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

Toshitaka Matsui,[†] Shin-ichi Ozaki,[†,§] and Yoshihito Watanabe*[,†,‡]

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

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Abstract: A His64 → Asp mutant of sperm whale myoglobin (Mb), H64D Mb, has been prepared to mimic the active site of chloroperoxidase from the marine fungus *Caldariomyces fumago*, in which distal glutamic acid is suggested to enhance compound I formation by H_2O_2. The H64D mutant allows us to see the accumulation of compound I in the reaction of Mb with H_2O_2 for the first time. The successful observation of compound I is due to at least 50-fold improvement in the formation rate of compound I as well as its stabilization upon the His64 → Asp replacement. Catalytic activity of wild-type Mb and a series of His64 Mb mutants (H64A, H64S, H64L, and H64D Mb) are examined for one-electron oxidation and oxygenation by using H_2O_2 as an oxidant. The H64D mutant is the best catalyst among the myoglobins and shows 50−70-fold and 600−800-fold higher activity than the wild type in the one-electron oxidations and peroxygenations, respectively. The origin of the varied activity upon the mutations is discussed on the basis of the formation rate and stability of compound I.

Introduction

Myoglobin (Mb),[1] a carrier of molecular oxygen, is one of the most extensively examined hemoproteins, and is a good model for elucidating the role of active site residues in the interaction of hemoproteins with small molecules such as dioxygen, carbon monoxide, and cyanide.[2−5] The reaction between Mb and H_2O_2 also has been studied over three decades from such perspectives as a possible source of myocardial reperfusion injury,[6,7] a model for the reactions catalyzed by peroxidase and catalase,[8−10] and, like cytochrome P-450, a catalyst for the oxygenation of olefins and sulfides.[11−14] Incubation of Mb with H_2O_2 causes slow conversion ($\sim 10^2$ M^{-1} s^{-1}) of the ferric heme to a ferryl heme (Fe^{IV}=O Por), similar to compound II in horseradish peroxidase (HRP).[8,15,16] Compound II has only one oxidizing equivalent above the ferric state despite two oxidizing equivalents of H_2O_2. One more oxidizing equivalent in Mb is found as a protein radical, leading to protein dimerization (Figure 1).[16,17] On the contrary, peroxidases and catalases readily react with H_2O_2 ($\sim 10^7$ M^{-1} s^{-1}) to give a two-electron oxidized heme (compound I), which is normally a ferryl porphyrin cation radical (Fe^{IV}=O $Por^{+\bullet}$).[18,19] Compound I in Mb has never been observed for the native and wild-type proteins. While partial homolysis of the peroxide bond can form compound II (Figure 1), heterolysis has been suggested to be a major process.[20] Thus, the absence of Mb compound I appears to be due to its rapid decay to compound II.

A histidine residue in a distal heme pocket (His64 in sperm whale Mb) has been shown as one of the radical sites of Mb treated by H_2O_2.[21,22] Recently, we have reported that His64 mutants (H64A, H64S, and H64L mutants) of sperm whale Mb

* To whom correspondence should be addressed. E-mail: yoshi@ims.ac.jp.
† Institute for Molecular Science.
§ Present address: Faculty of Education, Yamagata University, Kojirakawa, Yamagata 990-8560, Japan.
‡ The Graduate University for Advanced Studies.

(1) Abbreviations used: Mb, myoglobin; HRP, horseradish peroxidase; CPO, chloroperoxidase from the marine fungus *Caldariomyces fumago*; compound I, a ferryl porphyrin cation radical; compound II, a ferryl porphyrin; *m*CPBA, *m*-chloroperbenzoic acid; ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid).

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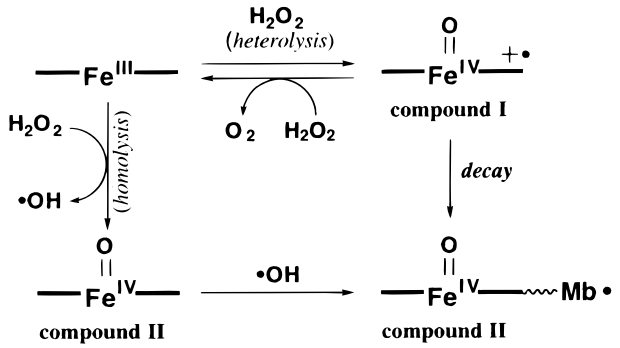

Figure 1. Proposed pathway for the reaction of Mb with H_2O_2.

