Communication

On the Formation and Reactivity of Compound I of the His-64 Myoglobin Mutants*

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Myoglobin (Mb) catalyzes various two-electron oxidations; however, ferryl porphyrin cation radical equivalent to peroxide compound I has not been identified yet. Distal histidine mutants of sperm whale Mb (His-64 → Ala, Ser, and Leu) afford an apparent intermediate followed by the formation of a ferryl heme (Mb-II) in the reaction with m-chloroperbenzoic acid. Because the intermediate exhibits characteristic absorption spectrum of compound I and bears two electron oxidizing equivalents above the ferric state, we have assigned the species as compound I of myoglobin (Mb-I). Although we have recently observed compound I of the F43H/H64L Mb mutant, F43H and wild type Mb react with m-chloroperbenzoic acid to give Mb-II without any accumulation of Mb-I. The results unambiguously indicate that His-64 plays a key role in destabilizing wild type Mb-I. Furthermore, Mb-I is found to be capable of performing two-electron oxidation of styrene, thioanisole, and H₂O₂.

The key intermediate in the catalytic cycles of heme-containing peroxidase and catalase are ferryl porphyrin cation radicals (FeIV = O Por •⁺) called compound I formed by the reaction of the resting ferric enzymes with peroxide (1, 2). By the two sequential one-electron transfers from substrates, compound I is reduced back to the ferric state via a ferryl heme (FeIV = O Por) known as compound II. The two-electron oxidation that is often associated with the ferryl oxygen transfer to substrates also takes place by compound I. The reactive species responsible for the oxygenation by cytochrome P-450, heme-containing monoxygenase is assumed to be compound I (3, 4).

Myoglobin (Mb),1 a carrier of molecular oxygen, can catalyze H₂O₂-dependent two-electron oxidations including styrene epoxidation (5–8); however, the reactions of Mb with peroxides are known to yield a ferryl heme (Mb-II) coupled to a transient oxidation (5–8); however, the reactions of Mb with peroxides have recently observed compound I of the F43H/H64L mutant was not detected in the presence of H₂O₂ due to the enhanced catalase activity of the mutant, whereas the reaction of F43H/H64L Mb with m-chloroperbenzoic acid (mCPBA) afforded compound I (Mb-I). We attributed the stabilization of Mb-I for the F43H/H64L double mutant to the replacement of His-64 with an unoxidizable amino acid like Leu but not to Phe-43 → His mutation (12). To examine the hypothesis, we have examined the reactions of mCPBA with H64A, H64S, H64L, and F43H mutants of sperm whale Mb. Because these single mutants except for F43H Mb afford Mb-I, His-64 is unambiguously identified as a critical residue for destabilizing wild type Mb-I. Furthermore, the reaction rates of H64A and H64S Mb-I with styrene, thioanisole, and H₂O₂ have been determined by single turnover kinetics.

EXPERIMENTAL PROCEDURES

Materials—mCPBA and thioanisole were obtained from Nakalai Tesque. Styrene and H₂O₂ (30%) were purchased from Wako. H64A, H64S, and H64L Mb mutants were constructed by cassette mutagenesis. The cassette including the desired His-64 substitution and a new silent His mutation (12). To examine the expression and purification of the mutants were performed according to the method described by Springer et al. (13).

Reaction of Myoglobin Mutants with m-Chloroperbenzoic Acid—The reactions between ferric Mb mutants and mCPBA were performed at 5.0 °C in 50 mM sodium acetate buffer (pH 5.3) or 50 mM sodium phosphate buffer (pH 7.0). Whole spectral changes during the reactions were recorded on a Hi-Tech SF-43 stopped flow apparatus equipped with a MG 6000 diode array spectrophotometer (5 μM of Mb, 100 μM of mCPBA with or without 4 mM of styrene). The expression and decay rates of Mb-I were determined at pH 7.0 using single wavelength mode (13).

As expected from the reaction scheme, observed formation rates of Mb-I were proportional to mCPBA concentration (Equation 3), whereas its decay rates to Mb-II were essentially constant.

H₂¹⁸O₂ resulted in only 20% ¹⁸O incorporation into the epoxide, and oxygen in the epoxide was derived primarily (80%) from molecular oxygen (5, 6). The incorporation of the molecular oxygen has been attributed to the co-oxidation by the protein-peroxo radical, which is formed by the reaction of molecular oxygen and the protein radical (Scheme I). Thus, Mb-I appears to be a branch to co-oxidation versus ferryl oxygen transfer mechanism.

