Kinetic Studies on the Formation and Decomposition of Compounds II and III

REATIONS OF LIGNIN PEROXIDASE WITH \( \text{H}_2\text{O}_2 \)*

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The present study characterizes the serial reactions of \( \text{H}_2\text{O}_2 \) with compounds I and II of lignin peroxidase isozyme H1. These two reactions constitute part of the pathway leading to formation of the oxy complex (compound III) from the ferric enzyme. Compounds II and III are the only complexes observed; no compound III* is observed. Compound III* is proposed to be an adduct of compound III with \( \text{H}_2\text{O}_2 \), formed from the complexation of compound III with \( \text{H}_2\text{O}_2 \) (Wariishi, H., and Gold, M. H. (1990) J. Biol. Chem. 265, 2070-2077). We provide evidence that demonstrates that the spectral data, on which the formation of compound III* is based, are merely an artifact caused by enzyme instability and, therefore, rule out the existence of compound III*. The reactions of compounds II and III with \( \text{H}_2\text{O}_2 \) are pH-dependent, similar to that observed for reactions of compounds I and II with the reducing substrate veratryl alcohol. The spontaneous decay of the compound III of lignin peroxidase results in the reduction of ferric cytochrome c. The reduction is inhibited by superoxide dismutase, indicating that superoxide is released during the decay. Therefore, the lignin peroxidase compound III decays to the ferric enzyme through the dissociation of superoxide. This mechanism is identical with that observed with oxymyoglobin and oxyhemoglobin but different from that for horseradish peroxidase. Compound III is capable of reacting with small molecules, such as tetranitromethane (a superoxide scavenger) and fluoride (a ligand for the ferric enzyme), resulting in ferric enzyme and fluoride complex formation, respectively.

The present study characterizes the reactions leading to the formation and decomposition of compound III of lignin peroxidase. Compound III, or oxy complex, is the dioxygen complex of ferrous peroxidase, similar to oxyhemoglobin. Compound III of peroxidases can be formed by three mechanisms: (i) complexation of ferrous peroxidase with dioxygen (Wittenberg et al., 1987), (ii) the complexation of superoxide anion with ferric peroxidase (Shimizu et al., 1989), and (iii) the reaction of ferryl peroxidase (compound II) with \( \text{H}_2\text{O}_2 \) (Noble and Gibson, 1970; Nakajima and Yamazaki, 1987). Although structurally similar to oxyhemoglobin, compound III of peroxidase is much more unstable than oxyhemoglobin (Cai and Tien, 1990). The ferrous iron is readily oxidized by dioxygen, which results in the conversion of compound III to the ferric peroxidase (Tamura and Yamazaki, 1972).

The formation of lignin peroxidase compound III from ferrous peroxidase and dioxygen has been characterized using transient kinetic methods (Cai and Tien, 1990). Lignin peroxidase compound III is exceedingly more stable than the compound III of other peroxidases (Cai and Tien, 1989, 1990). Similar to peroxidases and the globins, the compound III of the lignin peroxidase decays to the ferric state exponentially. In addition to the spontaneous decay, the compound III of the lignin peroxidase is rapidly converted back to the ferric state in the presence of \( \text{H}_2\text{O}_2 \) and a reducing substrate veratryl alcohol (Cai and Tien, 1989). The mechanism for such conversion is not clear. These reactions are of significance because excess \( \text{H}_2\text{O}_2 \) not only results in the formation of compound III but also causes irreversible inactivation of the enzyme.

The formation of compound III from ferric lignin peroxidase and excess \( \text{H}_2\text{O}_2 \) involves serial reactions of enzyme with \( \text{H}_2\text{O}_2 \). The first step is the formation of compound I, which has been characterized using transient kinetic methods (Andrawis et al., 1988). The second step is formation of compound II from the reaction of compound I with \( \text{H}_2\text{O}_2 \). This latter reaction has been suggested yet not demonstrated unambiguously (Tien et al., 1986). The last step is the reaction of compound II with \( \text{H}_2\text{O}_2 \), resulting in compound III formation. This reaction has been observed with horseradish peroxidase (Noble and Gibson, 1970; Nakajima and Yamazaki, 1987). Although the last reaction was reported for lignin peroxidase, the product obtained was not compound III but the so-called compound III*, proposed primarily based on its spectral properties in the Soret region (Wariishi and Gold, 1989; Wariishi et al., 1990). This was the first time that compound III* was introduced as one of the peroxidase intermediates; however, the existence of compound III* has by no means been unequivocally established.

