Catalase Reaction by Myoglobin Mutants and native Catalase: Mechanistic Investigation by Kinetic Isotope Effect

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Running title: KIE on Catalase Reactions
Abstract

The catalase reaction has been studied in detail by using myoglobin (Mb) mutants. Compound I of Mb mutants (Mb-I), a ferryl species (Fe$^{IV}$=O) paired with a porphyrin radical cation, is readily prepared by the reaction with a nearly stoichiometric amount of $m$-chloroperbenzoic acid ($m$CPBA). Upon the addition of H$_2$O$_2$ to an Mb-I solution, Mb-I is reduced back to the ferric state without forming any intermediates. This indicates that Mb-I is capable of performing two-electron oxidation of H$_2$O$_2$ (catalatic reaction). GC-MS analysis of the evolved O$_2$ from a 50:50 mixture of H$_2^{18}$O$_2$/H$_2^{16}$O$_2$ solution containing H64D or F43H/H64L Mb showed the formation of $^{18}$O$_2$ ($m/e = 36$) and $^{16}$O$_2$ ($m/e = 32$) but not $^{16}$O$^{18}$O ($m/e = 34$). This implies that O$_2$ is formed by two electron oxidation of H$_2$O$_2$ without breaking the O–O bond. Deuterium isotope effects on the catalatic reactions of Mb mutants and catalase suggest that the catalatic reactions of Micrococcus lysodeikticus Catalase (MLC) and F43H/H64L Mb proceed via an ionic mechanism with a small isotope effect of less than 4.0, since the distal histidine residue is located at a proper position to act as a general acid-base catalyst for the ionic reaction. In contrast, other Mb mutants such as H64X (X: A, S, and D) and L29H/H64L Mb oxidize H$_2$O$_2$ via a radical mechanism in which a hydrogen atom is abstracted by Mb-I with a large isotope effect in a range of 10 to 29, due to a lack of the general acid-base catalyst.
Introduction

Catalase is a heme enzyme, which catalyzes the disproportionation of hydrogen peroxide to H\textsubscript{2}O and O\textsubscript{2}. In the first step, H\textsubscript{2}O\textsubscript{2} serves as a two-electron oxidant to generate a ferryl porphyrin cation radical (O=Fe\textsuperscript{IV} Por\textsuperscript{•+}) called compound I and H\textsubscript{2}O (Eq. 1). In the second step, compound I serves as a two-electron oxidant of H\textsubscript{2}O\textsubscript{2} affording O\textsubscript{2} (catalatic reaction) accompanied by regeneration of the ferric form of catalase (Eq. 2) (1,2). Although the catalase reaction has been known since 1940s and it is suggested that the distal histidine is important for the deprotonation of hydrogen peroxide (1,2), the detailed mechanism for the oxidation of it by compound I has yet to be clarified, since difficulty in the preparation of catalase compound I by a stoichiometric amount of H\textsubscript{2}O\textsubscript{2} or alkyl peroxides has precluded direct observation of the reaction step of compound I with H\textsubscript{2}O\textsubscript{2} (1,3,4). It also remains uncertain why chloroperoxidase (5) and KatG (6) show catalatic activity among the heme peroxidases.

\[
\text{Catalase (Fe}^{\text{III}}\text{) + H}_2\text{O}_2 \xrightarrow{k_1} \text{Compound I (O=Fe}^{\text{IV}}\text{Por}^{\text{•+}}\text{) + H}_2\text{O} \quad \text{(Eq. 1)}
\]

\[
\text{Compound I + H}_2\text{O}_2 \xrightarrow{k_2} \text{Catalase (Fe}^{\text{III}}\text{) + H}_2\text{O} + \text{O}_2 \quad \text{(Eq. 2)}
\]

Myoglobin (Mb), a carrier of molecular oxygen, has been studied as a structural and/or functional model for elucidating the role of active site residues in heme enzymes (7-14). Recently, we have proven that the distal histidine (His64) in sperm whale Mb is a critical residue in destabilizing Mb compound I (Mb-I). In fact, His-64 deletion mutants of Mb gave Mb-I as an observable species under stopped-flow conditions. More importantly, the successful observation of Mb-I has allowed us the direct observation of the oxidation step of H\textsubscript{2}O\textsubscript{2}, olefins, thioethers, N-demethylation, and an aromatic ring by Mb-I (10,11,15,16). For example, H64X (X = A, S, and D) mutants are almost completely oxidized to Mb-I by \textit{m}-chloroperbenzoic acid (\textit{m}CPBA) and Mb-
I is completely reduced by \( \text{H}_2\text{O}_2 \), though the reaction is about 1,000–3,000 fold slower than that reported for catalase (11). The Mb mutants (F43H/H64L and L29H/H64L Mb) have also been found to dismutate \( \text{H}_2\text{O}_2 \) to \( \text{O}_2 \) and \( \text{H}_2\text{O} \) at the rate which is 50- and 5-fold faster than wild-type Mb, respectively (10). The crystal structures of them suggest that the reactivity is controlled by the presence or absence of distal histidine. (10)

We report herein the kinetic isotope effect (KIE) on catalatic reactions (Eq. 2) of catalase and Mb mutants in \( \text{H}_2\text{O} \) and \( \text{D}_2\text{O} \). The detailed kinetic studies provide valuable insight into the role of distal histidine for the \( \text{H}_2\text{O}_2 \) oxidation by compound I of catalase and Mb mutants.
Experimental procedures

