Characterization of the Oxycomplex of Lignin Peroxidases from *Phanerochaete chrysosporium*: Equilibrium and Kinetics Studies†

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ABSTRACT: The oxycomplexes (compound III, oxyperoxidase) of two lignin peroxidase isozymes, H1 (p*I* = 4.7) and H8 (p*I* = 3.5), were characterized in the present study. After generation of the ferroperoxidase by photochemical reduction with deazoflavin in the presence of EDTA, the oxycomplex is formed by mixing ferroperoxidase with O_2. The oxycomplex of isozyme H8 is very stable, with an autoxidation rate at 25 °C too slow to measure at pH 3.5 or 7.0. In contrast, the oxycomplex of isozyme H1 has a half-life of 52 min at pH 4.5 and 29 min at pH 7.5 at 25 °C. The decay of isozyme H1 oxycomplex follows a single exponential. The half-lives of lignin peroxidase oxycomplexes are much longer than those observed with other peroxidases. The binding of O_2 to ferroperoxidase to form the oxycomplex was studied by stopped-flow methods. At 20 °C, the second-order rate constants for O_2 binding are 2.3×10^5 and 8.9×10^5 M^{-1} s^{-1} for isozyme H1 and 6.2×10^4 and 3.5×10^5 M^{-1} s^{-1} for isozyme H8 at pH 3.6 and pH 6.8, respectively. The dissociation rate contants for the oxycomplex of isozyme H1 (3.8×10^{-3} s^{-1}) and isozyme H8 (1.0×10^{-3} s^{-1}) were measured at pH 3.6 by CO trapping. Thus, the equilibrium constants (K, calculated from k_{on}/k_{off}) for both isozymes H1 (7.0×10^7 M^{-1}) and H8 (6.2×10^7 M^{-1}) are higher than that of myoglobin (1.9×10^6 M^{-1}). O_2 binding to ferroperoxidase of isozyme H1 was found to increase upon ionization of a group with $pK_a = 6.63$ and $\Delta H_{ion} = 7.2$ kcal/mol, indicative of an imidazole group of a His residue. The characteristics of the oxycomplex of lignin peroxidases suggest that the electronic structure and geometric configuration in their heme environment are different from that in myoglobin or horseradish peroxidase.

The present paper describes the characterization of the oxycomplexes (compound III) of two lignin peroxidase isozymes. The secretion of lignin peroxidase isozymes, which are glycosylated heme proteins, by *Phanerochaete chrysosporium* imparts this wood-degrading fungus the ability to degrade lignin and lignin model compounds (Tien & Kirk, 1984; Glenn et al., 1983). These peroxidases catalyze the one-electron oxidation of aromatic rings found in lignin-like substrates to form aryl cation radicals (Kersten et al., 1985; Hammel et al., 1986; Schoemaker et al., 1985).

The catalytic mechanism of lignin peroxidases has been extensively studied. Resonance Raman spectroscopic studies indicated that the active sites of these isozymes are most similar to other peroxidases (Kuila et al., 1985). The catalytic cycle of peroxidases involves the two-electron oxidation of the heme by H_2O_2, forming compound I (reaction 1); a second intermediate, compound II, is then formed when compound I oxidizes the substrate by one electron (reaction 2). After oxidizing another molecule of the substrate by one electron, compound II returns to the resting state (reaction 3)

$$\text{resting enzyme} + H_2O_2 \rightarrow \text{compound I} + H_2O \quad (1)$$

$$\text{compound I} + AH \rightarrow \text{compound II} + A^{\bullet} \quad (2)$$

$$\text{compound II} + AH \rightarrow \text{resting enzyme} + A^{\bullet} \quad (3)$$

where AH stands for the substrate and $A^{\bullet}$ the cation radical. Compounds I and II have been detected in the catalytic cycle of lignin peroxidases (Tien et al., 1986; Renganathan & Gold, 1986). Furthermore, nucleotide sequencing of cDNA clones encoding isozyme H2 (de Boer et al., 1987), H8 (Tien & Tu, 1987), and other isozymes (Andrawis et al., 1989; Schalch et al., 1989) reveals the presence of the proximal and distal His residues which are highly conserved in the active site of peroxidases.

