes under the experimental conditions (data not shown). Thus, we have started from Scheme III which is a general formula for reaction systems involving metMb, ferryl Mb, and one unknown species (X) as well as all the possible reaction steps among the three species.

The application of Scheme III into the present reaction system does not necessarily mean that the system contains only three chemical species. Each step represented by one rate constant in the scheme may consist of a few chemical steps involving further intermediates each of which converts to others with an intrinsic rate constant. However, such intermediates were invisible in the present reaction system, indicating that the reaction steps to form them were very slow as compared with those of metMb, ferryl Mb, and X in Scheme III. In such a case, the intrinsic rate constants representing the invisible chemical species degenerate to one rate constant, as we exemplified in "Discussion". Therefore, Scheme III, which involves only three species and six rate constants, is sufficient to describe the kinetics of the present reaction system, regardless of possible involvement of more chemical species in the system.

Reaction systems represented by Scheme III were categorized into the competitive-consecutive reactions with reversible reaction steps by Szabó (37). When all rate constants in Scheme III are first- or pseudo first-order, the reaction system of Scheme III approaches from the initial condition ([metMb] = [metMb]$_0$, [ferryl Mb] = [ferryl Mb]$_0$, and [X] = [X]$_0$) to an equilibrium state by a first-order process with two apparent rate constants, $k_{1obs}$ and $k_{2obs}$, which relate with the rate constants for the elementary steps as in the following equations (Equations 8 and 9),

$$ k_{1obs} = \frac{S_1 + S_2}{2} + \frac{(S_1 - S_2)^2}{4} + (k_{12} - k_{13})(k_{21} - k_{31}) $$  \hspace{1cm} (Eq. 8)

$$ k_{2obs} = \frac{S_1 + S_2}{2} - \frac{(S_1 - S_2)^2}{4} + (k_{12} - k_{13})(k_{21} - k_{31}) $$  \hspace{1cm} (Eq. 9)

where $S_1$ and $S_2$ are further given by following Equations 10 and 11(37).

$$ S_1 = k_{12} + k_{13} + k_{31} $$  \hspace{1cm} (Eq. 10)

$$ S_2 = k_{21} + k_{23} + k_{32} $$  \hspace{1cm} (Eq. 11)

Then the combination of Equations 8–11 yields Equation 12.

$$ k_{1obs} + k_{2obs} = k_{12} + k_{13} + k_{21} + k_{23} + k_{31} + k_{32} $$  \hspace{1cm} (Eq. 12)

Equation 12 describes that the sum of the apparent rate constants is equivalent to that of the rate constants for the elementary steps. In the present stopped-flow rapid-scan experiments, the reaction of metMb with H$_2$O$_2$ approached from the initial ([metMb] = [metMb]$_0$, [ferryl Mb] = [X] = [X]$_0$) to the final ([metMb] = [X] = [X]$_0$, [ferryl Mb] = [ferryl Mb]$_0$) conditions. Thus, $k_{31}$ and $k_{32}$ are very small as compared with the other rate constants, practically being almost zero. If it is not, [metMb] $
eq$ 0 and [X] $
eq$ 0 at $t = \infty$. Accordingly, Equation 12 is reduced to Equation 13 under the present experimental conditions.

$$ k_{1obs} + k_{2obs} = k_{12} + k_{13} + k_{21} + k_{23} $$  \hspace{1cm} (Eq. 13)

If X in Scheme III can decompose to metMb upon reacting with a sulfide, $k_{23}$ is given as a sum of two rate constants, $k_{21}$ and $k_{23}$; the former is a pseudo first-order constant for the reaction between X and the sulfide, and the latter is independent of the sulfide. In the presence of an excess amount of the sulfide, then $k_{1obs} + k_{2obs}$ is given by Equation 14,

$$ k_{1obs} + k_{2obs} = k_{12} + k_{13} + k_{21} + k_{23} $$  \hspace{1cm} (Eq. 14)

where $k_{2obs}$ ( = $k_{21}$/[sulfide]) is a bimolecular rate constant between X and the sulfide. This equation, a linear equation in the variables, $k_{1obs} + k_{2obs}$ and [sulfide], means that a plot of $k_{1obs} + k_{2obs}$ versus [sulfide] gives a straight line with a slope of $k_{2obs}$.