Materials—Standard chemicals were obtained from Wako Chemicals and Nacalai Tesque. 99.9% Deuterium oxide was purchased from Aldrich and Cambridge Isotope Laboratories. mCPBA was obtained from Nacalai Tesque and purified as reported previously (17). H$_2^{18}$O$_2$ was prepared from $^{18}$O$_2$ as described by Foote et al. (18). $^{18}$O content in H$_2^{18}$O$_2$ was determined by GC-MS analysis of triphenylphosphine oxide formed by oxidation of triphenylphosphine with H$_2^{18}$O$_2$. Concentration of peroxide in a reaction solution was determined by oxidation of potassium iodide in the presence of horseradish peroxidase as a catalyst to produce I$_3^-$ ($\epsilon_{553} = 2.62 \times 10^4$ M$^{-1}$ cm$^{-1}$) (19,20).

Beef Liver Catalase (BLC) and Micrococcus lysodeikticus Catalase (MLC) were obtained from Sigma and Nagase ChemteX, respectively. Catalase was purified by Superdex 75 size exclusion chromatography. Concentration of BLC and MLC was determined by using a molar extinction coefficient of $\epsilon_{405} = 3.24 \times 10^5$ M$^{-1}$ cm$^{-1}$ (21) and $\epsilon_{406} = 1.03 \times 10^5$ M$^{-1}$ cm$^{-1}$ (22), respectively. Mutant genes of H64A, H64S, H64D, F43H/H64L and L29H/H64L sperm whale Mb were constructed by a method of Matsui et al. (10,23). Expression and purification of the mutants were performed according to a method described by Springer et al. (24).

Preparation of D$_2$O buffer—In a typical run, 50 mM sodium acetate buffer was prepared by adding an appropriate amount of acetate acid in D$_2$O and titrated with a concentrated sodium hydroxide solution of D$_2$O. The pD of the buffer solution was determined by using an equation of pD = pH$_{obs}$ + 0.3314 $n$ + 0.076 $n^2$, where $n$ represents the fraction of deuterium in the solution (25) and pH$_{obs}$ is apparent pH reading on an HM-30V pH meter (TOA electronics Ltd., Tokyo, Japan). D$_2$O samples were prepared as follows: To an H$_2$O solution (0.5 mL) containing a protein (1 mM) was added D$_2$O buffer (4.5 mL) and the resulting mixture was concentrated by ultrafiltration. The same procedure was repeated 5 times. After the completion of H–D
exchange, the solution was incubated overnight in D$_2$O buffer at 4 °C.

Spectroscopy– Electronic absorption spectra were recorded on a UV-2400 spectrophotometer (Shimadzu Co., Ltd., Kyoto, Japan). Spectral changes were monitored either on a SF-43 stopped-flow apparatus (HI-TECH Scientific Co., Wiltshire, UK) equipped with a MG 6000 diode array spectrophotometer or on a RSP-601 stopped-flow rapid-scan spectrometer (Unisoku Co., Ltd., Osaka, Japan).

Reaction of MLC and H$_2$O$_2$– For the determination of kinetic parameters, $k_1$ and $k_2$, the MLC compound I (MLC-I) formation and the dismutation of H$_2$O$_2$ were monitored by following changes in absorbance at 406 nm and 240 nm, respectively, by stopped-flow technique in 50 mM sodium phosphate buffer (pL 7.0) at 5 °C. In order to determine the apparent rate constant of MLC-I formation ($k_{\text{app}-\text{MLC-I}}$), the MLC (1.0–1.2 M) was mixed with a small excess amount (2.0–8.9 molar equiv. to MLC) of H$_2$O$_2$. The rate constant of the H$_2$O$_2$ dismutation by MLC ($k_{\text{app}-\text{H}_2\text{O}_2}$) was determined under steady-state conditions ([MLC]=50 nM, [H$_2$O$_2$]=1.8–16 mM).

Reactions of Mb mutants and mCPBA– In order to obtain the authentic absorption spectra of Mb compound I (Mb-I), Mb mutants (5.5–9.7 M) were oxidized by more than 10 equiv. of mCPBA. At the same time, the Mb-I formation rates by mCPBA were determined in 50 mM sodium acetate buffer (pL 5.0) at 5 °C by following the decay of absorbance at 408 nm. Bimolecular rate constants are determined by plotting observed pseudo-first-order rates versus oxidant concentration.

Reaction of Mb-I and H$_2$O$_2$– The reaction of Mb-I with H$_2$O$_2$ was directly monitored by use of a double-mixing stopped-flow technique. In order to avoid catalytic catalase reactions (Eq. 1 and 2), the first mixing of a Mb mutant (5.5–7.8 M) to prepare Mb-I
was carried out with 1.5 molar equiv. of \textit{m}CPBA. Subsequently, at least 100-fold excess of H$_2$O$_2$ was introduced to start the catalatic reaction (Eq. 2) in 50 mM sodium acetate buffer (pH 5.0) at 5 °C. The catalatic reaction was monitored by following an increase in absorbance at 408 nm. Delay time for the second mixing was in a range of 0.1–1.1 sec. The reproduction of Mb-I by remaining H$_2$O$_2$ is too slow to observe under the present experimental conditions. In order to examine the temperature dependence of H64A Mb-I reaction with H$_2$O$_2$, the catalatic reaction was repeated at 5, 10, 15, 20 and 25 °C. Activation parameters (A and $E_a$) were determined by an Arrhenius plot: $\ln k_2 = \ln A - \frac{E_a}{RT}$. All experiments were repeated at least twice.