The Mb mutants in which distal histidine (His-64, E7) is replaced by Leu or Val (Fig. 1) showed drastic increase in the ratio of ferryl oxygen transfer even though the Mb-I as well as Mb-II of those mutants has not been observed when H₂O₂ is used as an oxidant (6, 10, 11). We previously designed a F43H/H64L mutant to mimic the active site of peroxidases because the distal histidine of peroxidases functions as a general acid-base catalyst in the formation of compound I (12). Compound I of the F43H/H64L mutant was not detected in the presence of H₂O₂ due to the enhanced catalase activity of the mutant, whereas the reaction of F43H/H64L Mb with m-chloroperbenzoic acid (mCPBA) afforded compound I (Mb-I). We attributed the stabilization of Mb-I for the F43H/H64L double mutant to the replacement of His-64 with an unoxidizable amino acid like Leu but not to Phe-43 → His mutation (12). To examine the hypothesis, we have examined the reactions of mCPBA with H64A, H64S, H64L, and F43H mutants of sperm whale Mb. Because these single mutants except for F43H Mb afford Mb-I, His-64 is unambiguously identified as a critical residue for destabilizing wild type Mb-I. Furthermore, the reaction rates of H64A and H64S Mb-I with styrene, thioanisole, and H₂O₂ have been determined by single turnover kinetics.

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‡ The abbreviations used are: Mb, myoglobin; Mb-I, a ferryl porphyrin cation radical of myoglobin; Mb-II, a ferryl heme of myoglobin; mCPBA, m-chloroperbenzoic acid.

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Myoglobin Compound I

RESULTS AND DISCUSSION

Formation of Myoglobin Compound I—The reaction of ferric Mb with mCPBA was performed at pH 5.3 on a stopped flow apparatus, and Fig. 2A shows absorption spectra of H64A Mb during the reaction. Within 80 ms after the mixing, Soret absorption of H64A decreased to less than half and a broad visible band having a peak at 648 nm appeared. Then, the novel intermediate was slowly converted to a species with Soret absorption of 419 nm, which is readily assigned to Mb-II (FeIV = O Por). The formation of the first intermediate proceeded with distinct isosbestic points at 430, 462, and 540 nm (Por). The formation of the first intermediate proceeded with mechanisms.

The reaction rates were obtained by fitting the increase in absorbance at 406 nm to a single exponential function.

Means of a double-mixing, rapid scan technique at 5.0 °C in 50 mM sodium acetate buffer, pH 5.3. Ferric H64A or H64S mutant was mixed with 1.3 mol eq of mCPBA into an age loop to prepare compound I (aging time, 0.80 and 2.0 s, respectively), and then the aged solution was mixed with a substrate solution to monitor whole spectral changes. The final reaction mixture contained 2.2 × 10−3 M of Mb and varied concentrations of styrene (0.10–1.0 mM), thioanisole (25–100 mM), or H2O2 (0.25–2.0 mM). The reaction rates were obtained by fitting the increase in absorbance at 406 nm to a single exponential function.

**RESULTS AND DISCUSSION**

**Formation of Myoglobin Compound I**—The reaction of ferric Mb with mCPBA was performed at pH 5.3 on a stopped flow apparatus, and Fig. 2A shows absorption spectra of H64A Mb during the reaction. Within 80 ms after the mixing, Soret absorption of H64A decreased to less than half and a broad visible band having a peak at 648 nm appeared. Then, the novel intermediate was slowly converted to a species with Soret absorption of 419 nm, which is readily assigned to Mb-II (FeIV = O Por). The formation of the first intermediate proceeded with distinct isosbestic points at 430, 462, and 540 nm (kobs = 99 s⁻¹ at [mCPBA] = 100 μM), and its spectrum is characteristic of ferryl porphyrin cation radical (FeIV = O⁺), equivalent to peroxidase compound I (1). On the contrary, subsequent Mb-II formation seems to be accompanied by partial degradation of heme chromophore because the latter reaction did not give isosbestic points, and the Soret absorption of Mb-II was less intense than that of wild type Mb-II.

