The Mechanism of Oxyperoxidase Formation from Ferryl Peroxidase and Hydrogen Peroxide*

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Formation of oxyperoxidase from the reaction of ferryl horseradish peroxidase with H₂O₂ is inhibited by a small amount of tetranitromethane (TNM), a powerful scavenger of superoxide anion radical. The inhibition by TNM, however, does not exceed 35% as the TNM concentration is increased above 5 μM. The stoichiometry of the reaction in the presence of TNM suggests the following equation for TNM-sensitive formation of oxyperoxidase.

Ferryl peroxidase + H₂O₂ →
(ferric peroxidase + O₂ + R*) → oxyperoxidase

The kinetic study on the TNM-resistant formation of oxyperoxidase suggests that the displacement of the oxygen with H₂O₂ takes place at the sixth coordination position at maximal rates of 0.048 and 0.054 s⁻¹ for peroxidases A and C, respectively, at 5 °C. The TNM-sensitive and -resistant reactions are concluded to occur in parallel, and both yield oxyperoxidase. In either mechanism, the protonated form of ferryl peroxidase is active and the pKₐ value is 7.1 for peroxidase A and 8.6 for peroxidase C.

Oxyperoxidase decomposes spontaneously with a large activation energy (23.0 kcal/mol), and the reaction of ferryl peroxidase with H₂O₂ reaches a steady level of oxyperoxidase, which depends on pH and the concentration of H₂O₂.

Compound III of peroxidase was first reported by Keilin and Mann (1) in the reaction of horseradish peroxidase with a large excess of H₂O₂ and subsequently studied by many distinguished pioneers in the field of hemoprotein study (2-5). The formation of Compound III was then ascribed to the reaction of Compound II (ferryl form) of peroxidase with H₂O₂ (4, 5). It was also found that peroxidase is converted to Compound III when it catalyzes aerobic oxidation of dihydroxyfumarate (6, 7). Because of spectral similarity between Compound III and known oxyhemoproteins, Compound III was once assumed to serve as a catalytic intermediate in the aerobic oxidation of dihydroxyfumarate catalyzed by peroxidase (6, 8).

Yamazaki and Piette (9) proposed three reactions of peroxidase Compound III formation. These are reactions of ferrous peroxidase with oxygen, ferric peroxidase with superoxide anion, and ferryl peroxidase with H₂O₂. Since peroxidase Compound III is formed directly from a reaction of the ferrous enzyme with oxygen (10-13), it may be rather called oxyperoxidase.

The respective reactions of ferrous and ferric peroxidases with oxygen and superoxide anion can be regarded primarily as the binding of these oxygen species to the heme iron (11-15). However, ligand-exchange mechanisms have not been presented to explain the formation of oxyperoxidase from ferryl peroxidase and H₂O₂, probably because the oxygen atom is considered to be tightly bound to the heme iron in the ferryl form (16). And the mechanism has remained unsettled. Another important problem to be solved in the reaction of ferryl peroxidase with H₂O₂ is reversibility of the reaction. This paper will answer these problems.

MATERIALS AND METHODS

Peroxidases used in this experiment were peroxidases A and C, which were purified from horseradish roots according to the method of Shannon et al. (17) with slight modification. The ratio of A₄₅₀ nm to A₃₆₅ nm for peroxidase A (isoenzymes A₁ + A₂) was 3.6 and that of A₄₅₀ nm to A₃₆₅ nm for peroxidase C was 3.4. The concentrations of peroxidases A and C were calculated on the basis that the values of A₄₅₀ at 404 and 403 nm are 107 and 109, respectively, (18). Compound II (ferryl peroxidase) was formed by successive additions of 1.0 eq of ferrocyanide or ascorbate and 1.1 molar eq of H₂O₂. The content of ferryl peroxidase was more than 95%. The oxyperoxidase preparation used for measuring the rate of oxyperoxidase decomposition was prepared by photolysis of a CO/ferrous peroxidase solution which had been freed of excess dithionite by gel filtration under dark. The resultant solution contained about 85% oxyperoxidase and 15% ferric peroxidase. Tetranitromethane (TNM)¹ was purchased from Wako Pure Chemical Industry (Osaka, Japan).