**Identification of evolved oxygen**—An Mb mutant (200 mM, in 50 mM sodium acetate buffer (pH 5.0)) or BLC (25 mM, in 50 mM sodium phosphate buffer (pH 7.0)) was mixed with H$_2^{16}$O$_2$/H$_2^{18}$O$_2$ (1:1) (200 mM) at room temperature under N$_2$ for 5 min. Evolved three different molecular oxygens, $^{16}$O$_2$ ($m/e = 32$), $^{16}$O$^{18}$O ($m/e = 34$) and $^{18}$O$_2$ ($m/e = 36$), were separated and quantified by a Shimadzu GC-17A/GCMS-QP5000 (Shimadzu Co., Ltd., Kyoto, Japan) equipped with a VARIAN Molsieve 5A PLOT capillary column.
Results

Catalase reaction of MLC in H₂O and D₂O– UV-vis spectral changes of MLC by the addition of peroxides in H₂O and D₂O are shown in Fig. 1. The spectral changes with hydrogen peroxide are different from those with methyl hydroperoxide. The steady-state spectra in the presence of hydrogen peroxide show that 45 and 85% of MLC exist as MLC-I in H₂O and D₂O buffers, respectively (Fig. 1A). On the other hand, the spectra of MLC with methyl hydroperoxide indicate that MLC has been completely converted to MLC-I in both buffers (Fig. 1B). Fig. 2 shows the time course of absorbance change in the reactions of MLC and 2–8.9 equiv. molar of hydrogen peroxide. The steady-state concentration of MLC-I in each run is almost the same even though the amount of hydrogen peroxide was varied. Thus, we could determine the rate constants, $k_1$ and $k_2$, without considering the equilibrium steps (Eq. 3 and 5) under these experimental conditions (see Appendix 1 and 2). In order to obtain the apparent rate constants of MLC-I formation ($k_{app-MLC-I}$), the MLC (1.0–1.2 μM) was mixed with a small excess amount (2.0–8.9 molar equiv. to MLC) of H₂O₂. The rate constants of the H₂O₂ dismutation by MLC ($k_{app-H₂O₂}$) were determined under steady-state conditions ([MLC]=50 nM, [H₂O₂]=1.8–16 mM). The apparent second-order rate constants ($k_{app-MLC-I}$) for MLC-I formation by H₂O₂ were determined from the Soret absorbance change at 406 nm as $(2.11 \pm 0.06) \times 10^7$ and $(1.19 \pm 0.02) \times 10^7$ M⁻¹ s⁻¹ in H₂O and D₂O, respectively. In addition, the apparent rate constants ($k_{app-H₂O₂}$) of H₂O₂ dismutation by MLC-I were determined by the decrease of H₂O₂ absorption at 240 nm (Fig. 3) as $(9.33 \pm 0.10) \times 10^6$ and $(2.86 \pm 0.04) \times 10^6$ M⁻¹ s⁻¹ in H₂O and D₂O, respectively. The apparent rate constants $k_{app-MLC-I}$ and $k_{app-H₂O₂}$ are expressed as a function of $k_1$ (Eq. 1) and $k_2$ (Eq. 2): $k_{app-MLC-I} = k_1 + k_2$ (see Appendix 1) and $k_{app-H₂O₂} = 2k_1k_2/(k_1+k_2)$ (see Appendix 2). Thus, the $k_1$ and $k_2$ values are determined from the $k_{app-MLC-I}$ and $k_{app-H₂O₂}$ value and they are listed in Table I and II, respectively. On the other hand, the observed
concentrations of MLC in equilibrium is given by $[\text{MLC}]_{eq} = (k_2/k_1 + k_2)[\text{MLC}]_0$ ([8] in Appendix 1). Using this equation, the concentrations of MLC-I in H$_2$O and D$_2$O are calculated from the $k_1$ and $k_2$ values as 43.1 ± 6.8 % and 74.1 ± 7.7 %, respectively. These values agree with the concentrations of MLC-I estimated from the steady-state spectra in Fig. 1. Such agreement confirms the validity of our kinetic analysis. We have also determined the kinetic isotope effects (KIEs) on $k_1$ and $k_2$ of MLC to be 1.0 and 4.0, respectively.

