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On the Reactions of Lignin Peroxidase Compound III (Isozyme H8)

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Compound III (oxyperoxidase) of lignin peroxidase isozyme H8 (pI=3.5) is formed by either reduction of native ferric enzyme (to ferrous) followed by the reaction with dioxygen or by the addition of excess hydrogen peroxide to resting enzyme. When prepared from the ferrous enzyme, Compound III is stable for days. When formed from excess hydrogen peroxide, the enzyme is rapidly inactivated. However, if the hydrogen peroxide is removed by gel filtration, the resulting Compound III exhibits the same stability as when prepared from ferrous enzyme. Compound III of lignin peroxidase is also relatively unreactive to reducing substrates. Addition of veratryl alcohol to Compound III does not result in any reaction. However, when only 1 equivalent of hydrogen peroxide is added to Compound III in the presence of veratryl alcohol, Compound III is converted to resting enzyme and veratraldehyde formation is detected spectroscopically. © 1989 Academic Press, Inc.

Lignin peroxidase isozymes, heme-containing glycoproteins, have been isolated from the wood-degrading fungus *Phanerochaete chrysosporium* (1,2). These enzymes catalyze the oxidation of highly recalcitrant substrates at low pH (1-4). Biophysical studies have indicated that the heme active site of lignin peroxidase is most similar to those of peroxidases (5). The amino acid sequence, deduced from nucleotide sequencing of cDNA clones (6,7), has also indicated similarities, including residues thought to be essential for peroxidase activity. The lignin peroxidase isozymes are similar to other peroxidases in their catalytic cycle, undergoing the classical intermediates Compound I and Compound II (3). In the presence of H_2O_2 and in the absence of reducing substrate, lignin peroxidases readily form Compound III which is not involved in the catalytic cycle of peroxidases (8). Compound III of peroxidases can also be directly formed from a reaction of the ferrous enzyme with dioxygen. The structure of Compound III is proposed to be the following valence isomers: $Fe^{2+}O_2 \rightleftharpoons Fe^{3+}O_2^{\cdot-}$. The spectral properties of Compound III are very similar to oxyhemeproteins (9,10) so that Compound III has also been referred to as oxyperoxidase.

Marquez *et al.* (11) previously reported that lignin peroxidase Compound III is formed in the presence of 20 to 30 fold excess H_2O_2. Wariishi and Gold (12) have reported that the formation of

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lignin peroxidase Compound III can lead to inactivation of the enzyme. Also reported was that lignin peroxidase Compound III readily reacts with veratryl alcohol (a substrate made from ligninolytic cultures of *P. chrysosporium*) to form resting enzyme and superoxide:

$$Fe^{3+}O_2^- + \text{veratryl alcohol} \rightarrow Fe^{3+} + O_2^- + \text{veratryl alcohol}$$

In contrast, the present study clearly demonstrates that the formation of lignin peroxidase Compound III does not lead to inactivation of the enzyme. In fact Compound III is very stable. It is also demonstrated that lignin peroxidase Compound III does not react with veratryl alcohol in the absence of H_2O_2.

Materials and Methods

Materials. Veratryl alcohol was purchased from Aldrich Chemical Co and further purified by reverse phase HPLC. 3-Methyl-5-deazoflavin was kindly provided by Vincent Massey of University of Michigan. Catalase (thymol free) was purchased from Sigma Chemical Co. Hydrogen peroxide was quantitated by titration with potassium permanganate (13). All other chemicals were reagent grade and used without purification.

Enzyme Purification. Lignin peroxidase isozyme H8 (pI=3.5) was isolated and purified from *P. chrysosporium* strain BKM-F-1767 as previously described (14). The 409 nm to 280 nm absorbance ratio was typically equal to 4.0. Enzyme content was measured by 409 nm absorbance using an extinction coefficient of 168/mM/cm (3).

Formation of Compound III from ferroperoxidase. Ferriperoxidase was reduced by photoactivated deazoflavin (15). The reaction mixture contained ferriperoxidase, 0.5 μM deazoflavin and 2 mM EDTA in 25 mM sodium tartrate buffer, pH 3.5. The reduction was performed at room temperature in an anaerobic cell whose main body was constructed of quartz. The cell has two sidearms, and deazoflavin was added to one of the sidearms and mixed with ferriperoxidase solution only after anaerobic conditions were established. The light source was a camcorder light which was equipped with a 50 W halogen lamp. Reduction was monitored spectrophotometrically. After reduction, oxyperoxidase was formed readily by mixing ferroperoxidase with oxygen gas, air or oxygen-containing solutions.