Spectrophotometric measurements were performed with Hitachi 124 and Shimadzu UV-300 dual-beam spectrophotometers. These photometers were equipped with a cuvette compartment thermostatically controlled, and the compartment was kept dry with nitrogen gas to avoid blurring the cuvette surface when the reactions were carried out at low temperatures. The reactions were carried out in 50 mm buffer solutions at 5 °C, except as otherwise mentioned. Buffer systems used were potassium phosphate (pH 5.5-8.0), Tris-HCl (pH 8-9), and sodium carbonate or glycine-HCl (pH 9-10). The O₂ concentration was measured polarographically with a Clarke-type O₂ electrode.

RESULTS

When H₂O₂ was added to ferryl peroxidase C, the visible spectrum changed markedly, showing the formation of oxyperoxidase. The spectral change reached a maximum in about 3 min with isosbestic points at 496, 532, 566, and 594 nm at pH 8.9 (Fig. 1A). The extent of the change depended on the amount of H₂O₂ added. Fig. 1B shows a titration curve which approximated a first-order dissociation curve, and it seemed as if oxyperoxidase was a reversible complex of ferryl peroxidase and H₂O₂. The apparent dissociation constant was 59 μM. The formation of oxyperoxidase obeyed apparently first-

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¹The abbreviation used is: TNM, tetranitromethane.
and 1. Ha02. H202 was added stepwise to ferryl peroxidase and each spectrum was recorded 5 min after the addition of H2O2. The concentration added are noted. B, the fraction of oxyperoxidase was plotted against the logarithm of [H2O2] added. Each point was obtained with fresh ferryl peroxidase.

The rate was measured at 577 nm or at an isosbestic point between Compound I and ferryl peroxidase. The isoabsorbance point and its $\Delta A_{257}$ value varied slightly with pH (see Footnote 6 in Table II). Buffer systems used were potassium phosphate (○), Tris·HCl (●), sodium carbonate, and glycine·NaOH (▲).

Our attention was then focused on the problem as to whether the reaction of ferryl peroxidase with H2O2 is irreversible or not. As oxyperoxidase is known to decompose spontaneously to the ferric form (11-13), we examined a possibility that the apparent dissociation constant obtained from the titration curve in Fig. 1B might represent a dynamic feature of oxyperoxidase decomposition. The activation energy was calculated at 8.0, 6.9, and 23.0 kcal for curves a-c, respectively.

apparent dissociation constant in a wide pH range. This consistency was also seen when the temperature was varied (Table I). The O2 dissociation was found to be too slow to be detected from the displacement of O2 with CO in a CO-saturated and O2-free solution of oxyperoxidase.

In order to analyze the mechanism of oxyperoxidase formation, complications arising from the decomposition of oxyperoxidase were minimized by carrying out the reaction at 5°C. The decomposition of oxyperoxidase became relatively slower than its formation as the temperature was lowered because of its large temperature dependence (Fig. 4). The
powerful scavenger of superoxide radical (30, by the presence of 1 mM H2O2 (28, 29). TNM, which is a compound I to the ferryl form and further to oxyperoxidase in the ferryl form, serves as a strong oxidant (22), we assumed that it causes 1-electron oxidation of H2O2 as follows:

\[
\text{Ferryl peroxidase} + \text{H}_2\text{O}_2 \rightarrow \text{peroxidase} + \text{O}_2^- + \text{H}^+ \quad (2)
\]

The oxyperoxidase formation from peroxidase and superoxide radical has been verified (9, 14, 15, 27).

If oxyperoxidase is formed through Reactions 2 and 3, its formation should be inhibited by superoxide radical scavengers. The oxyperoxidase formation was partially inhibited by the presence of a large amount of superoxide dismutase (data not shown), but the use of superoxide dismutase appeared to be unsuitable for the present purpose because it is inactivated by the presence of 1 mM H2O2 (28, 29). TNM, which is a powerful scavenger of superoxide radical (30, 31), was found to inhibit the formation of oxyperoxidase at very low concentrations (Fig. 5A). No direct reactions of TNM with ferryl peroxidase, like Compound I, serves as a strong oxidant (22), we assumed that it causes 1-electron oxidation of H2O2 as follows:

\[
\text{Oxyperoxidase} + \text{O}_2^- + \text{H}^+ \rightarrow \text{oxyperoxidase} + \text{H}_2\text{O} \quad (3)
\]

activation energy was measured to be 23.0 kcal mol\(^{-1}\) for the decomposition of oxyperoxidase and 6.9 kcal mol\(^{-1}\) for its formation from ferryl peroxidase and H2O2.