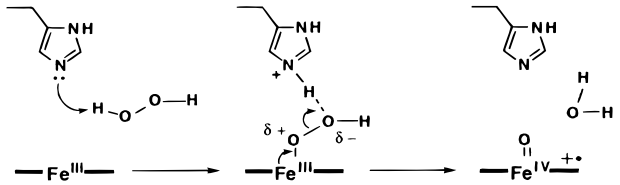

Figure 2. Proposed roles of distal histidine in the formation of compound I.

react with organic peracids including *m*-chloroperbenzoic acid (*m*CPBA) to accumulate compound I.[23] Thus, the instability of Mb compound I can be attributed to the rapid reduction by the distal histidine, which is located very close to the heme center.[21] In the reaction with H_2O_2, however, Mb compound I has never been observed, even in the His64 mutants.[23] Although we have attributed the absence of compound I to its rapid reduction by H_2O_2 (Figure 1), there is no direct evidence for the compound I formation in the reaction with H_2O_2. The direct observation of Mb compound I requires its faster formation and slower reduction by H_2O_2 as well as its stabilization. The enhancement of the compound I formation, which is often a rate-determining step in oxidation reactions catalyzed by Mb, is of particular interest from the viewpoint of constructing an efficient oxidation enzyme.[24]

In this paper, we report the preparation of a mutant of sperm whale Mb where the distal histidine is replaced by aspartic acid (H64D Mb), to mimic the active site of chloroperoxidase from the fungus *Caldariomyces fumago* (CPO). While classical peroxidases and catalases accelerate the formation of compound I by utilizing distal histidine, probably as a general acid−base catalyst (Figure 2), distal glutamic acid is suggested to be crucial for the rapid formation of compound I in CPO.[25−27] The H64D Mb mutant allows us to observe, for the first time, partial accumulation of compound I in the reaction with H_2O_2. The reactivity of ferric H64D Mb with H_2O_2 is suggested to be at least 50-fold higher than that of wild-type Mb. We have also examined catalytic activity of wild-type Mb and a series of His64 mutants (H64A, H64S, H64L, and H64D Mb) for one-electron oxidations and oxygenations by using H_2O_2 as an oxidant. The H64D mutant is the best catalyst among the myoglobins and shows 50−70-fold and 600−800-fold higher activity than the wild type in the one-electron oxidations and peroxygenations, respectively. The origin of the varied activity upon the mutations is discussed on the basis of the formation rate and stability of compound I.

Experimental Procedures

Materials. The mutant genes for H64D, H64A, H64S, and H64L sperm whale Mb were constructed by cassette mutagenesis. The cassette including the desired His-64 substitution and a new silent *Hpa*I restriction site was inserted between the *Bgl*II and *Hpa*I sites. The expression and purification of the mutants were performed according to the method described by Springer et al.[3] All the chemicals were obtained from Wako and Nakalai Tesque, and used without further purification. The buffers used for the reactions were 50 mM sodium phosphate (pH 7.0) and sodium acetate (pH 5.3).

Reaction with *m*CPBA and Hydrogen Peroxide. All the spectral changes were monitored on a Hi-Tech SF-43 stopped-flow apparatus equipped with a MG 6000 diode array spectrophotometer. An optical filter (360 nm) was used for avoiding possible photoreduction of compound I by UV light. The reactions with *m*CPBA were carried out at pH 5.3 for observing a complete accumulation of compound I and at pH 7.0 for determining the decay rate of compound I to compound II.[23] The reactivity of compound I with substrates was determined by means of a double-mixing rapid scan technique at 5.0 °C and pH 5.3. The first mixing of ferric Mb with a slight excess of *m*CPBA (1.3 mol equiv) resulted in approximately 80% accumulation of compound I, which was subsequently mixed with at least 10-fold excess of substrates. The bimolecular rate constants were calculated from observed rates at more than four different concentrations of the substrates. The reaction with H_2O_2 was performed at 20 °C in the absence and presence of 0.87 mM styrene. The compound I intermediate was best observed at pH 7.0 with H_2O_2 as an oxidant.

Assay of the One-Electron Oxidations. Activity for one-electron oxidation of guaiacol and ABTS was measured at 20 °C and pH 7.0 on a Shimadzu UV-2400 spectrophotometer. The formation rate of the guaiacol oxidation product was determined from the increase in the absorbance at 470 nm ($\epsilon = 3.8 \times 10^3$ M^{-1} cm^{-1}).[24] The 1 mL final assay volume contained 2 mM guaiacol, variable amounts of H_2O_2 (0.2−5 mM), and 1 μM Mb except for the H64D mutant (0.5 μM). The formation of the ABTS cation radical was monitored at 730 nm ($\epsilon = 1.4 \times 10^4$ M^{-1} cm^{-1}).[24] The reaction mixture contained 1 mM ABTS and variable amounts of H_2O_2 (0.1−2 mM). Final concentrations of Mb were 0.5 μM for wild type, H64A, and H64S Mb, 1 μM for H64L Mb, and 10 nM for H64D Mb.