Despite a number of physical and kinetic similarities between lignin peroxidases and other peroxidases, differences have been observed. In contrast to other peroxidases, pH has no effect on the rate of lignin peroxidase compound I formation (Andrawis et al., 1988; Harvey et al., 1989). Lignin peroxidases are also unique in their ability to oxidize substrates that cannot be oxidized by other peroxidases (Higuchi, 1985; Kamaya & Higuchi, 1984; Hammel et al., 1986). Consistent with this observation, our recent electrochemical study revealed that the redox potentials (of Fe^{2+}/Fe^{3+} couple) of lignin peroxidases are approximately 130 mV higher than that of horseradish peroxidase (Millis et al., 1989), suggesting a heme environment which is more electron deficient in lignin peroxidases.

To further characterize the active site of lignin peroxidases, we have studied O_2 binding to ferrous peroxidase of isozymes H1 and H8. Ligand binding studies have been useful in characterization of heme protein active sites. The binding of O_2 to ferrous peroxidase forms the oxycomplex (Wittenberg et al., 1967), which is equivalent to compound III, formed by the addition of excess H_2O_2 to resting enzyme (Keilin & Mann, 1937). O_2 binding is affected by the electronic density of the heme iron, the extent of the proximal His H-bonding, and the steric hindrance in both the proximal and distal sides in the heme environment (Chang & Traylor, 1975; Traylor & Traylor, 1982). Therefore, studies on O_2 binding can provide structural information on the heme active site, though the oxycomplex is not part of the peroxidase catalytic cycle. Peroxidases do not typically form stable oxycomplexes; for instance, the oxycomplex of horseradish peroxidase autode-

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composes to form ferriperoxidase with a half-life of 4 min at 20 °C (Wittenberg et al., 1967). As demonstrated in this paper, however, lignin peroxidase isozyme H8 exhibits a half-life too long to accurately measure, whereas isozyme H1 has a half-life of 52 min at 25 °C. We also demonstrate that the oxycomplex of the lignin peroxidases binds O_2 reversibly, in a manner similar to that of myoglobin (Antonini & Brunori, 1971) and chloroperoxidase (Sono et al., 1985). The kinetics of the oxycomplex formation and decay, in addition to the ionizable group which affects these processes, have also been determined as part of this work.

Materials and Methods

Chemicals and Enzyme Purification. 3-Methyl-5-deazoflavin was kindly provided by Dr. Vincent Massey of The University of Michigan. Sodium dithionite was purchased from Sigma Chemical Co. Lignin peroxidase isozyme H8 (p*I* = 3.5) was isolated and purified to homogeneity from *P. chrysosporium* strain BKM-F-1767 as previously described (Kirk et al., 1985). Isozyme H1 (p*I* = 4.7) was isolated and purified by the same method from an overproducing strain (Myer and Tien, unpublished data). The RZ values (A_{409}/A_{280}) were typically equal to or greater than 4. Concentrations of isozymes H1 and H8 were determined by using $\epsilon_{409} = 168$ and 169 mM^{-1} cm^{-1}, respectively (Tien & Kirk, 1984; Farrell et al., 1989).

Formation of Ferroperoxidase and Oxycomplex. Ferrous lignin peroxidase was generated by illuminating an anaerobic ferriperoxidase solution that contained 0.5 μM deazoflavin and 2 mM EDTA in a buffer as noted in the figure legends (Massey & Hemmerich, 1977). Illumination was provided by a camcorder light equipped with a 50-W halogen lamp. The reduction was carried out at room temperature in an anaerobic cell whose main body was constructed of quartz and which contained two side arms. Deazoflavin was added to one of the sidearms and mixed with ferriperoxidase solution only after the anaerobic conditions were established. Anaerobiosis was achieved as previously described (Millis et al., 1989). The oxycomplex is formed readily after ferroperoxidase solution is mixed with O_2 gas, air, or O_2-containing solution.