In the present report, we provide kinetic data showing sequential reactions of \( \text{H}_2\text{O}_2 \) with lignin peroxidase compound I and compound II. Evidence is also provided that demonstrates compound III is the final product from the reaction of compound II and \( \text{H}_2\text{O}_2 \). Our results show that the formation of "compound III*" is an artifact. The mechanism of compound III autoxidation to the ferric enzyme is also studied. These results provide an explanation of the reactivity of compound III in the presence of \( \text{H}_2\text{O}_2 \).

EXPERIMENTAL PROCEDURES

Materials—Lignin peroxidase isozyme H1 (pI = 4.7) was isolated from the extracellular fluid of 5-day cultures of a Phanerochaete...
chrysosporium strain P58L-1 (Tien and Myer, 1990). The purification procedure was as described by Tien and Kirk (1988). The concentration was determined using $e_{380} = 169$ mm$^-1$ cm$^-1$ (Farrell et al., 1989), and the RZ number ($A_{425}/A_{280}$) was always over 4. Hydrogen peroxide (30%) was purchased from Fisher. The H$_2$O$_2$ stock solutions were prepared daily, and the enzyme concentrations were determined using $e_{380} = 39.4$ mm$^-1$ cm$^-1$ (Nelson and Kiesow, 1972). Horse heart cytochrome c, tetramethylmethane (TNM) and superoxide dismutase were purchased from Sigma. Cytochrome c was acetylated as described by Green and Hill (1984). Stock solution of TNM was prepared fresh in 100% ethanol prior to use. The amounts of cytochrome c and TNM reduced were determined using $e_{450} = 18.5$ mm$^-1$ cm$^-1$ and $e_{350} = 14.8$ mm$^-1$ cm$^-1$, respectively (Green and Hill, 1984). Veratryl alcohol was purchased from Aldrich and vacuum-distilled. All other chemicals were reagent-grade and used without purification. Buffers were prepared daily, and the concentration was determined using $e_{380} = 0.218$ mm$^-1$ cm$^-1$ (Nelson and Kiesow, 1972). Horse heart cytochrome c was used for all experiments. All manipulations were at 4 °C.

Preparation of Enzyme Intermediates—Compound I was prepared by equimolar mixing of ferric enzyme with H$_2$O$_2$ in double-distilled, K$_2$MnO$_4$-treated water. Compound II was prepared by mixing the ferric enzyme with 2 molar eq of H$_2$O$_2$. The spectrum of the compound II was identical with that obtained from mixing the ferric enzyme with 1 molar eq each of H$_2$O$_2$ and ferrocyanide. Compound III was obtained by introducing air to the ferrous enzyme solution. Ferrous enzyme was prepared as described previously by reducing the ferric enzyme anaerobically in pH 7.0 sodium phosphate buffer containing EDTA and 5-deazoflavin (Cai and Tien, 1990). The resulting compound III solution was passed through an Econo-Pac 10 DG desalting column (Bio-Rad) to remove EDTA and 5-deazoflavin and to equilibrate with the desired buffer. All manipulations were at 4 °C.

Stopped-flow Measurements—Stopped-flow measurements were performed using an apparatus described by Johnson (1986) under pseudo-first order conditions. Rate constants and fitting of multivariate kinetic traces were performed as described by Johnson (1986). Kinetic difference spectra were constructed by plotting the amplitude of the spectral change as a function of wavelength. The concentration of H$_2$O$_2$ was 1.9 mM, and the enzyme concentrations were 3.8 and 14 PM, final, for the Soret and visible regions, respectively. For rate constant measurements, enzyme concentrations varied from 1.3 to 3.5 PM. Reactions were carried out at pH 7.2 or otherwise indicated. Reactions were carried out at pH 7.2 or otherwise indicated. All experiments were performed at 25 °C.