Assay of the Peroxygenation Reactions. Peroxygenations of thioanisole and styrene were performed at 25 °C and pH 7.0. The reaction mixture contained 10 μM Mb, 1 mM H_2O_2, and either 1 mM thioanisole or 8.7 mM styrene. For the thioanisole sulfoxidation, acetophenone was added as an internal standard, and the mixture was extracted with dichloromethane for HPLC analysis on a Dicel OD chiral-sensitive column installed on a Shimadzu SPD-10A spectrophotometer equipped with a Shimadzu LC-10AD pump system. For the styrene oxidation, an internal standard was 2-phenyl-2-propanol, and the dichloromethane extracts were analyzed by GC (Shimadzu GC-14B) equipped with a Chiraldex G-TA capillary column.

To determine total yields of the *m*CPBA-supported styrene oxidation, 100 μM *m*CPBA was added to a solution containing 10 μM Mb and 8.7 mM styrene at pH 5.3. Incubation time was 5 min for wild-type Mb and 0.5−1 min for the His64 mutants. The exhaustion of *m*CPBA was confirmed by the addition of excess potassium iodide before the extraction with dichloromethane.

Kinetic Measurements for Association of Cyanide The association rate of ferric Mb with cyanide was measured at 20 °C and pH 7.0 on a Hi-Tech stopped-flow apparatus. The kinetic traces at 408 nm were used for determining pseudo-first-order rates. The association rate constants were given by the slope of a plot of the observed rates versus cyanide concentration.

Reaction with Cumene Hydroperoxide The reaction mixture containing 10 μM Mb and 270 μM cumene hydroperoxide was incubated for 50 min at 20 °C and pH 7.0. Aliquots of the mixture

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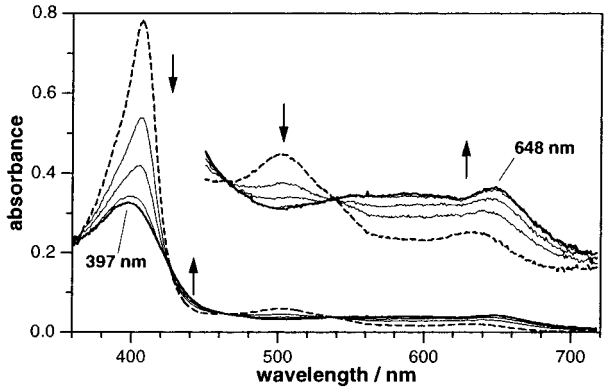

Figure 3. Rapid-scan absorption spectra of H64D Mb in the reaction with *m*CPBA in 50 mM sodium acetate buffer (pH 5.3) at 5.0 °C. Broken line represents the spectrum of the ferric mutant and solid lines are spectra recorded at 50, 100, 200, and 300 ms after mixing. Directions of the spectral change are indicated in the figure by arrows.

were analyzed by a Shimadzu HPLC system equipped with a Shimadzu CR-6A Data Module on a GL Sciences ODS-80A column. Phenethyl alcohol was employed as an internal standard. The column was eluted with 50% water/50% methanol at a flow rate of 1.0 mL/min and the effluent was monitored at 210 nm. Assignment of the components was based on the retention time of authentic samples.

Results

Stability and Reactivity of Compound I in H64D Mb. Figure 3 shows absorption spectral change during the reaction of ferric H64D Mb with *m*-chloroperbenzoic acid (*m*CPBA). Upon mixing, Soret absorption decreased to less than half and a broad visible band having a peak around 648 nm appeared. The spectral change is typical for the conversion of a ferric heme to a ferryl porphyrin cation radical (Fe^{IV}=O $Por^{+\bullet}$), equivalent of compound I in HRP.[18] Compound I in the H64D mutant spontaneously decayed to a ferryl heme (Fe^{IV}=O Por) and a protein radical.[15,28] Since wild-type Mb directly formed the ferryl heme, similar to compound II in peroxidases, coupled with a protein radical under the same condition,[15] the H64D mutation as well as H64A, H64S, and H64L mutations[23] appear to prolong the lifetime of compound I. The decay rate of H64D compound I (1.2 s^{-1}) was almost the same as those in the other His64 mutants (0.9−1.2 s^{-1}),[23] indicating a subtle effect of Asp64 on the stability of compound I. The elimination of the histidine residue is crucial for the stabilization as suggested earlier.[23]