Rate Constant for the Decay of Compound I to Ferryl Mb—In the presence of an excess amount of H$_2$O$_2$, $k_{12}$ and $k_{13}$ are given by Equations 15 and 16,
where \( k'_{12} \) and \( k'_{13} \) are bimolecular rate constants. On the other hand, when specific oxygen acceptors, such as sulfides, are absent in the present reaction system, only the reaction between compound I and \( \text{H}_2\text{O}_2 \) contributes to \( k_{21} \) in Scheme I. In such a case, \( k_{21} \) is also given by using a bimolecular rate constant as shown in Equation 17.

\[
k_{21} = k'_{13}[\text{H}_2\text{O}_2] \quad \text{(Eq. 17)}
\]

Substituting the above three equations into Equations 8 and 9, and setting \( k_{31} \) and \( k_{32} \) to zero, we can express \( k_{10}^{\text{obs}} \) and \( k_{20}^{\text{obs}} \) by Equations 2 and 3. The combination of Equations 2 and 3 yields Equation 7. Thus, a plot of \( k_{10}^{\text{obs}} + k_{20}^{\text{obs}} \) versus \([\text{H}_2\text{O}_2]\) gives a straight line whose slope and intercept on the \( k_{10}^{\text{obs}} + k_{20}^{\text{obs}} \) axis are \( k'_{12} + k'_{13} + k'_{21} \) and \( k'_{23} \), respectively.

Dependences of \( k_{10}^{\text{obs}} \) and \( k_{20}^{\text{obs}} \) on \([\text{H}_2\text{O}_2]\) under Condition That \( k'_{21} \) Is Negligible—If \( k'_{21} \) is negligible, then Equations 2 and 3 are reduced as shown in Equations 18 and 19.

\[
k_{10}^{\text{obs}} = \frac{(k'_{12} + k'_{13})[\text{H}_2\text{O}_2] + k_{23}}{2} + \frac{\langle (k'_{12} + k'_{13})[\text{H}_2\text{O}_2] - k_{23} \rangle^2}{4}
\]

\[
k_{20}^{\text{obs}} = \frac{(k'_{12} + k'_{13})[\text{H}_2\text{O}_2] + k_{23}}{2} - \frac{\langle (k'_{12} + k'_{13})[\text{H}_2\text{O}_2] - k_{23} \rangle^2}{4} = k_{23}
\]

Relationships between \( k_{10}^{\text{obs}} \) and \( k_{20}^{\text{obs}} \) and \( k'_{12}, k'_{13}, k'_{21}, \) and \( k'_{23} \) at a Higher Concentration of \( \text{H}_2\text{O}_2 \)—\( k_{10}^{\text{obs}} \) and \( k_{20}^{\text{obs}} \) are given by Equations 2 and 3. Among the terms in these equations, \( k_{23} \) is a constant, whereas others (\( k'_{12}, \text{[H}_2\text{O}_2] \) etc.) involve the parameter \([\text{H}_2\text{O}_2]\). Therefore \( k_{23} \) becomes negligible as compared with other terms when \([\text{H}_2\text{O}_2]\) becomes large. Then Equations 2 and 3 are reduced to Equations 20 and 21, respectively, at larger values of \([\text{H}_2\text{O}_2]\).

\[
k_{10}^{\text{obs}} = \frac{(k'_{12} + k'_{13} + k'_{21})[\text{H}_2\text{O}_2] + k_{23}}{2} + \frac{\langle (k'_{12} + k'_{13} + k'_{21})[\text{H}_2\text{O}_2] - k_{23} \rangle^2}{4}
\]

\[
k_{20}^{\text{obs}} = \frac{(k'_{12} + k'_{13} + k'_{21})[\text{H}_2\text{O}_2] + k_{23}}{2} - \frac{\langle (k'_{12} + k'_{13} + k'_{21})[\text{H}_2\text{O}_2] - k_{23} \rangle^2}{4}
\]

Substituting Equations 20 and 21 into the left hand of Equation 6, one can obtain the right hand.