Autoxidation of Compound III—The rate of compound III decay was determined as described previously (Cai and Tien, 1989), except that it was calculated using the absorbance at a Soret wavelength at which the change in absorbance was maximal. Absorption spectra were recorded using a Perkin-Elmer Lambda 6 UV-Vis spectrophotometer. A stopped-flow apparatus was used to follow the absorbance change when the decay of compound III became too fast to measure accurately using a spectrophotometer, as in the case when TNM was present in high concentration. All experiments were performed at 25 °C.

RESULTS

Formation of Compound II—Transient kinetic studies demonstrated that compound I is the first intermediate formed from the reaction of H$_2$O$_2$ with ferric lignin peroxidase (Tien et al., 1986; Andrawis et al., 1988). When the reaction is monitored over a longer period of time, a slower reaction where compound I reacts with H$_2$O$_2$ is observed. The kinetic difference spectrum for this slower reaction was constructed in both Soret and visible regions (Fig. 1A). This difference spectrum matches closely to the difference spectrum of compound II minus compound I, indicating that this reaction is the formation of compound II from compound I and H$_2$O$_2$.

The pseudo-first order rate for compound II formation is linearly dependent on H$_2$O$_2$ concentration, as shown in Fig. 2A, indicating a bimolecular reaction between compound I and H$_2$O$_2$ rather than the spontaneous reduction of compound I to compound II, as suggested previously (Harvey et al., 1989).

\[ \text{Compound I} + \text{H}_2\text{O}_2 \rightarrow \text{compound II} + \text{HO}_2^- \]  

The pseudo-first order rate for compound II formation is

\[ k_{\text{obs}} = \frac{\text{rate}}{[\text{H}_2\text{O}_2]} \]

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The plot of the pseudo-first order rate constants versus $H_2O_2$ concentration is again linear (Fig. 2B). The second order rate constants are $150 \text{ M}^{-1} \text{s}^{-1}$ at pH 3.5 and $70 \text{ M}^{-1} \text{s}^{-1}$ at pH 7.2.

The rate constant at neutral pH is in the same order of magnitude as that for the reaction of horseradish peroxidase compound II and $H_2O_2$. (Noble and Gibson, 1972). Compound III formation can also be observed from solutions of compound II (as the starting enzyme species) reacting with $H_2O_2$ (data not shown). The kinetic difference spectrum and the rate constants obtained from these reactions (where compound II is in one of the stopped-flow syringes) are identical with those when the ferric enzyme is used (as described above).

A typical stopped-flow trace where the absorbance change at 417 nm is monitored during the reaction of the ferric lignin peroxidase with $H_2O_2$ is shown in Fig. 3A. In the time domain shown, compound I formation is rapid and thereby not observed. The initial absorbance increase is due to the formation of compound II from compound I and $H_2O_2$. The second phase (also an increase in absorbance) corresponds to the formation of compound III from compound II and $H_2O_2$. Regardless of whether the ferric or ferrous enzyme was used, the formation of compound III is always accompanied by an increase in absorbance at 417 nm as opposed to a previous report by Wariishi et al. (1990).

Further characterization of the reaction of compound II with $H_2O_2$ revealed that the kinetics of lignin peroxidase compound II reacting with $H_2O_2$ is sensitive to the age of the enzyme preparation. When the change of absorbance at 417 nm was monitored, a different trace was obtained with an enzyme preparation that had been stored for months (Fig. 3B). The trace shown in Fig. 3B is similar to that observed by Wariishi et al. (1990) in their work describing the intermediate compound III*. Following the formation of compound II (the initial increase in absorbance, phase I), there is a decrease in absorbance (phase II), followed by a subsequent increase in absorbance (phase III). The kinetic difference spectrum and the rate constants of the slowest phase (phase III) were consistent with the formation of compound III. We further noted that the amplitude for the phase with a decrease in absorbance (phase II) varied from sample to sample. This phase was not observed when freshly prepared enzyme sample was used. Similar results were again obtained when compound II was used to start the reaction. Therefore, the decrease in absorbance associated with older enzyme preparations is most likely due to the partial bleaching of the enzyme by $H_2O_2$.