$$
\begin{align*}
\text{MLC} + \text{H}_2\text{O}_2 & \rightleftharpoons \text{MLC-H}_2\text{O}_2 & (\text{Eq. } 3) \\
\text{MLC-H}_2\text{O}_2 & \rightarrow \text{MLC-I} + \text{H}_2\text{O} & (\text{Eq. } 4) \\
\text{MLC-I} + \text{H}_2\text{O}_2 & \rightleftharpoons \text{MLC-I-H}_2\text{O}_2 & (\text{Eq. } 5) \\
\text{MLC-I-H}_2\text{O}_2 & \rightarrow \text{MLC} + \text{H}_2\text{O} + \text{O}_2 & (\text{Eq. } 6)
\end{align*}
$$
Fig. 1. UV-vis spectra of MLC with H$_2$O$_2$ (A) and methyl hydroperoxide (B) in the steady state. Black lines: MLC in H$_2$O, blue lines: addition of peroxide in H$_2$O and red lines: addition of peroxide in D$_2$O (50 mM sodium phosphate buffer solutions at pH 7.0 and 5 °C).
Fig. 2. Absorption changes in MLC reaction with deuterium peroxide (2–8.9 μM). The reactions were carried out in 50 mM sodium phosphate buffer solutions (pL 7.0) at 5 °C. The final MLC concentration was 1.0 μM. The inset shows a plot of $(\text{[H}_2\text{O}_2\text{]}_0 - \text{[MLC]}_0 + \text{[MLC]}_{\text{eq}})k_{\text{app-MLC-I}}$ versus $\text{[H}_2\text{O}_2\text{]}_0 - \text{[MLC]}_0 + \text{[MLC]}_{\text{eq}}$ for the reaction of MLC with hydrogen peroxide.
Fig. 3. Time-dependent decrease of H$_2$O$_2$ absorption due to the catalase activity of MLC. The catalase reactions were carried out in a 50 mM sodium phosphate buffer solution (pH 7.0) at 5 °C. The final MLC concentration was 50 nM. The inset shows a plot of $k_{\text{app-H}_2\text{O}_2}$ versus hydrogen peroxide concentration for the dismutation reactions of hydrogen peroxide by MLC.
Mb-I formation in H$_2$O and D$_2$O— The second-order rate constants ($k_1$) for the compound I formation of the Mb mutants by mCPBA were determined (vide supra) as listed in Table I (23,26). The H64A mutant affords Mb-I by the addition of mCPBA as shown in Fig. 4A. At 320 msec after the mixing, Soret absorption of H64A decreased to less than half and a broad band having a peak at 648 nm appeared. The rate constants of the Mb-I formation with mCPBA in H$_2$O and D$_2$O, indicate small $k_{1H}/k_{1D}$ values of 0.8–1.6. On the other hand, H64D Mb-I formation with H$_2$O$_2$ shows a larger $k_{1H}/k_{1D}$ value (Table 1). We attempted to observe the other Mb-I formation by H$_2$O$_2$, but the other Mb-Is could not be formed with H$_2$O$_2$ under the same conditions.

Catalatic reaction of Mb mutants in H$_2$O and D$_2$O— In order to observe the stoichiometric reaction of Mb-I with H$_2$O$_2$ spectroscopically, we have performed double-mixing stopped-flow experiments at 5.0 °C and pH 5.0. A small excess amount (1.5 molar equiv. to Mb) of mCPBA was used for the completion of Mb-I formation (23). For example, H64A Mb gave Mb-I up to 95% yield under these conditions (Fig. 4A). Decrease in the absorbance of the Soret band and increase in the absorbance at 648 nm are the clear indication of the Mb-I formation. Other Mb mutants are also oxidized to Mb-I under the same experimental conditions. Upon the addition of H$_2$O$_2$ to a H64A Mb-I solution (2nd mixing), Mb-I is reduced back to the ferric form without forming any intermediates (Fig. 4B). Similar spectral changes are also observed for the other mutants, revealing that the Mb-I is capable of performing two-electron oxidation of H$_2$O$_2$. The reproduction of compound I by the remaining H$_2$O$_2$ in the solution is too slow to observe under the present experimental conditions. The rate constants of the catalatic reaction in H$_2$O and D$_2$O ($k_{2H}$ and $k_{2D}$) were determined as described above and the $k_{2H}$ and $k_{2D}$ values are listed in Table II. Replacement of the H$_2$O solvent by D$_2$O caused 10–29 fold decrease in the rates of the catalatic reactions of H64X and L29H/H64L Mbs. Such large primary kinetic isotope effects suggest the occurrence of
tunneling. Accurate measurements of the primary kinetic isotope effects as a function of temperature are very important for the examination of the tunneling effect (27). The incidence of tunneling results in curvature of the Arrhenius plot \((\ln k = \ln A - E_a/RT)\) in a wide range of temperature, which is larger for hydrogen as compared to deuterium. In the temperature range of measurements (5–25 °C), a larger \(A_D\) value than \(A_H\) derived from an apparent linear Arrhenius plot can be used as criteria to recognize the tunneling \((A_H/A_D < 0.6)\) (Table III) (27). The \(A_H/A_D\) values (0.07) derived from the Arrhenius plots for H64A Mb, thus, indicates that large KIEs are ascribed to the linear tunneling effect (Fig. 5) (27). In contrast to H64A Mb, F43H/H64L Mb and MLC exhibited small KIEs of 2.1 and 4.0, respectively (we could not determine their thermodynamic parameters due to less stability and rapid reaction above 5 °C, respectively).

Identification of evolved oxygen—Preparation of \(^{18}\)O-labeled \(\text{H}_2\text{O}_2\) has allowed us to examine the source of the evolved \(\text{O}_2\). A GC-MS spectrum of the evolved \(\text{O}_2\) from a solution containing a 50:50 mixture of \(\text{H}_2^{18}\text{O}_2/\text{H}_2^{16}\text{O}_2\) and a Mb mutant (H64D or F43H/H64L) shows two peaks for \(^{18}\text{O}_2\) \((m/e = 36)\) and \(^{16}\text{O}_2\) \((m/e = 32)\) with no indication of \(^{16}\text{O}^{18}\text{O}\) \((m/e = 34)\) formation within an experimental error of ca. 3%. This demonstrates that the catalase reactions by the Mb mutants proceed as the cases of catalase and CPO (28,29).
Fig. 4. (A) Absorption spectral changes of H64A Mb upon the addition of 1.5 eq mCPBA at 5.0°C in 50 mM sodium acetate buffer, pH 5.0. Final concentration: 7.1 μM H64A and 10.7 μM mCPBA. Spectra were recorded before the addition of mCPBA (thick line) and after the mixing. (B) Spectral changes upon the addition of H2O2 to Mb-I. Final concentrations: 7.1 μM H64A, 10.7 μM mCPBA and 250 μM H2O2. Spectra were recorded at 0.40 (thick line) and 0.6–2.2 sec after mixing with a 0.20 sec interval. The direction of absorbance changes is indicated by arrows.
### TABLE I