Compound I is formed upon reactions of ferric peroxidase not only with H2O2 but also with alkyl hydroperoxides (23, 24) and peroxynitrobenzoate derivatives (24–26). Oxyperoxidase was formed from the reaction of ferryl peroxidase only with H2O2, but not with ethyl hydroperoxide and m-chloroperoxynitrobenzoate, which are both good substrates for Compound I formation. A property of H2O2 which differs from other Compound I-forming peroxides might be that H2O2 can serve as a reductant as well as an oxidant. H2O2 reacted with Compound I of peroxidase C with a rate constant of 5 \(\times\) 10\(^4\) M\(^{-1}\) s\(^{-1}\).

\[
\text{Compound I} + \text{H}_2\text{O}_2 \rightarrow \text{peroxidase} + \text{O}_2
\]

This means that horseradish peroxidase has a weak catalatic activity. The oxidation of H2O2 by Compound I was found to occur mostly through 2-electron reduction of Compound I. A few percent of the reduction, however, occurred by way of 1-electron transfer, which resulted in slow conversion of Compound I to the ferryl form and further to oxyperoxidase in the presence of excess H2O2. Since ferryl peroxidase, like Compound I, serves as a strong oxidant (22), we assumed that it causes 1-electron oxidation of H2O2 as follows:

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\[
\text{Oxyperoxidase} + \text{O}_2^- + \text{H}^+ \rightarrow \text{oxyperoxidase} + \text{H}_2\text{O} \quad (3)
\]

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\]

The oxyperoxidase formation from peroxidase and superoxide radical has been verified (9, 14, 15, 27).

If oxyperoxidase is formed through Reactions 2 and 3, its formation should be inhibited by superoxide radical scavengers. The oxyperoxidase formation was partially inhibited by the presence of a large amount of superoxide dismutase (data not shown), but the use of superoxide dismutase appeared to be unsuitable for the present purpose because it is inactivated by the presence of 1 mM H2O2 (28, 29). TNM, which is a powerful scavenger of superoxide radical (30, 31), was found to inhibit the formation of oxyperoxidase at very low concentrations (Fig. 5A). No direct reactions of TNM with ferryl and oxyperoxidases were observed at these TNM concentrations. The reaction between TNM and H2O2 was also negligibly slow at pH values below 8. Although the oxyperoxidase formation was very sensitive to TNM, it was not completely inhibited at higher concentrations of TNM. The inhibition reached a maximum (35%) at a TNM concentration of about 5 \(\mu\)M (Fig. 5B). This inhibition was accompanied by the increase of catalatic activity of peroxidase (Fig. 6). The effects of TNM concentration on the inhibition of oxyperoxidase formation (Fig. 5B) and the increase of catalatic activity suggested a close relation between the two phenomena. Curve c of Fig. 6 shows a weak catalatic reaction observed during the formation of oxyperoxidase from ferryl peroxidase and H2O2. The catalatic activity was maximal in the middle stage of oxyperoxidase formation. This might be explained by assuming that a small part of ferryl peroxidase was converted to the ferric form through the following reaction (13),

\[
\text{Ferryl peroxidase} + \text{oxyperoxidase} + \text{H}^+ \rightarrow \text{peroxidase} + \text{O}_2
\]

\[
+ \text{H}_2\text{O} \rightarrow \text{peroxidase} + \text{O}_2
\]

\[
(+ \text{H}_2\text{O} \rightarrow \text{peroxidase} + \text{O}_2
\]