**Effect of pH on Compound II and Compound III Formation**—The rates of compound II and compound III formation are both pH-dependent (Fig. 4). Both reactions are accelerated at low pH. The $pK_a$ could not be accurately determined due to insufficient data points in the acidic region ($pH < 2.5$). These pH profiles closely resemble those for the reactions of compounds I and II with veratryl alcohol (Marquez et al., 1988), implying that $H_2O_2$ reacts with similar ionized states of compounds I and II. It is interesting to note that compound II of horseradish peroxidase also reacts with $H_2O_2$ faster at low pH, but unlike lignin peroxidase, the $pK_a$ for horseradish peroxidase is near neutral (Nakajima and Yamazaki, 1987).

**Mechanism for Compound III Autoxidation**—In the absence of $H_2O_2$, compound III spontaneously decays to the ferric enzyme with no detectable intermediate (Cai and Tien, 1990). The decay of horseradish peroxidase compound III has been proposed to proceed through the dissociation of $H_2O_2$ from compound III generating compound II and $H_2O_2$ (Reaction 3) (Tamura and Yamazaki, 1972; Nakajima and Yamazaki, 1987).

$$\text{Compound III} + H^+ \rightarrow \text{compound II} + H_2O_2$$ (3)

The formation of compound II during the course of compound III decay can be tested by the addition of reducing substrate to the reaction mixture. These substrates should be oxidized, and compound III decay should, in turn, be accelerated. The decomposition of horseradish peroxidase compound III results in oxidation of a number of substrates (Tamura and Yamazaki, 1972). No veratraldehyde could be detected in incubations of lignin peroxidase compound III with veratryl alcohol at pH 3.5. Furthermore, the decay of lignin peroxidase compound III was also not accelerated by veratryl alcohol (data not shown).

An alternate pathway for the autoxidation $Fe^{3+}O_2$ of compound III is the dissociation of superoxide from compound III.

$$Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^-$$

The autoxidation of oxymyoglobins and oxyhemoglobins has
been well documented to proceed by this mechanism (Misra and Fridovich, 1972; Gotoh and Shikama, 1976; Wallace et al., 1982). Consistent with the above mechanism, superoxide could be detected during the course of compound III decay. The decay of the lignin peroxidase compound III resulted in concomitant cytochrome c reduction, as indicated by the absorbance increase at 550 nm (Fig. 5). This reduction is completely inhibited by superoxide dismutase, demonstrating the involvement of superoxide. Furthermore, the time course for the reduction of cytochrome c correlates with the oxidation of compound III and the stoichiometry of cytochrome c reduced and the ferric enzyme generated is approximately 1:1.

The rate of lignin peroxidase compound III autoxidation is greatly increased by TNM, a superoxide scavenger. Surprisingly, the rate of compound III decay is linearly dependent on TNM concentration (Fig. 6A). TNM is reduced in the process as indicated by the absorbance increase at 350 nm (data not shown). The amount of TNM reduced equals the amount of ferric enzyme generated. The rate of compound III decay is also accelerated by fluoride with the formation of the ferric-fluoride complex. The decay follows a single exponential, and the apparent decay rates are proportional to the concentration of fluoride (Fig. 6B). These results suggest that fluoride is able to displace superoxide from compound III. If fluoride only reacted with the free ferric enzyme after the dissociation of superoxide, the rate of the fluoride complex formation would be expected to be zero order in respect to fluoride concentration.