*Rate constants ($k_i$) of Mb and catalase compound I formation*

<table>
<thead>
<tr>
<th>Mb</th>
<th>Oxidants</th>
<th>$k_{1H}^a$ ($\text{M}^{-1}\text{s}^{-1}$)</th>
<th>$k_{1D}^b$ ($\text{M}^{-1}\text{s}^{-1}$)</th>
<th>$k_{1H}/k_{1D}$</th>
</tr>
</thead>
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<tr>
<td>H64A Mb</td>
<td>mCPBA</td>
<td>$9.87 \pm 0.01 \times 10^5$</td>
<td>$6.26 \pm 0.01 \times 10^5$</td>
<td>1.6</td>
</tr>
<tr>
<td>H64S Mb</td>
<td>mCPBA</td>
<td>$6.29 \pm 0.01 \times 10^5$</td>
<td>$4.42 \pm 0.01 \times 10^5$</td>
<td>1.4</td>
</tr>
<tr>
<td>H64D Mb</td>
<td>mCPBA</td>
<td>$5.26 \pm 0.01 \times 10^5$</td>
<td>$3.28 \pm 0.01 \times 10^5$</td>
<td>1.6</td>
</tr>
<tr>
<td>H64D Mb</td>
<td>$H_2O_2$</td>
<td>$12.4 \pm 0.1 \times 10^3$</td>
<td>$0.80 \pm 0.02 \times 10^3$</td>
<td>15</td>
</tr>
<tr>
<td>L29H/H64L</td>
<td>mCPBA</td>
<td>$4.18 \pm 0.03 \times 10^5$</td>
<td>$4.20 \pm 0.05 \times 10^5$</td>
<td>1.0</td>
</tr>
<tr>
<td>F43H/H64L</td>
<td>mCPBA</td>
<td>$6.67 \pm 0.03 \times 10^5$</td>
<td>$8.77 \pm 0.27 \times 10^5$</td>
<td>0.8</td>
</tr>
<tr>
<td>MLC</td>
<td>$H_2O_2$</td>
<td>$9.09 \pm 1.45 \times 10^6$</td>
<td>$8.82 \pm 0.92 \times 10^6$</td>
<td>1.0</td>
</tr>
</tbody>
</table>

*a* In H$_2$O buffer.  *b* In D$_2$O buffer.

Mb : 50mM sodium acetate buffer (pL 5.0) at 5.0 °C

MLC : 50mM sodium phosphate buffer (pL 7.0) at 5.0 °C

### TABLE II

*Rate constants ($k_2$) of the catalatic reaction*

<table>
<thead>
<tr>
<th>Mb</th>
<th>$k_{2H}^a$ ($\text{M}^{-1}\text{s}^{-1}$)</th>
<th>$k_{2D}^b$ ($\text{M}^{-1}\text{s}^{-1}$)</th>
<th>$k_{2H}/k_{2D}$</th>
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<tbody>
<tr>
<td>H64A Mb</td>
<td>$5.26 \pm 0.06 \times 10^3$</td>
<td>$2.32 \pm 0.02 \times 10^2$</td>
<td>23</td>
</tr>
<tr>
<td>H64S Mb</td>
<td>$6.91 \pm 0.01 \times 10^3$</td>
<td>$2.38 \pm 0.01 \times 10^2$</td>
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<tr>
<td>H64D Mb</td>
<td>$15.8 \pm 0.3 \times 10^3$</td>
<td>$8.18 \pm 0.02 \times 10^2$</td>
<td>18</td>
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<tr>
<td>L29H/H64L</td>
<td>$33.6 \pm 0.1 \times 10^3$</td>
<td>$3.39 \pm 0.03 \times 10^3$</td>
<td>10</td>
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<tr>
<td>F43H/H64L</td>
<td>$21.0 \pm 0.5 \times 10^3$</td>
<td>$10.3 \pm 0.1 \times 10^3$</td>
<td>2.1</td>
</tr>
<tr>
<td>MLC</td>
<td>$1.20 \pm 1.98 \times 10^7$</td>
<td>$3.08 \pm 0.32 \times 10^6$</td>
<td>4.0</td>
</tr>
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*a* In H$_2$O buffer.  *b* In D$_2$O buffer.