Ferric peroxidase is immediately converted to Compound I, exhibiting catalatic activity (Fig. 6, curve d). The TNM-induced acceleration of the catalatic activity in the ferryl peroxidase-H2O2 system was also ascribable to the Compound I formation caused by TNM. Table II shows stoichiometric relation in the formation of Compound I, oxyperoxidase, and reduced TNM. This stoichiometry was consistent with the following equation,

\[
\text{Ferryl peroxidase} + 2\text{H}_2\text{O}_2 + \text{C(NO}_2\text{)}_3 \rightarrow \text{Compound I}
\]

\[
+ \text{O}_2 + \text{C(NO}_2\text{)}_3 + \text{NO}_2 + 2\text{H}_2\text{O} + \text{H}^+
\]

\[
+ \text{O}_2 + \text{C(NO}_2\text{)}_3 + \text{NO}_2 + 2\text{H}_2\text{O} + \text{H}^+
\]

Fig. 6 shows the effect of TNM on double reciprocal plots of the initial rate of oxyperoxidase formation and the concentration of H2O2. To explain the incompleteness of TNM inhibition, we assumed that oxyperoxidase was formed through two parallel reactions, TNM-sensitive and TNM-resistant. The rate of TNM-sensitive reaction was therefore estimated from the difference of the two rates in the presence and absence of TNM, which are plotted in Fig. 7A. Reciprocal of the rate of TNM-sensitive reaction is plotted against reciprocal of [H2O2] in Fig. 7B. The rate was proportional to [H2O2]. We therefore concluded that the TNM-sensitive formation of oxyperoxidase occurs through a mechanism consisting of Reactions 2 and 3, the former being rate-determining. The second-order rate constant of Reaction 2 was thus estimated as 2.1 \(\text{M}^{-1}\) s\(^{-1}\) for peroxidase C.

The TNM-resistant formation of oxyperoxidase exhibited
**TABLE II**

Stoichiometry of the reactions of ferryl peroxidases A and C with H$_2$O$_2$ in the presence of TNM

<table>
<thead>
<tr>
<th>Peroxidase isoenzyme</th>
<th>pH</th>
<th>ΔOxyperoxidase$^*$</th>
<th>Compound I</th>
<th>Reduced TNM$^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>7</td>
<td>1.9</td>
<td>2.1</td>
<td>2.1</td>
</tr>
<tr>
<td>C</td>
<td>7</td>
<td>2.5</td>
<td>2.4</td>
<td>2.1</td>
</tr>
</tbody>
</table>

$^*$The difference in the amount of oxyperoxidase formed in the presence and absence of TNM is shown. The formation of oxyperoxidase was measured at an isosbestic point between Compound I and the ferryl form. The wavelengths were 456 nm at pH 7 and 462 nm at pH 8 for peroxidase A, and 455.5 nm at pH 7 and 8 for peroxidase C. The values in parentheses above show values of Δ$_{TNM}$ for the formation of oxyperoxidase.

The formation of Compound I was measured at 650 nm. The values for Δ$_{TNM}$ (Compound I-ferryl form) were 4.60 at pH 7 and 4.23 at pH 8 for peroxidase A, and 5.63 at pH 7 and 5.18 at pH 8 for peroxidase C. The values for Δ$_{TNM}$ (ferryl form-oxypseudoxidase) were 0.76 at pH 7 and 0.66 at pH 8 for peroxidase A, and 0.94 at pH 7 and 0.89 at pH 8 for peroxidase C.

The reduction of TNM was measured at 355 nm, where Δ$_{TNM}$ for the TNM reduction was 148 and those for the formation of Compound I from either ferryl or oxyperoxidase were 10.6 at pH 7 and 11.1 at pH 8 for peroxidase A, and 11.6 at pH 7 and 11.3 at pH 8 for peroxidase C. Using these data, corrections were made for the amount of Compound I formed.

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**FIG. 7.** Double reciprocal plots for initial rate of oxyperoxidase C formation and concentration of H$_2$O$_2$. A, 8 μM ferryl peroxidase C and pH 7.5. The initial velocity was measured from kinetic traces as shown in Fig. 5 (curve a) in the presence (O) and absence (C) of TNM (11 μM). B, the difference of the initial velocities (Δv) in the presence and absence of TNM was obtained from data in A.