Stopped-Flow Measurements of the Oxycomplex Formation Rate Constants. The stopped-flow apparatus used was described previously by Johnson (1986). For O_2 binding experiments, the syringes were soaked overnight in 10 mM sodium phosphate, pH 7, containing 10 mM sodium dithionite. The ferroperoxidase was transferred directly from the cuvette to one of the syringes to prevent contact with air. The other syringe was filled with O_2-containing water. The formation of the oxycomplex was followed by the disappearance of ferroperoxidase, indicated as the absorbance decrease at 437 nm. The second-order rate constants were determined under pseudo-first-order kinetic conditions by using O_2 concentrations that were at least 6 times that of the enzyme. Each kinetic trace consisted of 250 datum points, and each datum point was typically the average of three traces. Different O_2 concentrations were made by diluting air-saturated water (2.83×10^{-4} M O_2 at 20 °C) or O_2-saturated water (1.38×10^{-3} M O_2 at 20 °C) with deoxygenated water. Buffers used were sodium tartrate (pH 3.5–5.5) and sodium phosphate (pH 5.5–8.0). All buffers were adjusted to the same ionic strength ($\mu = 0.03$), and the pH of each buffer was corrected to that of the final reaction mixture.

Measurements of the Oxycomplex Dissociation Rate Constants. After the oxycomplex was formed, the solution was evacuated and purged with CO gas. The dissociation of O_2 from the oxycomplex was followed by formation of CO–ferroperoxidase. The first-order dissociation rate constants were calculated from the increase in absorbance at 426 nm (i.e., formation of CO–ferroperoxidase complex) and/or the decrease in absorbance at 417 nm (i.e., disappearance of the oxycomplex). All determinations were made in buffers as described above.

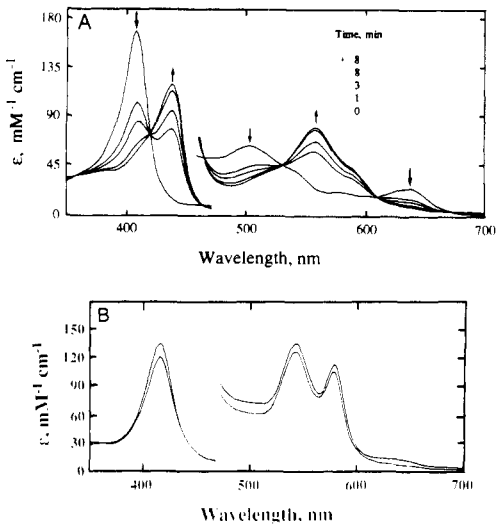


FIGURE 1: (A) Photoreduction of lignin peroxidase isozyme H1 by 5-deazoflavin. The sample contains 3.5 μM isozyme H1 and 2 mM EDTA in sodium phosphate ($\mu = 0.03$), pH 7.3. Ferric lignin peroxidase was reduced to the ferrous form via photoreduction. The sample was scanned after specified times of illumination as designated in figure. Arrows give the direction of the absorbance change during reduction. The scale for the visible region is 6-fold that for the Soret. Experimental details are as described under Materials and Methods. (B) Spectrum of isozyme H8 oxycomplex. The oxycomplex was formed either by mixing ferrous lignin peroxidase with air (lower spectrum) in sodium phosphate ($\mu = 0.03$), pH 6.8, or by reacting ferric lignin peroxidase with a 60-fold excess of H_2O_2, followed by passage of the enzyme through a Sephadex G-25 column (upper spectrum) in the same buffer. The absorbance in the visible region is expanded 10-fold.

Decay of the Oxycomplex. The decay of oxycomplex isozyme H1 to its ferric form was monitored spectrophotometrically by the decrease in absorbance at 545 or 417 nm. The decay fits a single exponential. Buffers used to determine its pH dependency were sodium tartrate (pH 3.5–5.5) or sodium phosphate (pH 5.5–7.5) with constant ionic strength ($\mu = 0.06$).

Miscellaneous Methods. All spectrophotometric measurements were performed by using a Perkin-Elmer Lambda 4B UV/vis spectrophotometer. Veratryl alcohol oxidizing activity was measured at pH 3.5; other conditions are as previously described (Tien & Kirk, 1988). All of the data were analyzed by an iterative, nonlinear least-squares fit program using a modification of the method of moments (Dyson & Isenberg, 1971; Johnson, 1986).