Mb : 50mM sodium acetate buffer (pL 5.0) at 5.0 °C

MLC : 50mM sodium phosphate buffer (pL 7.0) at 5.0 °C
<table>
<thead>
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<th>parameter</th>
<th>H64A</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_H$ (M$^{-1}$ s$^{-1}$)</td>
<td>5260</td>
</tr>
<tr>
<td>$k_D$ (M$^{-1}$ s$^{-1}$)</td>
<td>232</td>
</tr>
<tr>
<td>KIE</td>
<td>23</td>
</tr>
<tr>
<td>$\Delta E_{\text{a(H)}}$ (kcal mol$^{-1}$)</td>
<td>0.47</td>
</tr>
<tr>
<td>$\Delta E_{\text{a(D)}}$ (kcal mol$^{-1}$)</td>
<td>1.3</td>
</tr>
<tr>
<td>$A_H / A_D$</td>
<td>0.07</td>
</tr>
<tr>
<td>$\Delta H^\circ$ (O-H) (kcal mol$^{-1}$)</td>
<td>0.33</td>
</tr>
<tr>
<td>$\Delta H^\circ$ (O-D) (kcal mol$^{-1}$)</td>
<td>1.2</td>
</tr>
</tbody>
</table>

* $a$ 50 mM sodium acetate buffer (pL 5.0) at 5 °C. $b$ 50 mM sodium acetate buffer (pL 5.0) at 5, 10, 15, 20 and 25 °C.
Fig. 5. Arrhenius plot of the reaction of H64A Mb-I with H$_2$O$_2$. These reactions were carried out in 50 mM sodium acetate buffer (pL 5.0) at 5, 10, 15, 20 and 25 °C. The final concentration was 7.1 μM H64A, 10.7 μM mCPBA and 100-fold excess of H$_2$O$_2$. 
Discussion

In early studies, the rate constants of compound I formation for human erythrocyte, horse liver and *Micrococcus lysodeikiticus* catalases (MLC) were reported to be in a range of $1.2 \sim 6 \times 10^7 \text{M}^{-1}\text{s}^{-1}$ (30-33). On the other hand, the $k_1$ and $k_2$ values of bovine liver catalases (BLC) in the presence of 0.25–1.00 M ethanol are $5.6 \times 10^6 \text{M}^{-1}\text{s}^{-1}$ and $1.2 \times 10^7 \text{M}^{-1}\text{s}^{-1}$, respectively (4). The $k_1$ of BLC, which is smaller than those of the other catalases, indicates possible inhibition by acetaldehyde or denaturation of BLC under these conditions. Thus, we have employed MLC to determine $k_1$ and $k_2$ in the absence of ethanol in order to elucidate a detailed mechanism of the catalatic reaction.

In the case of MLC, high catalatic activity has precluded the complete accumulation of MLC-I by $\text{H}_2\text{O}_2$ and the preparation of MLC-I required a large excess of the oxidant even by using methyl hydroperoxide. The rate constant ($k_2$) of the catalatic reaction of MLC could not be determined directly due to a rapid reformation of MLC-I by $\text{H}_2\text{O}_2$ remaining in the reaction solution. Thus, we have determined $k_1$ and $k_2$ by the measurement of the apparent MLC-I formation and its reduction rates by hydrogen peroxide as $9.1 \times 10^6$ and $1.2 \times 10^7 \text{M}^{-1}\text{s}^{-1}$, respectively (*vide supra*).

In the case of MLC, a small KIE (1.0) for the formation of MLC-I has been observed. This value is very similar to that reported for HRP-I formation (1.6) by Dunford *et al.* (34). Therefore, distal histidine in MLC could serve as a general acid-base catalyst for the formation of MLC-I as proposed for peroxidases. On the other hand, the reaction of H64D Mb and $\text{H}_2\text{O}_2$ shows a large KIE ($k_w/k_D = 15$). Since the compound I formation mechanism includes deprotonation from $\text{H}_2\text{O}_2$ to afford a Fe-OOH intermediate, we have assigned that the deprotonation of hydrogen peroxide is a great barrier for the formation of H64D Mb-I due to a lack of the distal histidine. These results suggest that the distal histidine might reduce the $\text{pK}_a$ value of $\text{H}_2\text{O}_2$ (11.6) by ca. 4 $\text{pK}_a$ unit in the active site of MLC.

While the reaction of MLC-I with hydrogen peroxide gave a mixture of MLC and
MLC-I in the steady state because the resulting MLC is immediately converted to MLC-I by excess of H$_2$O$_2$ remaining in the solution, we have successfully determined the deuterium isotope effect on the catalatic reaction of MLC to be 4.0 on the basis of apparent MLC-I formation and MLC-I reduction rates. (Table II). F43H/H64L Mb also gave a small KIE (2.1) determined by the direct observation of the reaction of Mb-I and H$_2$O$_2$. These results indicate that hydrogen peroxide is easily deprotonated by the distal histidine and then reacts with compound I of MLC and F43H/H64L Mb. On the other hand, in the reactions of H$_2$O$_2$ and Mb-I of L29H/H64L and H64X (X = D, A, and S), the KIEs are extremely larger (10–29) than those of F43H/H64L Mb and MLC (Table II). Large KIEs are due to the tunneling effect, which is confirmed by the Arrhenius parameter ratio (Table III) (27).

In previous studies, we have proven that the distal histidine (His43) in F43H/H64L Mb serves as a general acid-base catalyst to form compound I in the reaction with H$_2$O$_2$. On the other hand, distal aspartic acid (Asp64) in H64D and the distal histidine (His29) in L29H/H64L Mb hardly participate as the catalyst (10,11). The crystal structure of F43H/H64L Mb shows the distance between the N$^\text{e}$ of His43 and the ferric heme iron to be 5.7 Å, being similar to structurally known peroxidases and MLC (Fig. 6A, C and D) (2,10,35). On the other hand, the distance in L29H/H64L Mb is too far from the heme iron (6.6 Å) to serve as the general acid-base catalyst (Fig. 6B) (10). Thus, the catalatic reaction of MLC and F43H/H64L Mb, in which the general acid-base catalyst is located at a proper position, could proceed via the ionic mechanism with a small KIE (<4) (Scheme 1A), while the other Mb mutants oxidize H$_2$O$_2$ via a mechanism which could be different from the ionic mechanism with exhibiting a large KIE (10–29) due to a lack of the general acid-base catalyst.