The reactions of compound I, prepared by *m*CPBA, with substrates were examined by using a double mixing stopped-flow technique, in which all the His64 mutants showed similar spectral changes. Addition of guaiacol resulted in sequential reduction of compound I to compound II and the ferric state (Figure 4A). On the contrary, thioanisole, styrene, and H_2O_2 reduced compound I directly to the ferric state (Figure 4B). Thus, compound I in the Mb mutants appears to be capable of one-electron oxidations, oxygenations, and oxidation of H_2O_2 to O_2 as catalyzed by peroxidase, P450, and catalase, respectively. The reduction rates of compound I summarized in Table 1 are essentially the same among the mutants except for 7-fold decrease in the thioanisole sulfoxidation by H64D compound I.

Direct Observation of H64D Mb Compound I in the Reaction with H_2O_2. In the reaction with H_2O_2, ferric H64D Mb was oxidized to compound II with a biphasic spectral change (Figure 5), which clearly indicates accumulation of an intermediate species prior to the compound II formation. The initial Soret decrease coupled with increase in visible absorption appears to be due to partial accumulation of compound I (approximately 40% based on the Soret absorbance). In fact, incubation with guaiacol and H_2O_2 resulted in the direct formation of compound II, which is consistent with the rapid reduction of compound I to compound II by guaiacol (Figure 4A, Table 1). The addition of thioanisole or styrene also prevented us from observing the compound I-like intermediate and significantly retarded the compound II formation. This observation can be rationalized by the rapid reduction of compound I to the ferric state by thioanisole and styrene (Figure 4B, Table 1) by a two-electron process. Thus, it is now convincing that the observed intermediate is compound I, and this is the first observation of Mb compound I by use of H_2O_2 as an oxidant.

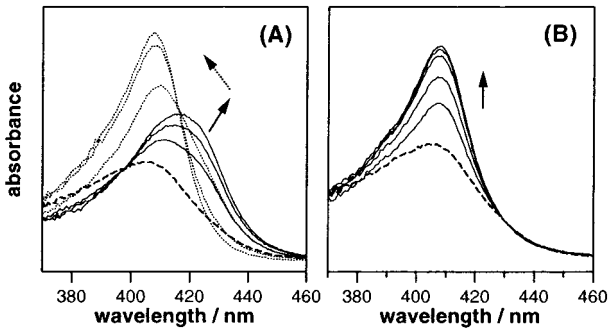

Figure 4. Soret absorbance changes during the reaction of compound I in H64D Mb, prepared by *m*CPBA, with guaiacol and styrene at pH 5.3 and 5.0 °C. (A) The reaction with guaiacol was monitored before mixing (broken line), at 2, 8, 20 ms (solid lines), and at 1, 3, 5 s (dotted lines) after mixing. (B) The reaction with styrene was monitored before mixing (broken line), and at 0.1, 0.3, 0.5, and 0.7 s after mixing. The directions of absorbance changes are indicated by arrows.

Table 1. Bimolecular Rate Constants for One- and Two-Electron Reduction of Compound I (M^{-1} s^{-1})[a]

myoglobin	guaiacol[b]	thioanisole[c]	styrene[c]	H_2O_2[c]
H64A	3.1×10^6	1.5×10^6	2.2×10^4	5.9×10^3
H64S	1.6×10^6	1.5×10^6	2.6×10^4	6.2×10^3
H64D	8.8×10^5	2.2×10^5	2.1×10^4	1.8×10^4

[a] Determined in 50 mM sodium acetate buffer (pH 5.3) at 5.0 °C. [b] Reduction rates of compound I to compound II. [c] Reduction rates of compound I to the ferric form.

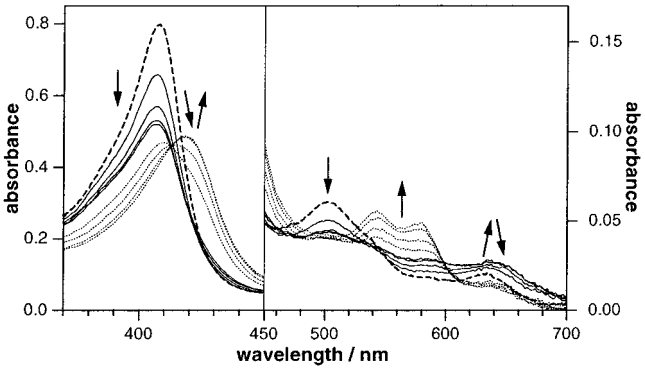

Figure 5. Spectral changes of H64D Mb in the reaction with 1 mM H_2O_2 at pH 7.0 and 20 °C. The spectra were recorded before mixing (broken line) and at 10, 30, 60, 100, and 150 ms (solid line) and 1, 2, 4, and 6 s (dotted line) after mixing. Directions of absorbance changes are indicated by arrows. For clarity, Soret and visible regions are expanded and magnified, respectively.