Results

Spectrum of the Oxycomplex. Ferroperoxidase was generated under anaerobic condition by photoreduction of ferriperoxidase in a solution containing 5-deazoflavin as a catalyst and EDTA as a photoreductant (Massey & Hemmerich, 1977). The reduction of ferriperoxidase to ferroperoxidase produced spectra having well-defined isosbestic points at 354, 419, 464, 531, 608, and 695 nm (Figure 1A), similar to the spectra observed during electrochemical reduction (Millis et al., 1989). The ferroperoxidases of both isozymes H1 and H8 exhibit λ_{max} values at 437 nm in Soret and at 558 nm in the visible region.

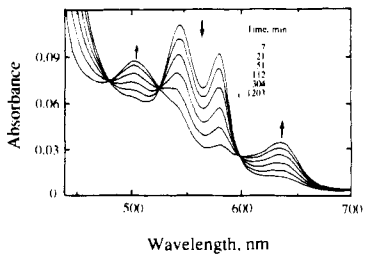


FIGURE 2: Autoxidation of isozyme H1 oxycomplex. Oxycomplex (8 μM) was formed in sodium tartrate (μ = 0.06), pH 3.6. The spectra in the visible region were taken after incubation at 25 °C for 7 min, 21 min, 51 min, 1 h 52 min, 5 h 4 min, and 17 h 3 min. Arrows indicate the direction of the absorbance change during the incubation.

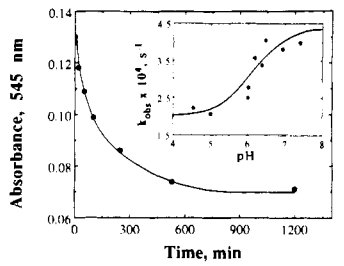


FIGURE 3: Absorbance change at 545 nm during autoxidation of oxycomplex. Conditions are the same as described in Figure 2. The inset shows the effect of pH on the rate of autoxidation. The solid line is drawn from computer fitting and is described by a pK_a = 6.11.

The oxycomplex is readily formed by mixing ferroperoxidase with O_2 gas, air, or an O_2-containing buffer (Figure 1B). The oxycomplex exhibits a spectrum with characteristic peaks at 355.7, 417, 545, and 580 nm for isozyme H1 and at 356, 417, 543, and 578 nm for isozyme H8. This spectrum is identical with that of compound III formed from ferriperoxidase in the presence of excess H_2O_2, followed by passage through a Sephadex G-25 column to remove H_2O_2 (Figure 1B; Cai & Tien, 1989). These absorption spectra are also similar to the spectrum obtained earlier (Renganathan et al., 1985).

Autoxidation of the Oxycomplex. The oxycomplex of isozyme H1 is more stable than that of horseradish peroxidase. Its decomposition results in formation of ferric enzyme. Spectral characterization of this autoxidation reveals well-defined isosbestic points, indicating a lack of long-life intermediates in this conversion process (Figure 2). This autoxidation reaction fits a single exponential (Figure 3). The rate of autoxidation increases as the pH increases from 3.5 to 7.0; the pH effect is opposite to that observed with horseradish peroxidase (Tamura & Yamazaki, 1972). The oxycomplex of isozyme H1 has a half-life ranging from 52 min at pH 4.5 to 29 min at pH 7.5. This pH dependency shows that an ionizable group having a pK_a = 6.11 at 25 °C controls the rate of decay (Figure 3, inset).

The oxycomplex of isozyme H8 is more stable than the isozyme H1 oxycomplex. Very little spectral change in the visible region of the oxycomplex was detected after overnight incubation at pH 3.5, 25 °C (data not shown). The rate of autoxidation is too low to accurately measure. The unusual stability of the isozyme H8 complex was previously observed when the oxycomplex was formed from the ferric enzyme and H_2O_2 and freed of excess H_2O_2 by passage through a Sephadex G-25 column (Cai & Tien, 1989). Similar to results observed with isozyme H1, increasing pH also increases the rate of isozyme H8 oxycomplex autoxidation, independent of how the oxycomplex is formed. The oxycomplex formed from ferric enzyme and excess H_2O_2, followed by purification through a Sephadex G-25 column, exhibited the same pH dependency.