Exclusive formation of $^{18}$O$_2$ and $^{16}$O$_2$ from a 50:50 mixture of H$_2^{16}$O$_2$ and H$_2^{18}$O$_2$ indicates that O$_2$ is formed by two electron oxidation of H$_2$O$_2$ without breaking the O–O bond. There are two possible mechanisms on the formation of O$_2$ in the reaction of
H₂O₂ and compound I without showing ¹⁸O/¹⁶O scrambling as depicted in Scheme 1. Mechanism A shows an ionic reaction via initial proton abstraction with the help of the distal histidine acting as a general acid-base catalyst for MLC and F43H/H64L Mbs (Scheme 1A). A similar deprotonation process is involved in the formation of compound I (Scheme 1C) (36). In mechanism B, the reaction starts by a hydrogen atom transfer from H₂O₂ to the ferryl species to yield a radical intermediate. The hydrogen abstraction by the ferryl intermediate has been proposed for the alkane hydroxylation by cytochrome P450, its model complexes, and even in non-heme enzymes (37,38). In these reactions, large KIEs in a range of 9–29 are commonly observed due to a tunneling effect (27). We have also observed the tunneling effect (ΔH/ΔD = 0.07) for the catalatic reaction of H64A Mb-I with KIE of 23 at 5 °C, suggesting the involvement of hydrogen abstraction through a tunneling process.

While the histidine residue at the position 43 helps the ionic oxidation of hydrogen peroxide, the k₂ value of F43H/H64L Mb is virtually the same as that for H64D Mb and smaller than that of L29H/H64L (Table II). The crystal structure of H64D Mb suggests that an Asp residue at the position 64 could help to incorporate hydrogen peroxide in the active site due to the enlargement of the active site as well as hydrogen bonding (39). Although His at position 29 of L29H/H64L Mb is located too far from the heme iron to play as the acid-base catalyst, L29H/H64L Mb provides larger space for the accommodation of hydrogen peroxide than the F43H/H64L mutant and His29 might help to stabilize the Mb-I form by a polar effect (10). Our results suggest that important factors for the catalatic reaction are not only the deprotonation but also an enough space and polarity for the accommodation of hydrogen peroxide at a suitable position in the active site. In fact, distal cavity mutation of a Trp residue in catalase-peroxidase completely depressed its catalase activity but retained peroxidase activity. It has been concluded that the indole ring is involved in the binding of a H₂O₂ molecule (40,41).

In summary, we have provided solid evidence of the importance of a general acid
base catalyst for the ionic catalatic reaction of MLC-I and Mb-I. The KIEs on the reaction of Mb-I mutants with H$_2$O$_2$ indicate that there are two different mechanisms for the catalatic reaction, i.e., ionic and radical mechanisms, depending on the presence and absence of the distal histidine acting as a general acid-base catalyst. In addition, Mb mutants indicate that the reaction of compound I with hydrogen peroxide can be accelerated not only by the acid-base catalyst but also an enough space and polarity by amino acid residues in the active site.
Scheme 1. Proposed mechanisms for the catalytic reaction. (A): Ionic mechanism by utilizing a general acid-base catalyst. (B): Radical mechanism. (C): Role of a general acid-base catalyst on the formation of compound I.
**Fig. 6.** Active site structures of (A) F43H/H64L Mb (10), (B) L29H/H64L Mb (10), (C) MLC and (D) cytochrome c peroxidase.
References


(i) footnotes

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1 The abbreviations used are: Mb, myoglobin; Mb-I, compound I of Mb; BLCase; beef liver catalase; BLCase-I, compound-I of BLCase; mCPBA, *m*-chloroperbenzoic acid; KIE, kinetic isotope effect.

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Appendix 1. Steady-state reaction with small excess hydrogen peroxide for MLC-I formation

Reactions:

[1] \[ \text{MLC} + \text{H}_2\text{O}_2 \rightleftharpoons \text{MLC-} I + \text{H}_2\text{O} \]

[2] \[ \text{MLC-} I + \text{H}_2\text{O}_2 \rightleftharpoons \text{MLC} + \text{O}_2 + \text{H}_2\text{O} \]

The formation rate of Compound I is given by equation [3].

\[ \frac{d[\text{MLC}]}{dt} = k_1[\text{MLC}][\text{H}_2\text{O}_2] - k_2[\text{MLC-} I][\text{H}_2\text{O}_2] \]

Conservation relation:

[4] \[ [\text{MLC}]_0 = [\text{MLC}] + [\text{MLC-} I] \]

Equation [3] is rewritten by equation [5].

\[ \frac{d[\text{MLC}]}{dt} = (k_1 + k_2)[\text{MLC}] - \frac{k_2}{k_1 + k_2} [\text{MLC}]_0 [\text{H}_2\text{O}_2] \]

In equilibrium

[6] \[ k_1[\text{MLC}]_{eq}[\text{H}_2\text{O}_2] - k_2[\text{MLC-} I]_{eq}[\text{H}_2\text{O}_2] = 0 \]