Although Equation 6 is a reasonable approximation under an excess \([\text{H}_2\text{O}_2]\), there must be some discrepancies between \( k_{10}^{\text{obs}} \) and \( k_{20}^{\text{obs}} \), causing certain uncertainties for calculated \( k'_{21} \), \( k'_{12} \), and \( k'_{13} \) values when they were obtained by using Equation 6 with experimental \( k_{10}^{\text{obs}} \) and \( k_{20}^{\text{obs}} \) data. We thus estimated how \( k_{10}^{\text{obs}} \) and \( k_{20}^{\text{obs}} \) were different from \( k'_{21} \), \( k'_{12} \), and \( k'_{13} \). Here, for convenience, the approximate \( k_{10}^{\text{obs}} \) and \( k_{20}^{\text{obs}} \) defined by Equations 20 and 21 were denoted by \( k'_{10} \) and \( k'_{20} \), respectively.

Under a given \([\text{H}_2\text{O}_2]\), these parameters are different from the actual \( k_{10}^{\text{obs}} \) and \( k_{20}^{\text{obs}} \) by small values as \( k_{10}^{\text{obs}} = k'_{10} + \alpha_1 \) and \( k_{20}^{\text{obs}} = k'_{20} + \alpha_2 \). Obviously, \( k'_{10} + k'_{12} < k'_{13} \). Observe that \( \alpha_1 + \alpha_2 = k_{23} \) from Equations 7, 20, and 21. The difference between \( k_{10}^{\text{obs}} \) and \( k_{20}^{\text{obs}} \) is then given as follows in Equation 22.
By comparing the Equation 29 to Equation 1, we can find relationships given by Equations 30–32,

\[
c_i^4 = [\text{metMb}]d\left(\frac{e_{\text{mol}}^1}{l_2} - \frac{1}{l_2 - l_1} - e_{\text{core}}^1\frac{l_2 - 1}{l_2 - l_1}\right) \quad (\text{Eq. 30})
\]

\[
c_i^3 = [\text{metMb}]d\left(-e_{\text{mol}}^1\frac{l_1}{l_1 - l_2} + \frac{1}{l_2 - l_1} + e_{\text{core}}^1\frac{l_1 - 1}{l_2 - l_1}\right) \quad (\text{Eq. 31})
\]

\[
c_i^2 = [\text{metMb}]d\frac{d e_{\text{core}}}{l_1} \quad (\text{Eq. 32})
\]

By combining Equations 30–32, we can obtain Equation 4. On the other hand, the combination of Equations 26 and 27 yields Equation 5.