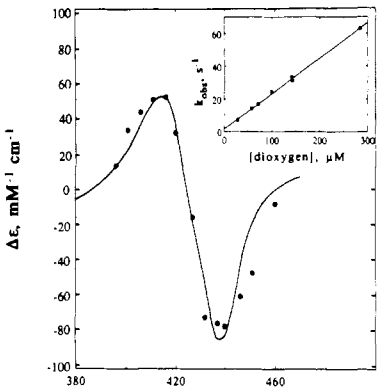


FIGURE 4: Kinetic difference spectrum of the reaction of ferroperoxidase with O_2. Ferroperoxidase of isozyme H1 (3.5 μM) in sodium tartrate (μ = 0.03), pH 3.6, was mixed with air-saturated water in a stopped-flow apparatus at 20 °C. Each point represents the amplitude at the specified wavelength. The smooth line is the difference spectrum of oxycomplex minus ferroperoxidase of isozyme H1. The inset shows a linear relationship between the observed rate constant and O_2 concentrations.

However, the autoxidation rate is still too slow to measure accurately.

Veratryl Alcohol Oxidation by the Oxycomplex. The ability of lignin peroxidase oxycomplexes to retain its activity during the course of its autoxidation was determined by measuring their veratryl alcohol oxidizing activity. Oxycomplexes of isozymes H1 and H8 were formed and sampled at different time intervals for their veratryl alcohol oxidizing activity. The results indicated very little (<7%) loss in activity for the isozyme H1 oxycomplex after a 40-min incubation at 25 °C, during which period the decay of the isozyme H1 oxycomplex undergoes approximately 1 half-life (data not shown). Even less activity loss was observed with isozyme H8. Because isozyme H8 forms a much more stable oxycomplex than isozyme H1, the low level of inactivation observed with isozyme H1 could be due to the autoxidation process.

These results substantiate our earlier finding that the formation of the oxycomplex does not lead to enzyme inactivation (Cai & Tien, 1989). This is in contrast to a previous paper by Wariishi and Gold (1989) on the same isozyme. Furthermore, the oxidation of veratryl alcohol by the oxycomplexes requires H_2O_2 as it does for resting enzymes (Cai & Tien, 1989). It is also interesting to note that no lag is observed in the oxidation of veratryl alcohol by the oxycomplex, suggesting that veratryl alcohol oxidation is initiated by the direct reaction of H_2O_2 with the oxycomplex. A lag would be expected if H_2O_2 reacted with free ferrous or ferric enzyme formed from the first-order decay of the oxycomplex.

Kinetics of the Oxycomplex Formation. The rate constant for the reaction of O_2 with the ferroperoxidase forms of isozymes H1 and H8 to form the oxycomplex was determined by following the decrease in absorbance at 437 nm with a stopped-flow apparatus. To demonstrate that the oxycomplex is the initial product from the reaction of ferroperoxidase with O_2, a kinetic difference spectrum was obtained with the stopped-flow apparatus in the Soret region of isozyme H1 by plotting the change in absorbance amplitudes as a function of wavelength (Figure 4). This difference spectrum agrees with the difference spectrum obtained by subtracting the ferroperoxidase spectrum from that of the oxycomplex. Similar results were obtained with isozyme H8 (data not shown).

The inset of Figure 4 shows a linear plot of the rates of the oxycomplex formation as a function of O_2 concentrations.

Table I. Kinetics Parameters of O_2 Binding to Ferroperoxidases and Myoglobin at 20 °C

heme protein	pH	k_1 (M^{-1} s^{-1})	k_{-1} (s^{-1})	K (M^{-1})	ref
lignin peroxidase					
isozyme H1	3.6	2.3×10^5	3.3×10^{-3}	7.0×10^7	this work
	6.8	8.9×10^5	*a*	*a*	this work
isozyme H8	3.6	6.2×10^4	1.0×10^{-3}	6.2×10^7	this work
	6.8	3.5×10^5	8.6×10^{-4}	4.1×10^8	this work
horseradish peroxidase	7.4	5.8×10^4	*b*	*b*	Wittenberg et al. (1967) Nakajima and Yamazaki (1987)
myoglobin					
sperm whale	7.0	1.9×10^7	10	1.9×10^6	Antonini and Brunori (1971)
Aplysia	7.0	1.5×10^7	70	2.2×10^5	Wittenberg et al. (1965)

[a] Not determined. [b] Cannot be determined.