[7] \[ [\text{MLC}]_0 = [\text{MLC}]_{eq} + [\text{MLC-} I]_{eq} \]

Therefore

[8] \[ [\text{MLC}]_{eq} = \frac{k_2}{k_1 + k_2} [\text{MLC}]_0 \]

Equation [5] is rewritten by equation [9].

\[ \frac{d[\text{MLC}]}{dt} = (k_1 + k_2)([\text{MLC}] - [\text{MLC}]_{eq})[\text{H}_2\text{O}_2] \]

At internal reaction of conservation relation:
At internal reaction, the back reaction is negligible, then

\[ [\text{MLC} - I] = [\text{H}_2\text{O}_2]_{\text{used}} \]

Combining [4], [10] and [11]

\[ [\text{H}_2\text{O}_2] = [\text{H}_2\text{O}_2]_0 + [\text{MLC}] - [\text{MLC}]_0 \]

Therefore equation [9] is rewritten by equation [13].

\[
\frac{d[\text{MLC}]}{dt} = (k_1 + k_2)([\text{MLC}] - [\text{MLC}]_\text{eq})([\text{H}_2\text{O}_2]_0 + [\text{MLC}] - [\text{MLC}]_0)
\]

or

\[
\frac{d[\text{MLC}]}{([\text{MLC}] - [\text{MLC}]_\text{eq})([\text{H}_2\text{O}_2]_0 + [\text{MLC}] - [\text{MLC}]_0)} = (k_1 + k_2) \, dt
\]

Integration of equation [13] gives equation [14],

\[
\ln\left(\frac{[\text{H}_2\text{O}_2]_0 + [\text{MLC}] - [\text{MLC}]_0}{[\text{MLC}] - [\text{MLC}]_\text{eq}}\right) = ([\text{H}_2\text{O}_2]_0 - [\text{MLC}]_0 + [\text{MLC}]_\text{eq})(k_1 + k_2) \, t
\]

Where \((k_1 + k_2) = k_{\text{app}} \cdot \text{MLC} - I\), equation [14] becomes [15]

\[
\ln\left(\frac{[\text{H}_2\text{O}_2]_0 + [\text{MLC}] - [\text{MLC}]_0}{[\text{MLC}] - [\text{MLC}]_\text{eq}}\right) = ([\text{H}_2\text{O}_2]_0 - [\text{MLC}]_0 + [\text{MLC}]_\text{eq})k_{\text{app}} \cdot \text{MLC} - I \, t
\]

Thus, \(([\text{H}_2\text{O}_2]_0 - [\text{MLC}]_0 + [\text{MLC}]_\text{eq})k_{\text{app}} \cdot \text{MLC} - I\) is determined by the trace of

\[
\ln\left(\frac{[\text{H}_2\text{O}_2]_0 + [\text{MLC}] - [\text{MLC}]_0}{[\text{MLC}] - [\text{MLC}]_\text{eq}}\right) \text{ versus } t
\]

And \(k_{\text{app}} \cdot \text{MLC} - I\) is determined by the plot of

\[
([\text{H}_2\text{O}_2]_0 - [\text{MLC}]_0 + [\text{MLC}]_\text{eq})k_{\text{app}} \cdot \text{MLC} - I \text{ versus } ([\text{H}_2\text{O}_2]_0 - [\text{MLC}]_0 + [\text{MLC}]_\text{eq})
Appendix 2. Steady-state equation for H$_2$O$_2$ dismutation

Reactions:

[1] \[ \text{MLC} + \text{H}_2\text{O}_2 \xrightleftharpoons{} k_1 \text{MLC - I} + \text{H}_2\text{O} \]

[2] \[ \text{MLC - I} + \text{H}_2\text{O}_2 \xrightleftharpoons{} k_2 \text{MLC} + \text{O}_2 + \text{H}_2\text{O} \]

H$_2$O$_2$ dismutation rate:

[16] \[ \frac{d[H_2O_2]}{dt} = k_1[\text{MLC}][\text{H}_2\text{O}_2] + k_2[\text{MLC - I}][\text{H}_2\text{O}_2] \]

At equilibrium

[17] \[ k_1[\text{MLC}][\text{H}_2\text{O}_2] - k_2[\text{MLC - I}][\text{H}_2\text{O}_2] = 0 \]

[18] \[ [\text{MLC}]_0 = [\text{MLC}] + [\text{MLC - I}] \]

Equation [16] becomes [19]

[19] \[ \frac{d[H_2O_2]}{dt} = \frac{2k_1k_2}{k_1 + k_2}[\text{MLC}]_0[H_2O_2] \]

\[ \frac{d[H_2O_2]}{[H_2O_2]} = \frac{2k_1k_2}{k_1 + k_2}[\text{MLC}]_0dt \]

[20] \[ \ln[H_2O_2] = \frac{2k_1k_2}{k_1 + k_2}[\text{MLC}]_0t \]

Where \[ \frac{2k_1k_2}{k_1 + k_2} = k_{\text{app-H}_2\text{O}_2} \]

[21] \[ \ln[H_2O_2] = [\text{MLC}]_0k_{\text{app-H}_2\text{O}_2}t \]

Thus, \([\text{MLC}]_0k_{\text{app-H}_2\text{O}_2}\) is determined by the trace of \(\ln[H_2O_2]\) versus \(t\)

\([\text{MLC}]_0\) is the initial concentration of MLC
Thus, \(k_{\text{app-H}_2\text{O}_2}\) is determined.