REFERENCES

1. Grisham, M. B. (1985) J. Free Radicals Biol. Med. 1, 227–232
2. Galaris, D., Sevanian, A., Cadenas, E., and Hochstein, P. (1990) Arch. Biochem. Biophys. 281, 163–169
3. Ortiz de Montellano, P. R., and Catalano, C. E. (1985) J. Biol. Chem. 260, 32735–32738
4. Rao, S. I., Wilks, A., and Ortiz de Montellano, P. R. (1993) J. Biol. Chem. 268, 3273–3275
5. Adachi, S., Nagano, S., Ishimori, K., Watanabe, Y., Morishima, I., Egawa, T., and Kitagawa, T. (1995) Biochemistry 34, 345–362
6. Ozaki, S., Matsui, T., and Watanabe, Y. (1996) J. Am. Chem. Soc. 118, 9784–9785
7. Ozaki, S., Matsui, T., and Watanabe, Y. (1997) J. Am. Chem. Soc. 119, 6666–6667
8. Matsui, T., Ozaki, S., and Watanabe, Y. (1997) J. Biol. Chem. 272, 32735–32738
9. Matsui, T., Ozaki, S., and Watanabe, Y. (1999) J. Am. Chem. Soc. 121, 9952–9957
10. King, N. K., Looney, F. D., and Winfield, M. E. (1967) Biochim. Biophys. Acta 133, 65–82
11. Yonetani, T., and Schleyer, H. (1967) J. Biol. Chem. 242, 1974–1979
12. Gibson, J. P., Ingrom, D. J. E., and Nicholls, P. (1985) Nature 311, 1398–1399
13. Wilks, A., and Ortiz de Montellano, P. R. (1992) J. Biol. Chem. 267, 8827–8833
14. Fenwick, C. W., and English, A. M. (1996) J. Am. Chem. Soc. 118, 12236–12237
15. George, P., and Irvine, D. H. (1952) Biochem. J. 52, 511–517
16. Piersch, J., Blumberg, W. E., Wittenberg, B. A., and Wittenberg, J. B. (1968) J. Biol. Chem. 243, 1871–1880
17. Harami, T., Mueda, Y., Morita, Y., Trautwein, A., and Gosner, U. (1977) J. Chem. Phys. 67, 1164–1169
18. Morishima, I., and Ogawa, S. (1978) Biochemistry 17, 4384–4388
19. Sitter, A. J., Rezek, C. M., and Terness, J. (1985) Biochim. Biophys. Acta 828, 229–235
20. Salomaa, P., Schalenger, L. L., and Long, F. A. (1964) J. Am. Chem. Soc. 86, 1–7
21. Egawa, T., Shimada, H., and Ishimura, Y. (1994) Biochem. Biophys. Res. Commun. 201, 1464–1469
22. Hofrichter, J., Henry, E. R., Sommer, J. H., Deutsch, R., Ikeda-Saito, M., Yonetani, T., and Eaton, W. A. (1985) Biochemistry 24, 2667–2679
23. Hug, S. J., Lewis, J. W., Kinterz, C. M., Thorgerisson, T. R., and Kliger, D. S. (1990) Biochemistry 29, 1475–1485
24. Schowen, R. L. (1977) in Isotope Effects on Enzyme-catalyzed Reactions (Cleland, W. W., O’Leary, M. H., and Northrop, D. B., eds) pp. 64–99, University Park Press, Baltimore, MD
25. Dunford, H. B., and Stillman, J. S. (1976) Coord. Chem. Rev. 19, 187–251
26. Schonbaum, G. R., and Chance, B. (1976) Enzymes 13, 363–408
27. Hes, W. D., and Hager, L. P. (1979) in The Porphyrens (Dolphin, D., ed) Vol. 7, pp. 295–332, Academic Press, New York
28. Miki, H., Harada, K., Yamaizaki, I., Tamura, M., and Watanabe, H. (1989) Arch. Biochem. Biophys. 275, 345–362
29. Poulas, T. L., and Kraut, J. (1980) J. Biol. Chem. 255, 8199–8205
30. Vitello, L. B., Erman, J. E., Miller, M. A., Wang, J., and Kraut, J. (1993) Biochemistry 32, 9807–9818
31. Rodriguez-Lopez, J. N., Smith, A. T., and Thorneley, R. N. F. (1996) J. Biol. Chem. 271, 4023–4030
32. Phillips, S. E. V. (1980) J. Mol. Biol. 142, 531–554
33. Baek, H. K., and Van Wart, H. E. (1989) Biochemistry 28, 5714–5719
34. Erman, J. E., Vitello, L. B., Miller, M. A., Shaw, A., Brown, K. A., and Kraut, J. (1993) Biochemistry 32, 9798–9806
35. Newmyer, S. L., and Ortiz de Montellano, P. R. (1995) J. Biol. Chem. 270, 19420–19426
36. Golub, G. H., and Kahan, W. (1965) SIAM J. Numer. Anal. 2, 205–224
37. Sauzé, Z. G. (1969) in Comprehensive Chemical Kinetics (Bamford, C. H., and Tipper, C. F. H., eds) Vol. 2, pp. 31–32, Elsevier Science Publishing Co., Inc., New York