Summarized in Table I are the rate of constants for the reaction of ferroperoxidase with O_2 for isozymes H1 and H8 at pH 3.6 and pH 6.8 along with those for horseradish peroxidase and myoglobin. The rate constant for lignin peroxidases is faster than that for horseradish peroxidase, but slower than that for myoglobin. The rate measurements for the oxycomplex formation are independent of the choice of wavelength and the source of oxygenated water (either from dilution of air-saturated or O_2-saturated water). The formation rate is also not significantly affected by the ionic strength of the buffer (data not shown). However, it is altered when the pH is changed (Table I).

Effect of pH on the Oxycomplex Formation of Isozyme H1. As shown in Figure 5 both the protonated and unprotonated forms of ferroperoxidase of isozyme H1 react with O_2 to form the oxycomplex. The unprotonated form reacts at a rate approximately 5 times faster than the protonated form at 20 °C. To account for this, the scheme

$$\begin{array}{ccc} Pr \cdot Fe^{2+} + O_2 & \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} & Pr \cdot Fe^{2+} \cdot O_2 \\ \Big\| K_a & & \Big\| K_a' \\ HPr \cdot Fe^{2+} + O_2 & \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} & HPr \cdot Fe^{2+} \cdot O_2 \end{array} \quad (4)\ (5)$$

is proposed, where Pr stands for the protein moiety. Whether K_a is equal to K_a' is presently not determined. Accordingly, the overall formation rate constant (k_{obs}) can be given as

$$k_{obs} = \frac{k_1}{1 + [H^+]/K_a} + \frac{k_2}{1 + K_a/[H^+]} \quad (6)$$

A least-squares fit of the data with eq 6 yields the following values: $k_1 = 1.3 \times 10^6$ M^{-1} s^{-1}, $k_2 = 2.1 \times 10^5$ M^{-1} s^{-1}, and $pK_a = 6.63$ at 20 °C. A pK_a value of 6.63 is within the range expected for an imidazole side chain of a His residue.

To further identify this ionizable group, the pK_a was determined at 8, 20, (see above) and 34 °C (Figure 5). At 8 °C, $k_1 = 7.2 \times 10^5$ M^{-1} s^{-1}, $k_2 = 1.0 \times 10^5$ M^{-1} s^{-1}, $pK_a = 6.86$; at 34 °C, $k_1 = 4.1 \times 10^6$ M^{-1} s^{-1}, $k_2 = 8.5 \times 10^5$ M^{-1} s^{-1}, and $pK_a = 6.38$. By use of the formulas given below, ΔH_{ion} and the activation energies for both unprotonated and protonated oxycomplex formation (designated Ea_1 and Ea_2, respectively) can be calculated

$$-pK_a = \frac{-\Delta H_{ion}}{2.303RT} + \frac{\Delta S}{2.303R} \quad (7)$$

$$\log k_1 = \frac{-Ea_1}{2.303RT} + \log A_1 \quad (8)$$

$$\log k_2 = \frac{-Ea_2}{2.303RT} + \log A_2 \quad (9)$$

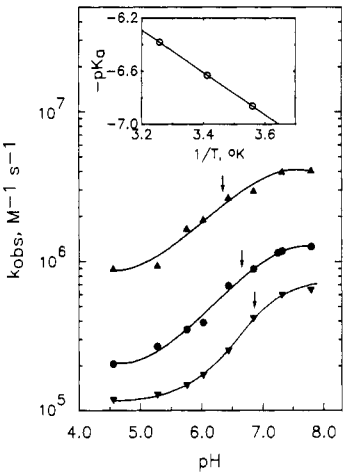


FIGURE 5: Effect of pH on the association rate of O_2 binding to ferrous isozyme H1. The association rate for each pH was determined at three different temperatures: 8 (solid inverse triangle), 20 (solid circle), and 34 °C (solid triangle). A semilogarithmic plot is constructed for each temperature. The smooth lines are theoretical, fitted by a least-squares program to eq 6. A pK_a of 6.86, 6.63, and 6.38 was calculated for 8, 20, and 34 °C, respectively. The inset shows the effect of temperature on the pK_a for ionization. The $\Delta H_{ion} = 7.2$ kcal/mol is calculated from the slope, which is equal to $\Delta H_{ion}/(2.303R)$.

where R is the gas constant and T is the absolute temperature. Thus, $\Delta H_{ion} = 7.2$ kcal/mol (Figure 5, inset), $Ea_1 = 11.3$ kcal/mol, and $Ea_2 = 13.8$ kcal/mol. A ΔH_{ion} of 7.2 kcal/mol confirms the prediction from the pK_a value that this ionizable group is the imidazole group of a His residue.