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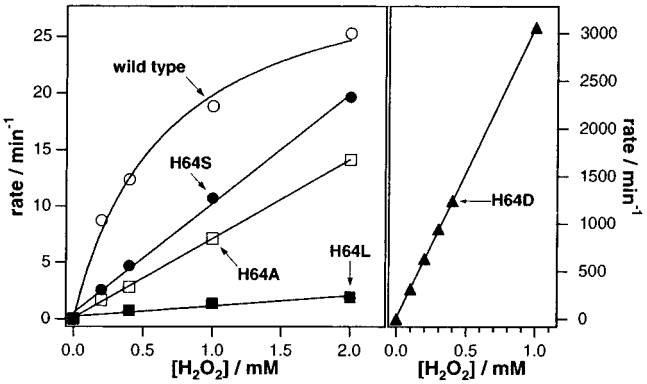

Figure 6. Initial oxidation rates of ABTS as a function of the concentration of H_2O_2 at 20 °C in 50 mM sodium phosphate buffer (pH 7.0).

Compound I of the H64A, H64S, and H64L mutants could not be prepared by H_2O_2, possibly because its formation is slower than its reduction by H_2O_2 (Table 1).[23] The rates of compound I reduction by H_2O_2 do not greatly differ between the H64D mutant and the others (Table 1). Therefore, the formation rate of H64D compound I should be much higher than those in other His64 mutants. Unfortunately, we have failed in kinetic analysis of the reactions between the His64 mutants and H_2O_2 due to the complex reaction mechanism (Figure 1). The exact reaction rates of ferric Mb with H_2O_2 could be determined only for the wild type as 5.1×10^2 M^{-1} s^{-1}, where compound I formation was kinetically negligible.[24] Since the 40% accumulation of H64D compound I requires that the formation rate of compound I is similar to its reduction rate by H_2O_2 (1.8×10^4 M^{-1} s^{-1}, Table 1), the His64 → Asp replacement is strongly suggested to promote the formation of compound I by H_2O_2.

One-Electron Oxidation of Guaiacol and ABTS. Catalytic one-electron oxidation (peroxidation) of guaiacol and ABTS was examined for the wild type and His64 mutants at 20 °C and pH 7.0 in the presence of H_2O_2. A possible oxidation of H_2O_2 by compound I is negligible during the peroxidation because of the higher reactivity of guaiacol and ABTS than H_2O_2 (Table 1). With increasing concentrations of H_2O_2, all the His64 Mb mutants exhibited a linear increase in the peroxidation activity (Figure 6). Thus, the major rate-determining step of the mutants is the reaction of ferric Mb with H_2O_2. Wild-type Mb showed hyperbolic dependency due to the transition of the rate-determining step as reported earlier.[24] In the presence of a small amount of H_2O_2, the slowest step for the wild type is also the reaction of the ferric Mb with H_2O_2. Therefore, the slopes of the [H_2O_2]−rate plots (for the wild type, determined at [H_2O_2] = 0.2 mM) mainly depend on the reactivity of ferric Mb with H_2O_2. Each Mb afforded similar slopes for guaiacol and ABTS (Table 2).

The peroxidation activity appears to be affected by the polarity of the residues at position 64; i.e., the apolar replacement by Leu resulted in more than 100-fold decrease in activity, compared to approximately 6- and 4-fold decrease in activity in H64A and H64S Mb, respectively, but 50−70-fold increase in activity in H64D Mb (Table 2). The results indicate that the His64 → Asp replacement significantly improves the activation rate of H_2O_2 by ferric Mb while the other His64 mutants are less reactive with H_2O_2 than the wild type.

The rate constant for the H_2O_2 activation can be calculated from the peroxidation activity assuming that (1) compound I formation is the sole rate-determining step and (2) two molecules of the substrate are oxidized by one electron upon the activation of one molecule of H_2O_2. Any incomplete fit to the assumption affords lower values than real rate constants. The ABTS oxidation activity gives a reasonable value for the wild type, 3.7×10^2 M^{-1} s^{-1}, which is roughly the same as that directly determined from the formation rate of compound II (5.1×10^2 M^{-1} s^{-1}). An estimated rate constant for the H64D mutant is 2.5×10^4 M^{-1} s^{-1}, and thus, ferric H64D Mb is found to react with H_2O_2 at least 50-fold faster than wild-type Mb.