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

Yields of Mb-I for the H64A mutant with varied amounts of mCPBA at pH 5.3 were shown in Fig. 3. An equimolar amount of mCPBA could convert 85% of H64A Mb to Mb-I at [Mb] = 50 μM. When the concentration of Mb was 10 μM, approximately 2 and 7 mol eq of mCPBA were required to accumulate 80 and 100% of Mb-I, respectively. Because simulations based on equations 1 and 2 roughly reproduced the curves (Fig. 3), the excess peracetic acid is necessary for the complete accumulation of Mb-I under the condition probably due to the decay of Mb-I to Mb-II.

H64S and H64L mutants exhibited similar spectral changes upon mixing with mCPBA and sequentially afforded Mb-I and Mb-II in the absence of substrates (Equations 1 and 2). Although we have reported the first observation of Mb-I in F43H/H64L double mutant (12), F43H Mb as well as the wild type was directly oxidized to Mb-II without any observable intermediate. On the basis of these findings, His-64 so called distal histidine in Mb is unambiguously identified as a critical residue for destabilizing Mb-I. It has been proposed that transiently formed wild type Mb-I is immediately reduced to Mb-II by neighboring amino acid residues, and the resulted protein radical has been shown to be centered at least on Tyr-103, Trp-14, Lys-42, and His-64 (14–16). Our present result strongly suggests that the diffusion of one oxidizing equivalent from wild type Mb-I is mainly initiated by the oxidation of His-64, which lies close vicinity of the heme (4.3 Å above heme.
iron, Fig. 1). Therefore, the substitutions of His-64 with unoxidizable amino acids drastically prolong the life-time of Mb-I, even though there may be other leak pathways of the oxidizing equivalent. On the other hand, the histidine found in the active site of peroxidases is located 5.6–5.9 Å above heme iron and free from the oxidation by compound I.

**Formation and Decay Rates of Myoglobin Compound I**—At pH 5.3, the H64A and H64S mutants were almost completely oxidized to Mb-I at similar rates (99 and 104 s⁻¹ at [mCPBA] = 100 μM, respectively). Only the H64L mutant did not show 100% accumulation of Mb-I due to at least 10-fold lower activity with mCPBA. Even though the stability of Mb-I appears to be essentially the same among the His-64 mutants, the concomitant heme degradation and instability of Mb-II disabled us to measure decay rates of Mb-I to Mb-II at pH 5.3 (17). Thus, we have examined optimal pH levels for the reactions.

As pH increases, the reaction rates of the ferric H64A with mCPBA showed roughly linear decreases (Fig. 4), which implies the presence of plural ionizable groups affecting the reactivities with mCPBA. At pH 9 or higher, the H64A was directly oxidized to Mb-II without the accumulation of Mb-I (Fig. 4) because the decay rates of Mb-I to Mb-II (k_{dec}) appeared to be greater at higher pH. The k_{dec} values were measurable between pH 7 and 8 (1.2 and 2.7 s⁻¹, respectively) where the heme degradation was negligible as judged from isosbestic conversion of Mb-I to Mb-II (Fig. 5). Thus, the formation rates of Mb-I (k₁) and its decay rates to Mb-II (k_{dec}) in the Mb mutants were determined at pH 7.0 (Table I).

The H64A and H64S mutants exhibited similar k₁ values, which are about 50-fold lower than that of horseradish peroxidase. In comparison with the H64A and H64S, 30-fold decrease in the k₁ was observed for H64L. Although reasons for the poor reactivity of the H64L are unclear, it is not due to the steric bulkiness of Leu because the reaction rate with peracetic acid was also 45-fold lower in the H64L than the H64A (7 × 10⁷ and 3.2 × 10⁷ M⁻¹ s⁻¹, respectively). The decay rates of Mb-I to Mb-II (k_{dec}) are essentially identical (0.9–1.2 s⁻¹ at pH 7.0) for the three His-64 mutants (Table I). As compared with these single mutants, F43H/H64L exhibited about 10-fold higher k_{dec} value even at pH 5.3 (9.2 s⁻¹), and the Mb-I formation in the double mutant was not apparent at pH 7.0 (12). Therefore, His-43 in F43H/H64L might be also oxidized by Mb-I (Fig. 1) possibly due to the close location to the β-meso heme edge, whereas the distance of His-43 from the iron is expected to be at least 1 Å longer than that of His-64 in wild type Mb (Fig. 1) and similar to that of distal histidine in peroxidase.