Oxycomplex Dissociation. The reversibility of the O_2 binding to ferroperoxidase of isozymes H1 and H8 was tested by CO trapping under O_2-free conditions. If O_2 dissociates from the oxycomplex to form free ferroperoxidase in an atmosphere composed solely of CO gas, the CO–ferroperoxidase complex will be formed (reactions 4 and 10): When k_1 [O_2]

$$\begin{array}{ccc} Pr \cdot Fe^{2+} \cdot O_2 & & \\ k_1(k_{on}) \Big\| \; O_2 \quad k_{-1}(k_{off}) \Big\| \; O_2 & & \\ Pr \cdot Fe^{2+} & \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} & Pr \cdot Fe^{2+} \cdot CO \\ & CO \quad CO & \end{array} \quad (10)$$

$\ll k_3$ [CO] $\gg k_{-1}$ (which can be satisfied by creating a CO-saturated and O_2-free atmosphere) and the autoxidation rate constant $\ll k_{-1}$ in the case of isozyme H1, k_{obs} for this reaction is equal to the dissociation rate constant k_{-1} for O_2.

To achieve such conditions, solutions of the oxycomplex were evacuated and purged with CO gas. The conversion of the oxycomplex to the CO–ferroperoxidase complex was followed

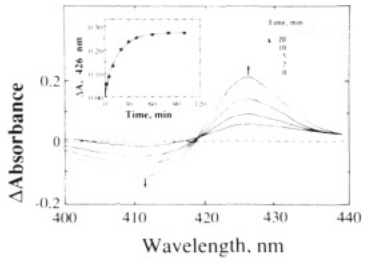


FIGURE 6: Difference spectrum of the conversion from oxycomplex to CO–ferroperoxidase complex. Oxycomplex of isozyme H8 (5.5 μM) was prepared in sodium tartrate (μ = 0.03), pH 3.6, containing 2 mM EDTA. Half of the solution was evacuated and purged with CO gas; the other half was used as the reference. Spectra are taken 2, 5, 10, and 20 min after the oxycomplex solution was mixed with CO. The inset shows the time course of the absorbance change at 426 nm. A first-order rate contant of 1×10^{-3} s^{-1} can be calculated from this single-exponential conversion.

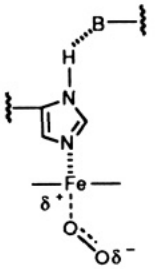
spectrophotometrically by repetitive scanning. A difference spectrum for isozyme H8, shown in Figure 6, is obtained from repetitive scanning using a reference cuvette containing the oxycomplex and an anerobic sample cuvette containing the oxycomplex purged with CO gas. A decrease in absorbance at 417 nm and an increase in absorbance at 426 nm were observed coincidentally, corresponding to the dissociation of the oxycomplex and the formation of the CO–ferroperoxidase complex, respectively. Consequently, an isosbestic point at 420 nm was obtained during this conversion. Similar results were observed for isozyme H1 (data not shown). k_{-1} (k_{off}) was estimated from the increase in absorbance at 426 nm (Figure 6, inset) or the decrease at 417 nm; a k_{-1} value of 3.8 $\times 10^{-3}$ s^{-1} was calculated for isozyme H1 and 1.0×10^{-3} s^{-1} for the isozyme H8 at pH 3.6 (Table I). Under the same conditions, the rate constant for the autoxidation of the isozyme H1 oxycomplex is 2.3×10^{-4} s^{-1}, more than 10 times slower than its dissociation. Thus, the k_{-1} values measured here are valid for both isozymes. The dissociation rate of the isozyme H8 oxycomplex was also determined at pH 6.8. The results indicate no apparent pH effect on the dissociation rate (Table I).

After the k_1 (k_{on}) and k_{-1} (k_{off}) for O_2 binding were determined, the equilibrium constant K for the oxycomplex of both isozymes H1 and H8 was calculated (Table I). These equilibrium constant values indicate that the ferrous forms of lignin peroxidase have higher affinities toward O_2 than myoglobin.