H$_2$O$_2$-saturated kinetics which gave an intercept on the ordinate. From the intercept shown in Fig. 7A, the maximum rate was estimated at 0.054 s$^{-1}$ for peroxidase C at 5 °C. This value did not change in the pH range between 6 and 8. The measurement at higher pH above 8 was impossible because direct reactions occurred between TNM and H$_2$O$_2$. Fig. 8A shows pH dependence of the maximum rate for TNM-resistant formation of oxyperoxidase A. The rate was measured from a series of experiments at varied pH values, one of which is shown in Fig. 8B. The rate was 0.048 s$^{-1}$ at pH 5.5 and decreased with the increase of pH according to a first-order dissociation curve. The pK$_a$ value was about 7 and consistent with that for ferryl peroxidase A shown in Fig. 2.

The formation of oxyperoxidase from ferryl peroxidase and H$_2$O$_2$ has been reported also for lactoperoxidase (33). The oxyform of lactoperoxidase was more stable than those of horseradish peroxidases, and the apparent dissociation constant was determined to be 55 μM at pH 7 and 20 °C. Although the catalytic activity of lactoperoxidase was stronger than those of horseradish peroxidases, there was no essential difference in the mechanism of oxyperoxidase formation between the two peroxidases. Fig. 9 shows effects of TNM on the formation of oxyperoxidase and the catalytic activity in the case of lactoperoxidase. The catalytic activity was so strong in the presence of TNM that it was difficult to attempt the kinetic analysis of the TNM-sensitive and TNM-resistant formation of oxylactoperoxidase.

**DISCUSSION**

It can be concluded from Fig. 2 that the protonated form of ferryl peroxidase reacts with H$_2$O$_2$ to form oxyperoxidase. Fig. 10 shows two parallel paths which may explain our experimental results. Path 1 is TNM-resistant, and path 2 is TNM-sensitive. In path 2, H$_2$O$_2$ is oxidized by ferryl peroxidase to a superoxide radical, which reacts with ferric peroxidase to form oxyperoxidase (Reactions 2 and 5). The rate constant for the reaction between ferric peroxidase and superoxide radical has been reported to be about 10$^7$ M$^{-1}$ s$^{-1}$ (15, 27). TNM acts as a powerful scavenger of superoxide radical through the following reaction and for it, a rate constant of 1.9 × 10$^9$ M$^{-1}$ s$^{-1}$ has been given (30).

$$\text{O}_2^- + C(\text{NO}_3^-)_2 \rightarrow \text{O}_2 + C(\text{NO}_3^-)_2 + \text{NO}_2$$ (6)
The complete inhibition of path 2 by the presence of less than 10 μM TNM can be explained by difference in the two rate constants.

In path 1, the reaction of ferryl peroxidase with H₂O₂ is preceded by dissociation of hydroxyl anion. It is evident from Fig. 8A that such dissociation occurs only in the protonated form of ferryl peroxidase. This mechanism is related to recent topics in the resonance Raman measurement of ferryl peroxidases. Using ³¹O-labeled H₂O₂, Hashimoto et al. (34) have demonstrated that the oxygen in Fe(IV)=O is exchangeable in a few minutes with that of bulk water in the protonated form of ferryl peroxidase C, but not in its proton-dissociated form. A similar result can be seen in the paper of Sitter et al. (35), although the occurrence of such exchange is not mentioned in their paper. The resonance Raman measurement has not yet given an exact value for the exchange rate, but the results appear not to contradict our explanation for kinetic inhibition of path 2 in Figs. 7A and 8B. The value is roughly 10 and 5 mM H₂O₂ for peroxidases A and C, respectively.

It is of interest to compare peroxidase and myoglobin in respect to the ability of conversion from ferryl to oxy form. Metmyoglobin is easily oxidized to the ferryl form, but ferryl myoglobin is not converted to oxymyoglobin in the presence of excess H₂O₂. Reasonable explanations for the inertness of ferryl myoglobin would be: 1) for path 1, the ferryl oxygen is not exchangeable or the ferryl form has a very weak affinity for H₂O₂ (high Kₐ value); and 2) for path 2, ferryl myoglobin cannot oxidize H₂O₂ and/or metmyoglobin does not combine with superoxide anion radical.

REFERENCES