**Conversion of Compound III to the Ferric Enzyme by H2O2 and Veratryl Alcohol**—We previously demonstrated that compound III of lignin peroxidase isozyme H8 is exceedingly stable (Cai and Tien, 1989). Addition of veratryl alcohol or H2O2 alone has no effect on the stability of compound III (Cai and Tien, 1989). The addition of H2O2, however, causes a slight red shift in the Soret region. The addition of veratryl alcohol and 1 eq of H2O2 caused a rapid conversion of compound III to the ferric enzyme (Cai and Tien, 1989). Veratraldehyde is generated in this reaction. This result was observed again with isozyme H1 (data not shown).

The conversion of compound III to ferric enzyme upon the addition of veratryl alcohol and H2O2 occurs much faster than the autoxidation of compound III. Consequently, we speculated that compound III could react directly with H2O2, forming either compound I or compound II, which then oxidizes veratryl alcohol. We tested this mechanism by trapping experiments with cyanide, a technique similar to those used by Wariishi and Gold (1990). The presence of cyanide assures single turnover conditions. Ferric enzyme, formed from enzyme turnover, would be readily trapped as the cyanide complex in the presence of 80 mM KCN. In the absence of H2O2, cyanide had no effect on the decay of compound III (not shown). Thus, if H2O2 reacts directly with compound III, then its addition should cause an immediate conversion of compound III to either compound I or compound II. Compound II (formally Fe4+ and shown below) is formed if H2O2 reacts with ferroperoxidase displacing dioxygen; compound I (Fe4+ and a porphyrin cation radical) is formed if it reacts with ferreroxidase displacing superoxide.

\[
\text{H}_2\text{O}_2 + \text{Fe}^{4+} = \text{O}_2 + \text{Fe}^{3+} + \text{H}_2\text{O} 
\]

(5)

\[
\text{H}_2\text{O}_2 + \text{Fe}^{3+} = \text{O}_2 + \text{Fe}^{4+} + \text{H}_2\text{O} 
\]

(6)

Compound I or II would readily react with veratryl alcohol, resulting in formation of ferric enzyme, which would then be trapped as the cyanide complex.

\[
\text{RH} + \text{Fe}^{4+} \rightarrow \text{Fe}^{3+} + \text{CN} 
\]

(7)

Initially, we tested the effect of cyanide on compound III, in the absence of veratryl alcohol, to determine if cyanide could be used as a trapping agent. Although Wariishi and Gold (1990) previously showed a spectrum of compound III in the presence of cyanide, it was necessary to determine that cyanide did not accelerate the decay of compound III. The addition of cyanide to compound III resulted in spectral changes as shown in Fig. 7A. Over the course of the experiment, compound III gradually disappears with concomitant formation of the cyanide complex. This can be attributed to trapping of the ferric enzyme by cyanide upon its formation from compound III decay. The cyanide complex exhibits a Soret maximum at 423 nm and a visible maximum at 540 nm (Andersson et al., 1985). The results of a similar experiment performed in the presence of veratryl alcohol is shown in Fig. 7B. Contrary to the results of Wariishi and Gold (1990), veratryl alcohol does not cause an immediate conversion of compound III (or the putative compound III*) to ferric enzyme. In fact, the rate of compound III decay in the presence of veratryl alcohol to yield the cyanide complex appears to be similar to that in the absence of veratryl alcohol. This was more clearly demonstrated when the decrease in absorbance at 420 nm, which is associated with the conversion of compound III to cyanide complex, was monitored over time (Fig.
Lignin Peroxidase Compound III

8). No difference in rate was observed in incubations with and without veratryl alcohol.

Conversion of Compound III to the Ferric Enzyme by H$_2$O$_2$ and Other Substrates—We previously demonstrated that the addition of just 1 eq of H$_2$O$_2$ to compound III in the presence of veratryl alcohol caused a rapid conversion of compound III to ferric enzyme with the formation of veratraldehyde (Cai and Tien 1989). This rapid conversion was also observed with 3,4-dimethoxybenzene. The addition of 400 $\mu$M 3,4-dimethoxybenzene to compound III in the presence of 73 $\mu$M H$_2$O$_2$ quickly resulted in the formation of ferric enzyme (Fig. 9A). This facile conversion was not observed with iodide as a substrate. Again, compound III was generated by the addition of 73 $\mu$M H$_2$O$_2$ to ferric enzyme. The addition of potassium iodide (400 $\mu$M) to this solution caused a rapid formation of triiodide product, as demonstrated by the increase in absorbance at 350 nm (Fig. 9B). Even with multiple turnovers (the initial H$_2$O$_2$ concentration was 25 molar excess over that of enzyme concentration), a significant amount of compound III was still present in the reaction mix (Fig. 9B). The addition of another aliquot of H$_2$O$_2$ (12.5 molar excess) still did not result in complete conversion of compound III to ferric enzyme (not shown).