Table 2. Slopes of the One-Electron Oxidation Activity of Mb against the Concentration of H_2O_2[a]

myoglobin	guaiacol	ABTS
wild type	38	44
H64L	0.12	0.73
H64A	6.8	7.0
H64S	8.9	9.5
H64D	1920	3050

[a] All the assays were performed in 50 mM sodium phosphate buffer (pH 7.0) at 20 °C. The values for the wild type were the rates at [H_2O_2] = 0.2 mM divided by 0.2 mM. The unit is nmol product/nmol Mb·min·[H_2O_2] mM.

Table 3. H_2O_2-Dependent Sulfoxidation of Thioanisole[a]

myoglobin	rate/min^{-1}	ee/*R* %	^{18}O from $H_2{}^{18}O_2$/%
wild type	0.25	25	92
H64L	0.072	27	89
H64A	0.74	6	98
H64S	1.7	9	99
H64D	145	6	99

[a] Determined in 50 mM sodium phosphate buffer (pH 7.0) at 20 °C.

Table 4. Peroxide-Ddependent Styrene Oxidation Catalyzed by Mb

	H_2O_2[a]				*m*CPBA[b]
myoglobin	rate/ min^{-1}	SO: PAA:BA[c]	ee(SO[c])/ *R* %	^{18}O in SO[c] from $H_2{}^{18}O_2$/%	yield/ %[d]
wild type	0.022	67:22:11	9	20	6.7
H64L	0.023	87:6:7	34	73	93
H64A	0.49	15:80:5	75	87	111
H64S	0.71	17:79:4	75	86	104
H64D	18	19:79:2	88	95	102

[a] Determined in 50 mM sodium phosphate buffer (pH 7.0) at 20 °C. [b] Determined in 50 mM sodium acetate buffer (pH 5.3) at 20 °C. [c] Abbreviations: SO, styrene oxide; PAA, phenylacetoaldehyde; BA, benzaldehyde. [d] nmol total products/nmol *m*CPBA added.

Peroxygenation of Thioanisole and Styrene. The H_2O_2-dependent oxygenation (peroxygenation) of thioanisole and styrene was examined at 20 °C and pH 7.0 (Tables 3 and 4). The H_2O_2 oxidation by compound I is also negligible due to the higher reactivity or higher concentration of thioanisole and styrene than H_2O_2 (Table 1). As reported earlier, thioanisole was exclusively converted to the corresponding sulfoxide, and styrene was oxidized to styrene oxide, phenylacetaldehyde, and benzaldehyde.[13,28−30] A major product for the styrene oxidation was styrene oxide in wild type and H64L Mb, and phenylacetaldehyde in the other His64 mutants (Table 4).

The peroxygenation activity of the mutants is lowest with H64L Mb and highest with H64D Mb as in the peroxidations (Tables 2−4). Relative activities of the mutants versus wild-type Mb, however, are much higher than those in the peroxidations. For example, the H64D replacement caused 50−70-

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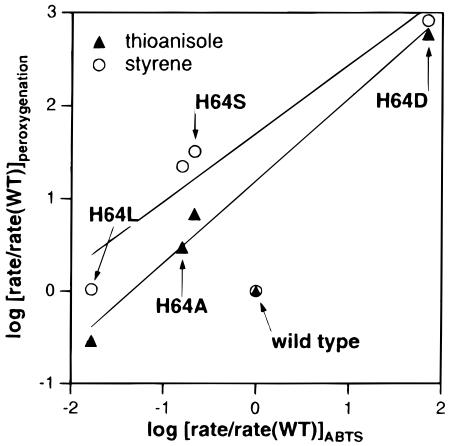

Figure 7. Relationships between ABTS oxidation activity and peroxygenation activity of wild-type Mb and the His64 mutants. All the values were normalized by comparison with corresponding rates of the wild type.

fold rate increase in the peroxidations but 600−800-fold increase in the peroxygenations. Figure 7 depicts plots of the peroxygenation activity against ABTS oxidation activity compared with that of the wild type. The His64 mutants exhibited roughly linear correlation of the peroxidation to the peroxygenation activity (slope: 0.89 for thioanisole and 0.74 for styrene). As noted in the previous section, the ABTS oxidation activity is a good measure of the activation rate of H_2O_2 by Mb. Thus, a major rate-determining step of the peroxygenation by the His64 mutants should be the reaction of ferric Mb with H_2O_2. In contrast, wild-type Mb showed 15- and 50-fold lower activity for the thioanisole and styrene oxidation, respectively, than those expected from the lines (Figure 7). The large deviation suggests a different peroxygenation mechanism of the wild type from those of the mutants.