Formation of Compound III from reactions with excess hydrogen peroxide. Compound III was also generated by reacting ferric enzyme (9.2 nmol) with 50 fold excess hydrogen peroxide in 25 mM sodium phosphate buffer, pH 7.0 in a total volume of 55 μl. The reaction was stopped at 30 s by the addition of 14 U catalase (5 μg). The reaction mix was then passed over a Sephadex G-25 column equilibrated with 25 mM sodium tartrate, pH 3.5, to ensure total removal of hydrogen peroxide.

Reactions of Compound III. Reactions of Compound III were monitored spectrophotometrically. Addition of hydrogen peroxide or veratryl alcohol to Compound III is as described in the figure legends. Veratraldehyde formation was monitored by its 310 nm absorbance and quantitated with an extinction coefficient of 93,000/M/cm. All spectrophotometric measurements were performed with a Perkin-Elmer Lambda 4B UV/VIS spectrophotometer.

Results

Stability of lignin peroxidase Compound III. Similar to horseradish peroxidase, lignin peroxidase Compound III can be formed via the reduction of native enzyme to the ferrous enzyme, followed by addition of dioxygen, or by the reaction of ferryl peroxidase with H_2O_2. The latter method is achieved by adding excess H_2O_2 to native enzyme. Figure 1 shows the absorption spectrum of lignin peroxidase Compound III formed by such a method. The spectrum is similar (see Discussion) to those reported previously by Renganathan and Gold (8) and similar to Compound III of horseradish peroxidase (9). It exhibits absorption maxima at 416, 543 and 578 nm. Unreacted H_2O_2 was removed by treatment with catalase and then by gel filtration with Sephadex G-25. Shown in Figure 1 are three

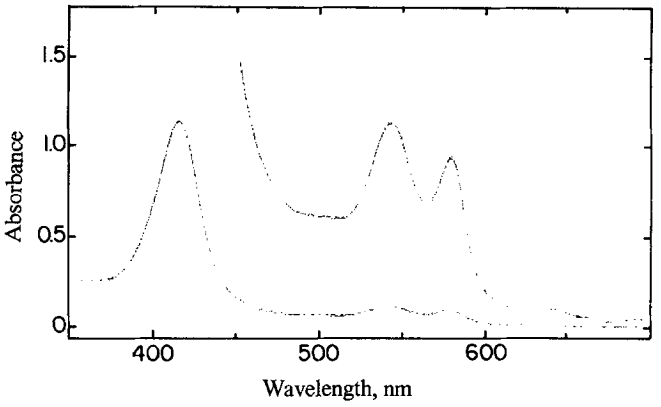


Figure 1. Stability of lignin peroxidase Compound III. Compound III was formed by addition of excess hydrogen peroxide and passed through a Sephadex G-25 column as described in Materials and Methods. Three scans are shown, the first was obtained immediately after elution from the Sephadex column. The next two were taken at 5 and 10 min. Absorbance in the visible region is multiplied by ten.

scans, the first of which was obtained immediately after the sample passed through the Sephadex column. Subsequent scans were obtained at 5 and 10 min. Little if any spectral changes are observed during this time period. The sample is actually stable for days (16).

Effect of veratryl alcohol on Compound III. Compound III does not react with veratryl alcohol since no spectral changes were observed upon addition of veratryl alcohol to Compound III (Figure 2). Figure 2 A shows the Soret absorbance of Compound III prepared by reduction of the enzyme followed by addition of dioxygen. Also shown in Figure 2 A is the Soret absorbance of the same sample 5 min and 10 min after addition of 1.5 mM veratryl alcohol. Little if any spectral changes can be detected. Figure 2 B shows a similar experiment except that Compound III was prepared by reacting with excess H_2O_2 followed by passage through Sephadex G-25. Again, no spectral changes can be observed at 3, 5 or 10 min after the addition of veratryl alcohol.