**Reaction of Compound I with Styrene, Thioanisole, and H₂O₂**—Two-electron oxidation ability of Mb-I was examined by means of a double mixing rapid scan technique at pH 5.3. Although Mb-I was prepared by a slight excess amount of mCPBA for avoiding its catalytic regeneration, H64L Mb-I was not apparent due to its poor reactivity with mCPBA. Thus, reactions of Mb-I with styrene, thioanisole, and H₂O₂ were examined for H64A and H64S mutants (approximately 80% yield of Mb-I). Upon mixing with the substrates, both H64A and H64S Mb-I were directly reduced to the ferric states with complete recovery of the Soret intensity. These spectral changes clearly reveal that Mb-I is capable of performing two-electron oxidation of styrene, thioanisole, and H₂O₂. The time course of Mb-I reduction using the absorbance increase at 406 nm obeyed pseudo-first-order kinetics. The reduction rates

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**Table I**

<table>
<thead>
<tr>
<th>Compound</th>
<th>k₁</th>
<th>k_{dec}</th>
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</thead>
<tbody>
<tr>
<td>H64A</td>
<td>4.2 × 10⁷</td>
<td>1.2</td>
</tr>
<tr>
<td>H64S</td>
<td>3.0 × 10⁷</td>
<td>1.2</td>
</tr>
<tr>
<td>H64L</td>
<td>1.1 × 10⁷</td>
<td>0.9</td>
</tr>
<tr>
<td>Horseradish peroxidase</td>
<td>1.9 × 10⁷</td>
<td></td>
</tr>
</tbody>
</table>

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were proportional to the substrate concentrations, and the second-order rate constants are listed in Table II. There was no significant difference in the reactivities of the heme enzymes of Mb-I.

The H$_2$O$_2$ oxidation by Mb-I is about 3000-fold slower than that reported for catalase compound I (18). Nevertheless, the complete reduction of Mb-I by H$_2$O$_2$ indicates that the resulting ferric Mb does not react with H$_2$O$_2$ in the time scale of the reduction, i.e., Mb-I appears to be reduced by H$_2$O$_2$ much faster than its formation by H$_2$O$_2$. Due to the catalase-like reaction, the direct observation of Mb-I in these mutants was failed when H$_2$O$_2$ was employed as the oxidant.

Styrene oxidation by Mb-I was about 4-fold faster than the H$_2$O$_2$ oxidation (Table II). Thioanisole was oxidized to methyl phenyl sulfoxide at further 60-fold higher rates (Table II), which are comparable with guaiacol and iodide oxidation rates (21). We have examined the reactions of horseradish compound I of hemoproteins even though chloroperoxidase has received relatively little study because of its difficulty of isolation. Few reports on the oxygenation rates of styrene and thioanisole in Mb-I are available (22). Styrene oxidation by Mb-I is about 4-fold faster than the H$_2$O$_2$ oxidation (Table II). Thioanisole was oxidized to methyl phenyl sulfoxide at further 60-fold higher rates (Table II), which are comparable with guaiacol and iodide oxidation rates (21). We have examined the reactions of horseradish compound I of hemoproteins even though chloroperoxidase has received relatively little study because of its difficulty of isolation. Few reports on the oxygenation rates of styrene and thioanisole in Mb-I are available (22).}

### Table II

<table>
<thead>
<tr>
<th>Myoglobin Compound I</th>
<th>Myoglobin</th>
<th>Styrene</th>
<th>Thioanisole</th>
<th>H$_2$O$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myoglobin Styrene Thioanisole H$_2$O$_2$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mb-I</td>
<td>H64A</td>
<td>2.2 × 10$^4$</td>
<td>1.5 × 10$^6$</td>
<td>5.9 × 10$^3$</td>
</tr>
<tr>
<td>Mb-I</td>
<td>H64S</td>
<td>2.6 × 10$^4$</td>
<td>1.5 × 10$^6$</td>
<td>6.2 × 10$^3$</td>
</tr>
</tbody>
</table>

In summary, the replacement of His-64 by unoxidizable amino acids enables us to observe compound I of Mb (Mb-I) in the reaction with mCPBA, and Mb-I is capable of performing two-electron oxidation of styrene, thioanisole, and H$_2$O$_2$. Our results clearly indicate that His-64 is a critical residue for destabilizing Mb-I, and the location of distal histidine is suggested to be important to control the stability of compound I in heme enzymes.

**Acknowledgment**—We thank Dr. John S. Olson (Rice University) for providing cDNA of sperm whale myoglobin.

**REFERENCES**

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