In the incubation with ^{18}O-labeled H_2O_2, the His64 mutants mainly incorporated ^{18}O into the sulfoxide and epoxide (Tables 3 and 4). High enantioselectivity of the mutants in the styrene epoxidation ruled out the involvement of hydroxy radical (Table 4). Thus, the His64 mutants appear to perform the peroxygenation through a ferryl oxygen transfer mechanism. The reactive species in H64D Mb is unambiguously compound I because compound I of the mutant, which was clearly observed in the reaction with H_2O_2 (Figure 5), was not accumulated under the catalytic condition (data not shown). In contrast, styrene epoxidation by the wild type resulted in only 20% incorporation of the peroxide oxygen although the oxygen source of the sulfoxidation was the peroxide (Tables 3 and 4). A major epoxidation process of wild-type Mb has been shown to be the incorporation of an oxygen atom of molecular oxygen mediated by a protein radical (co-oxidation mechanism),[12] which is consistent with the instability of compound I in the wild-type protein. It is likely that compound I in the wild type oxidizes amino acids rather than the exogenous substrates.

To examine the peroxygenation efficiency, the styrene oxidation was performed at pH 5.3 by employing *m*CPBA as an oxidant. Under the conditions used, *m*CPBA was consumed within 2 min by the wild type and within less than a few seconds by the mutants. The peroxygenation efficiency could be easily determined from total yields of the products after the exhaustion of *m*CPBA. It should be noted that a similar product distribution was obtained for *m*CPBA- and H_2O_2-supported peroxygenation (data not shown), suggesting the same mechanism for the two peroxides. The product yields for the His64 mutants were 100% within experimental errors (Table 4). On the contrary, the yield for the wild type was only 7% (Table 4), indicating that most of the reactive intermediate formed is not utilized for the oxidation of styrene. Thus, the low peroxygenation activity of wild-type Mb is due to the low efficiency of compound I (or its equivalents) to oxidize external substrates.

Table 5. Association Rate Constants of Cyanide and Product Analysis of the Reaction with Cumene Hydroperoxide[a]

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

[a] Determined in 50 mM sodium phosphate buffer (pH 7.0) at 20 °C.

General Acid−Base Functions of the Residues at Position 64. Finally, we have examined roles of Asp64 in H64D Mb in the activation of H_2O_2. The ferric H64D mutant was found to react with H_2O_2 at least 50-fold faster than the wild type and the other mutants. As proposed for distal histidine in classical peroxidases and distal glutamate in chloroperoxidase,[27,31] Asp64 may facilitate the reaction as a general acid−base catalyst. To evaluate the capability of Asp64 as a general base, association rates of cyanide (k_{CN}) to the ferric heme were measured (Table 5). At neutral pH, cyanide is dominantly protonated (HCN, pK_a ∼ 9) and the crucial step for cyanide association has been shown to be the deprotonation of HCN in the distal heme pocket as suggested for the binding of H_2O_2 (pK_a = 11.6).[4,5,31] The His64 → Asp replacement, however, retarded the cyanide association by 10-fold, indicating less basicity of Asp64 than His64.

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

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

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

The ability of Asp64 to function as a general acid was estimated from product analysis in the reaction of ferric Mb with cumene hydroperoxide (Table 5).[24] Heterolytic and homolytic cleavage of the O−O bond of the peroxide affords cumyl alcohol and acetophenone, respectively (eqs 1−3).[32] The acid catalyst is expected to selectively enhance the heterolysis and raise the ratio of heterolysis over homolysis (cumyl alcohol/acetophenone). The H64D mutant showed a slightly higher ratio than the wild type (Table 5); however, the improvement does not appear to be large enough for rationalizing the increased reactivity of H64D Mb with H_2O_2. The H64S mutant exhibited the highest ratio among the myoglobins but was less reactive with H_2O_2 than wild type and H64D Mb. Thus, we conclude that the residues at position 64 including Asp64 are not an effective acid catalyst in the reaction with cumene hydroperoxide.