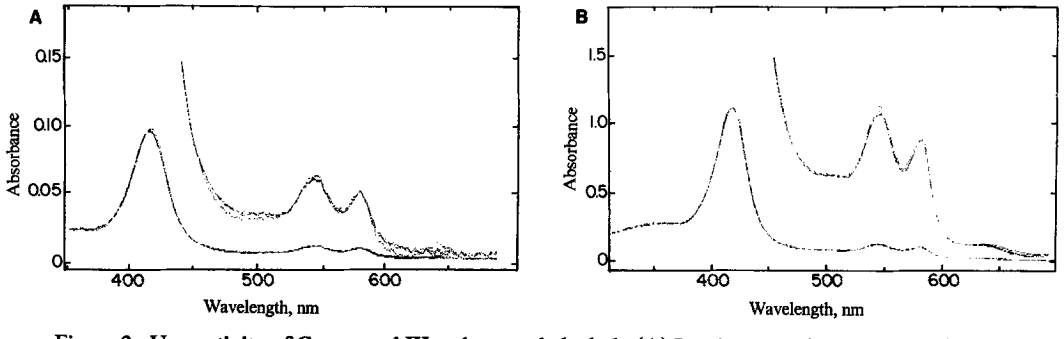
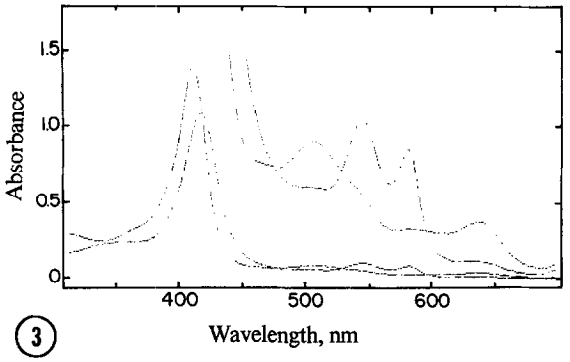


Figure 2. Unreactivity of Compound III and veratryl alcohol. (A) Lignin peroxidase Compound III was prepared by reduction of ferric enzyme to ferrous followed by addition of dioxygen as described in Materials and Methods. Three scans are shown, the first is of Compound III and the next two were obtained at 5 and 10 min after addition of veratryl alcohol (1.5 mM). Absorbance in the visible region is multiplied by six. (B) Lignin peroxidase Compound III was prepared as described in Figure 1. Four scans are shown, the first is of Compound III, the next three were obtained at 3, 5, and 10 min. after addition of veratryl alcohol (1.5 mM). Absorbance in the visible region is multiplied by ten.



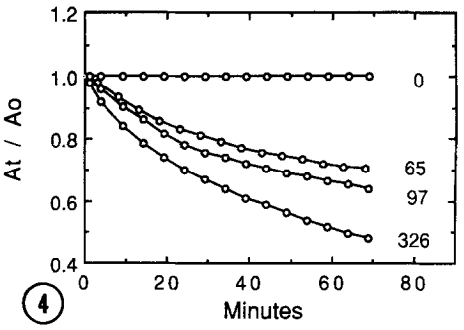


Figure 3. Conversion of Compound III to resting enzyme. Two scans are shown in the figure. One scan shows Compound III (with 416 nm, 543 nm and 578 nm maxima) in the presence of 1.5 mM veratryl alcohol. Compound III was prepared as described in Figure 1. The other scan was obtained immediately after the addition of 1 equivalent of hydrogen peroxide to Compound III and shows ferric enzyme (with 409 nm, 502 nm and 636 nm maxima). Absorbance in the visible region is multiplied by ten.

Figure 4. Time course of enzyme inactivation by excess hydrogen peroxide. Lignin peroxidase (7.3 or 8.3 μM) was incubated with hydrogen peroxide at 25°C in 25 mM sodium tartrate, pH 3.5. Enzyme inactivation is expressed as 419 nm absorbance at specfied times (At) divided by 419 nm absorbance at zero time (Ao). Number to right of lines give hydrogen peroxide concentration as equivalent of enzyme.

Reactivity of Compound III in the presence of hydrogen peroxide. Figure 3 shows the effect of H_2O_2 on lignin peroxidase Compound III in the presence of veratryl alcohol. Compound III is quickly converted back to resting enzyme with the addition of only 1 equivalent of H_2O_2. Concomitant with this conversion is the formation of veratraldehyde as demonstrated by increase in 310 nm absorbance. Addition of another equivalent of H_2O_2 resulted in a further increase in veratraldehyde formation (not shown).

Reaction of Compound III with hydrogen peroxide. Although lignin peroxidase Compound III does not react with veratryl alcohol, it undergoes further reactions with excess H_2O_2, which results in enzyme inactivation. The effect of H_2O_2 concentration on enzyme inactivation is shown in Figure 4. The enzyme inactivation by excess H_2O_2 is demonstrated by decrease in Soret absorbance, in a concentration-dependent manner. This inactivation is similar to that observed by other workers (17,18).