DISCUSSION

Compound I formation from the reaction of ferric enzyme and H$_2$O$_2$ has been well studied. The reactions of compound II and III with H$_2$O$_2$ have not been as extensively characterized. We have clearly demonstrated here the formation of compound II from compound I and H$_2$O$_2$ from stopped-flow kinetic difference spectra. The ability of the lignin peroxidase compound I to react with H$_2$O$_2$ has been previously suggested, based mainly on the change of the Soret absorption spectrum (Tien et al., 1986). In this study, the kinetic difference spec-
trum is more definitive proof for the occurrence of the reaction. The reaction of compound I and \( \text{H}_2\text{O}_2 \) also occurs with myeloperoxidase with rates comparable with those with the lignin peroxidase (Hoogland et al., 1988), whereas for horse-radish peroxidase, the reaction is very slow (Nakajima and Yamazaki, 1987).

The reaction of compound II with \( \text{H}_2\text{O}_2 \) to yield compound III has been well documented by Noble and Gibson (1970) in their work with horseradish peroxidase. This reaction was characterized spectrophotometrically where the difference spectrum was obtained in rapid kinetic experiments. We have obtained similar results here with lignin peroxidase. However, a different result was reported recently where compound III\( ^* \) was proposed to be the final reaction product of lignin peroxidase compound II with \( \text{H}_2\text{O}_2 \) (Wariishi et al., 1990). In partial agreement with our results, these workers showed that initial product of the reaction between lignin peroxidase compound II and \( \text{H}_2\text{O}_2 \) is compound III. However, they proposed a further reaction of compound III with \( \text{H}_2\text{O}_2 \), where another molecule of \( \text{H}_2\text{O}_2 \) formed an adduct with compound III, to form a complex that they referred to as compound III\( ^* \).

Compound III\( ^* \) has a relatively long and complicated history. Prior to the proposed existence of compound III\( ^* \), Wariishi and Gold (1989) proposed that compound III-reacts with veratryl alcohol resulting in its oxidation to form veratraldehyde. The compound III used in their study was prepared by the addition of excess \( \text{H}_2\text{O}_2 \) to ferric enzyme. We then demonstrated that compound III, when prepared in the absence of \( \text{H}_2\text{O}_2 \) by the addition of dioxygen to ferric peroxidase, is inert and does not oxidize veratryl alcohol (Cai and Tien, 1989). We also demonstrated that the addition of just 1 (enzyme) eq of \( \text{H}_2\text{O}_2 \) to this preparation of compound III and veratral alcohol results in the rapid conversion of compound III to ferric enzyme and the formation of veratraldehyde. Therefore, despite the presence of compound III in their preparation, some other intermediate must be responsible for the oxidation of veratryl alcohol observed by Wariishi and Gold (1989). Wariishi and Gold (1990) subsequently confirmed our findings on the inertness of compound III. At this time, these workers revised their mechanism and proposed that compound III, when prepared in the presence of excess \( \text{H}_2\text{O}_2 \), formed an adduct (compound III\( ^* \)). In light of the active site similarities between lignin peroxidase and other peroxi-
dases (Banci et al., 1991), it is difficult to envision where a second \( \text{H}_2\text{O}_2 \) molecule would bind. Nevertheless, they proposed that although veratryl alcohol is not oxidized by compound III\( ^* \), it does react with compound III\( ^* \) (but not compound III) by displacing superoxide from the active site.