Discussion

Reaction with Hydrogen Peroxide. The His64 → Asp substitution enables us to observe compound I of Mb in the

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reaction with H_2O_2 for the first time (Figure 5). The successful observation is due to at least 50-fold faster formation of compound I as well as stabilization of compound I. The other His64 mutants (H64A, H64S, and H64L) are less reactive with H_2O_2 than the wild type. It also has been reported that His64 → Gly, Thr, Val, Phe, and Tyr substitutions in Mb depress the reaction with H_2O_2.[33,34] Therefore, the carboxylate group of the distal Asp is crucial for the facile formation of compound I in H64D Mb. This result strongly supports the importance of a distal glutamic acid in CPO. Although the distal glutamate in CPO has been suggested to be a critical residue for the rapid formation of compound I,[27] its catalytic importance has not been examined yet using CPO mutants. The enhancement by a distal carboxylate group was also pointed out from a mutagenesis study on horseradish peroxidase (HRP).[35]

Of particular interest is the role of the distal carboxylate group in helping the reaction with H_2O_2. We examined the capability of Asp64 in H64D Mb as a general acid−base catalyst in the reactions with cyanide and cumene hydroperoxide (Table 5) and concluded that Asp64 was not an efficient general acid−base catalyst in these reactions. Although the role of Asp64 in the mutant remains unclear at this point, we might have to consider functions other than general acid−base. The reactivity with H_2O_2 appears to be affected by polarity of the residues at position 64 rather than their steric bulkiness (Table 2). It might be possible that the highly polar distal heme pocket of the H64D mutant raises the affinity of H_2O_2 to the pocket and/or Asp64 fixes H_2O_2 through a hydrogen bonding to have a preferable position to bind to the heme iron.

Catalytic Activity and Mechanism. Wild-type and native myoglobins have been shown to catalyze H_2O_2-dependent epoxidation of styrene mainly through a co-oxidation mechanism, in which the polypeptide radical at a protein surface mediates incorporation of one oxygen atom of molecular oxygen.[11] This is in contrast to the ferryl oxygen transfer mechanism suggested for oxygenation reactions by cytochrome P-450. Ortiz de Montellano and colleagues reported that a His64 → Val Mb mutant dominantly incorporated an oxygen atom of H_2O_2 in forming an epoxide.[12] Although the result implies a ferryl oxygen transfer mechanism for the epoxidation catalyzed by H64V Mb, the reactive species was not identified.[12] We have shown that the replacement of His64 by unoxidizable amino acids stabilizes compound I of Mb, and that the Mb compound I generated with *m*CPBA oxidizes styrene and thioanisole to the corresponding oxygenated products.[23,28] The H64D Mb mutant in this study provides the first direct evidence for the reactive intermediate being compound I in the reaction with H_2O_2. During the catalytic oxidation of styrene and thioanisole, the H64D mutant did not give detectable amounts of compound I (data not shown), which is easily observable in the absence of the substrates (Figure 5).

The plots of peroxygenation activity against peroxidation activity (Figure 7) suggest that the factors controlling peroxygenation activity of Mb are the formation rate and stability of compound I. In fact, under the assay conditions, the reduction rates of compound I by styrene and thioanisole calculated from the corresponding bimolecular rate constants (Table 1) are much higher than the formation rates of compound I even in the H64D mutant. Furthermore, while the efficiency of the *m*CPBA-supported styrene oxidation was only 7% in the wild type due to its very short lifetime of compound I, the His64 mutants completely transferred the oxidizing equivalents to styrene (Table 4). The His64 → Asp replacement improves the two critical factors to promote the catalytic activity for both peroxidation and peroxygenation reactions (Tables 2−4).

The present study provides a guide to construct further active enzymes based on Mb. Despite the high reactivity of the H64D mutant with H_2O_2, the rate-determining step in its catalytic cycle is the formation process of compound I. Thus, additional mutations on H64D Mb to help compound I formation will produce more efficient oxidation enzymes. Active site residues, which can directly perturb the reaction between heme and H_2O_2, are strong candidates to be replaced. Recently, Wan et al.[36] showed on the basis of random mutagenesis study on horse heart Mb that residues at the outer sphere of the active site can also influence the reactivity of Mb with H_2O_2. Thus, as proposed in their paper, the combined application of the rational design of the active site and random mutagenesis technique may produce a truly effective oxidation enzyme.

In summary, we have prepared the H64D mutant of sperm whale myoglobin to mimic the active site of chloroperoxidase. The His64 → Asp replacement is found to significantly improve the reactivity of ferric Mb with H_2O_2 as well as the stability of compound I. The two advances in H64D Mb allowed us to observe compound I for the first time in the reaction of Mb with H_2O_2, and to identify compound I as the reactive intermediate in the H_2O_2-dependent oxidations. The H64D mutant showed 50−70-fold higher peroxidation activity and 600−800-fold higher peroxygenation activity than the wild-type protein. These changes in the oxidation activity are rationalized by the enhanced stability and formation rate of compound I.

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