Discussion

In this report, we have provided compelling evidence that lignin peroxidase Compound III is exceedingly stable. Lignin peroxidase Compound III can be prepared by either the reaction of the ferrous enzyme with dioxygen or by reaction of resting enzyme with excess H_2O_2. The former preparation exhibits a half-life which is too long (days) for accurate measurement. Further characterization of Compound III is the topic of another paper (16). In contrast, the latter preparation is not very stable due to the presence of the H_2O_2. Its half-life is measured in minutes and the enzyme is ultimately denatured while removal of the H_2O_2 by gel filtration results in a preparation that is just as

stable as that prepared from the ferrous enzyme. The mechanism of inactivation by H_2O_2 is not known; however, it exhibits a rate which is dependent upon H_2O_2 concentration suggesting that inactivation results from sequential reactions with H_2O_2. Previous studies suggested that the formation of lignin peroxidase Compound III leads to inactivation of the enzyme (12). However, based on our observations, these studies were most likely observing H_2O_2-dependent inactivation of enzyme. As is shown here, Compound III is actually very stable whether it is prepared from the ferrous enzyme or from excess H_2O_2 followed by its removal.

It has been proposed that veratryl alcohol displaced superoxide from Compound III without veratraldehyde formation (12). This type of reaction with Compound III is not unprecedented. Gross *et al.* (19) demonstrated conversion of horseradish peroxidase Compound III to resting enzyme by phenolic substrates. In great contrast, the present study clearly shows that lignin peroxidase Compound III, prepared from the ferrous enzyme or from excess H_2O_2 followed by the removal of excess H_2O_2 through a Sephadex G-25 column, does not react with veratryl alcohol (Figure 2). It is also shown that if trace H_2O_2 is added to Compound III, even at an amount as low as 1 equivalent to enzyme, Compound III is converted back to resting enzyme in the presence of veratryl alcohol. In previous study which implicatied the reaction between veratryl alcohol and lignin peroxidase Compound III prepared with excess H_2O_2 at pH 3.0, the excess H_2O_2 was removed by the addition of catalase. It is highly unlikely that catalase, with a pH optimum of 7 (20), can efficiently scavenge all of the H_2O_2 at pH 3.0. Thus the reaction they observed was most likely dependent upon contaminating H_2O_2.

Furthermore, formation of superoxide in this previous study was demonstrated by reduction of tetranitromethane to form the nitroform anion. However, close examination of the stoichiometry indicates that the amount of superoxide formed in their reaction mixture [approximately 14 μM, based on absorbance change of 0.2 at 350 nm and an extinction coefficient of $1.48 \times 10^4 M^{-1}cm^{-1}$ in dimethylformamide for the nitroform anion (21)] is twice as much as enzyme (5.5 μM) present in the reaction mixture. This result is inconsistent with their mechanism. Moreover, the reduction of tetranitromethane is complete within 15 s whereas Compound III is not completely converted to resting enzyme until 30 s. To unequivocally demonstrate superoxide involvement, sensitivity to superoxide dismutase should be demonstrated.

There remains the question of how 1 equivalent of H_2O_2 can convert Compound III to resting enzyme in the presence of veratryl alcohol with veratraldehyde formation. Nakajima and Yamazaki (22) demonstrated that Compound III of horseradish peroxidase, formed by addition of excess H_2O_2 to resting enzyme, is actually an equilibrium mixture of Compound III and Compound II. Thus, when prepared from excess H_2O_2, Compound III exists in a dynamic steady state. Based on spectral data, a similar equilibrium probably exists for lignin peroxidase. Compound II has a Soret absorbance maximum near 420 nm (8). The Soret maximum for lignin peroxidase Compound III, prepared from the reaction of the ferrous enzyme with dioxygen or from excess H_2O_2 followed by its removal by gel filtration, is approximately 416 nm. However, the maximum is 419 nm if the H_2O_2 is not removed (8), which suggests that in the presence of H_2O_2 Compound III and Compound II are both present. Therefore, we propose that Compound II is readily formed from Compound III upon addition of H_2O_2 and it is Compound II that oxidizes veratryl alcohol. The mechanistic details of these reactions is a topic of on-going research in our laboratory.

Acknowledgments

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