\[
\text{Fe}^{3+}\text{O}_2^- + \text{RH} \rightarrow \text{Fe}^{3+} + \text{RH} + \text{O}_2
\]

Evidence for this reaction comes from the single turnover type of experiments in which cyanide was used to trap the ferric enzyme formed from this reaction. They found that the addition of veratryl alcohol to compound III\( ^* \) in the presence of cyanide resulted in rapid formation of the cyanide complex (from the ferric enzyme) (Wariishi and Gold, 1990). The addition of TNM to this reaction mixture resulted in its reduction, suggesting the formation of superoxide. However, inhibition by superoxide dismutase was not tested. The proposal that veratryl alcohol is not oxidized comes from negative evidence; no veratraldehyde could be detected in the reaction mix.

The results presented here should help clarify many of the seemingly contradictory aspects of compound III\( ^* \). First, the inability to detect veratraldehyde can be explained by the fact that cyanide is a substrate for lignin peroxidase (Shah et al., 1991). At concentrations used by Wariishi and Gold (1990), veratraldehyde would not be detected in any lignin peroxidase reaction mix. Second, in contrast to results of Wariishi and Gold (1990), we have shown that veratryl alcohol has no effect on the rate of the conversion of compound III to the cyanide complex in the presence of \( \text{H}_2\text{O}_2 \). The rate of compound III decay in the presence of \( \text{H}_2\text{O}_2 \) and cyanide is not changed by the presence of veratryl alcohol. Third, we have shown that the use of TNM for detection of superoxide with lignin peroxidase compound III is problematic. TNM increases the rate of compound III decay in a concentration-dependent manner. TNM is a small molecule and can conceivably diffuse to the active site and react directly with compound III.

\[
\text{Fe}^{3+}\text{O}_2^- + \text{C(NO)}_2^- \rightarrow \text{Fe}^{3+} + \text{O}_2 + \text{C(NO)}_2^- + \text{NO}
\]

Finally, we have also shown that spectral evidence for the existence of compound III\( ^* \) is dependent on the history of the enzyme sample. Wariishi and Gold (1990) monitored the reaction of compound II and \( \text{H}_2\text{O}_2 \) at 417 nm and observed two spectral phases, an initial phase with a decrease in absorbance followed by a slower phase with an increase in absorbance. The initial phase was attributed to the formation of compound III, whereas the slower one to the formation of compound III\( ^* \). We found that older samples of enzyme that had been stored for months yielded the compound III\( ^* \)-type spectrum. The amplitude for the initial absorbance decrease phase varied from sample to sample, and this phase was diminished completely when freshly prepared enzyme sample was used. Therefore, the spectral intermediates may be due to parallel reactions occurring with altered forms of lignin peroxidase.

Indeed, there are spectral differences between compound III prepared by the addition of excess \( \text{H}_2\text{O}_2 \) to ferric enzyme or by the addition of dioxygen to ferrous peroxidase, especially in the Soret region (Cai and Tien, 1989). Compound III prepared by the former method has an absorbance maximum in the Soret at 419 nm, whereas the latter method has a maximum at 416 nm (Cai and Tien, 1989). We previously attributed this difference to the fact that compound III, when prepared in the presence of excess \( \text{H}_2\text{O}_2 \) is not a static preparation (Cai and Tien, 1989). These preparations are rapidly turning over \( \text{H}_2\text{O}_2 \) as demonstrated by the rapid rate of enzyme inactivation (Cai and Tien, 1989) and by measurement of oxygen evolution (data not shown). Consequently, the spectral signature of such a preparation would not only reflect the compound III present but also other intermediates such as compound II, which has an absorbance maximum at 419 nm (Renganathan and Gold, 1986). In fact, spectral simulations of mixtures of compound II with III resemble compound III preparations in the presence of excess \( \text{H}_2\text{O}_2 \) (Cai and Tien, 1991).

Wariishi and co-workers also performed resonance Raman studies on compound III\( ^* \) (Muthusamy et al., 1990). Although there were no observable differences in the resonance Raman spectrum of compound III with compound III\( ^* \), these workers stated that the results supported their previous work, indicating that compound III\( ^* \) is a simple adduct of compound III with \( \text{H}_2\text{O}_2 \).

In contrast to the mechanism of compound III formation, the decomposition of compound III is relatively simple. The lignin peroxidase decays to the ferric enzyme by dissociation of superoxide. This mechanism appears to be the same as that for the autoxidation of oxymyoglobins and oxyhemoglobins (Mansouri and Winterhalter, 1973) but different from that of compound III of horseradish peroxidase (Nakajima and Yamazaki, 1987). With horseradish peroxidase, Nakajima and
Yamazaki have shown that compound III can yield compound II and \( \text{H}_2\text{O}_2 \). The decay of the lignin peroxidase compound III is promoted by small molecules such as fluoride, a ligand of the ferric enzyme, or TNM, a superoxide scavenger. Because the scavenging of superoxide by superoxide dismutase does not increase the rate of decay, the acceleration of compound III decay by TNM is consistent with the direct reaction of TNM with compound III. Fluoride most likely functions by displacing the superoxide from compound III, whereas TNM does not increase the rate of decay, the acceleration of compound III decay by TNM is consistent with the direct reaction of TNM with compound III. Fluoride most likely functions by displacing the superoxide from compound III, whereas TNM does not increase the rate of decay, the acceleration of compound III decay by TNM is consistent with the direct reaction of TNM with compound III. Fluoride most likely functions by displacing the superoxide from compound III, whereas TNM does not increase the rate of decay, the acceleration of compound III decay by TNM is consistent with the direct reaction of TNM with compound III.

\[
\text{Fe}^{3+}\text{O}_2^- + \text{H}^+ \rightarrow \text{Fe}^{2+} + \text{O}_2 + \text{H}_2\text{O}_2
\]  

(10)

Since superoxide dismutase scavenges free superoxide, the apparent decay rate is thus reduced. In fact, the reaction of superoxide and compound III has been demonstrated with hemoglobin and found to occur at sufficiently high rates (Sutton et al., 1976).

Finally, there remains the question of how compound III in the presence of veratryl alcohol rapidly decomposes to yield ferric enzyme upon the addition \( \text{H}_2\text{O}_2 \). Our results negate certain possibilities but do not allow us define the mechanism. Because the rate of this conversion is much faster than the rate of compound III autoxidation, we previously suggested that \( \text{H}_2\text{O}_2 \) reacts directly with compound III to yield either compound I or II (Reactions 5 and 6). Based on the results presented here, we can safely state that this is not true. If \( \text{H}_2\text{O}_2 \) reacted directly with compound III to yield compound I or II, cyanide, which is a substrate for lignin peroxidase (Shah et al., 1991) would act similarly to veratryl alcohol and also serve to rapidly convert compound I or II to resting enzyme. The conversion of compound III to resting enzyme is dependent upon the nature of the reducing substrate. Both veratryl alcohol and dimethoxybenzene cause a rapid conversion of compound III to resting enzyme upon addition of \( \text{H}_2\text{O}_2 \), and both are also known to be oxidized by one electron to form free radical products (Marquez et al., 1988; Kersten et al., 1985). In contrast, iodide, which is not oxidized to free radical products, does not cause the rapid decay of compound III. Iodide is known to be oxidized by two electrons to yield formally \( \text{I}^- \), which in turn, complexes with two other iodide molecules to form triiodide (Roman and Dunford, 1972). Although it would be attractive to propose that radical intermediates are involved in this conversion, more experimentation is required to define this mechanism.

In conclusion, we have shown some differences, but also similarities between compound III of lignin peroxidase and other peroxidases in reactions leading to their formation and decomposition. We have also discounted the existence of a putative complex between compound III and \( \text{H}_2\text{O}_2 \) referred to as compound III* (Wariishi and Gold, 1990). Consistent with our previous findings, the catalytic differences between lignin peroxidases and other peroxidases can be attributed to minor structural differences. These minor differences would account for kinetic and catalytic differences, but are not sufficient to cause formation of new peroxidase complexes.

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