Discussion

The ligand (O_2) binding studies, described in the present paper, demonstrate that the oxycomplex of isozyme H8 is very stable, whereas the H1 oxycomplex exhibits a half-life of approximately 52 min at pH 4.5. No intermediates are detected spectrophotometrically during the conversion of the lignin peroxidase isozyme H1 oxycomplex to its ferric enzyme. In the autoxidation of the horseradish peroxidase oxycomplex, the initial reaction, dissociation of the oxycomplex to H_2O_2 and compound II, is rate limiting (Tamura & Yamazaki, 1972). The autoxidation of the isozyme H1 oxycomplex follows a single exponential, indicative of a single rate-limiting step, and is dependent on an ionizable group with a pK_a of 6.11. The rate of the lignin peroxidase oxycomplex autoxidation increases with increasing pH, a property not observed for the horseradish peroxidase (Tamura & Yamazaki, 1972) or hemoglobin oxycomplex (Mansouri & Winterhalter, 1973). In contrast, the autoxidation rate for these two heme proteins decreases with increasing pH and no pK_a values were reported. This suggests a different mechanism or involvement of different active-site groups for the autoxidation of the lignin peroxidase oxycomplex. We speculate that the initial product of the lignin peroxidase oxycomplex autoxidation may be ferric enzyme and superoxide, a hypothesis presently being investigated in our laboratory.

Numerous factors have been identified which affect O_2 binding to heme proteins and heme model compounds. Particular attention has been given to the role of the axial His in O_2 binding. The observation that the axial imidazole ligand of heme proteins is H-bonded to a proximal base (B as shown in the diagram) has led to the hypothesis that the extent of



H-bonding increases the basicity of the axial His. The basicity has a major effect on the electron density of the heme (Quinn et al., 1984). Studies with chelated iron porphyrin complexes show that increased basicity of the axial ligand (i) stabilizes the higher oxidation states of the heme iron (Doeff et al., 1983; Quinn et al., 1984); (ii) increases O_2 affinity, presumably by decreasing the dissociation rate of O_2 (Traylor et al., 1981b; Suslick et al., 1984); (iii) polarizes the covalent Fe(II)–O_2 bond to cause the transfer of electron density from the iron to the oxygen imparting the oxycomplex with greater Fe^{3+}·O_2^- character (Traylor & Popovitz-Biro, 1988), which can eventually promote the oxidation of the heme iron by O_2 to form higher valence intermediates (Mincey & Traylor, 1979).

Studies with heme proteins support the conclusions from iron porphyrin complexes. In myoglobin, the N_δ proton forms a weak H-bond with the carbonyl of Leu89 (Takano, 1977); it forms a stronger H-bond in cytochrome c peroxidase by bonding to Gln239 which, in turn, is H-bonded to Glu187 (Poulos & Kraut, 1980). Structural details are not available for horseradish peroxidase; however, ^{1}H NMR studies indicate that the H-bonding of the N_δ proton is even stronger in horseradish peroxidase (La Mar & de Ropp, 1982). In accord with the H-bonding strength, the redox potentials of metmyoglobin, cytochrome c peroxidase, and horseradish peroxidase are +46, −194, and −278 mV at pH 7, respectively, and myoglobin forms a more stable oxycomplex than horseradish peroxidase (Wittenberg et al., 1967). These results support the fact that increased H-bonding increases the propensity of the oxycomplex to autoxidize. This is also consistent with the studies on reconstituted horseradish peroxidases showing that a decrease in the electron density of the iron atom decreases the autoxidation rate of the oxycomplex (Makino et al., 1976). Results described in the present paper show that lignin peroxidases form more stable oxycomplexes than horseradish peroxidase, suggesting that the electron density of the heme iron is decreased in lignin peroxidases as compared with horseradish peroxidase and is increased relative to metmyoglobin. This is in close agreement with conclusions from our recent electrochemical studies which suggested that the H-bonding of the N_δ proton in lignin peroxidase is intermediate between horseradish peroxidase and myoglobin (Millis et al., 1989).

The His in the distal pocket of heme proteins has also been shown to affect O_2 binding. Analogous results are obtained

here with lignin peroxidases. A pH dependency for the association rate (k_{on}) in O_2 binding to ferrous isozyme H1 is shown in our stopped-flow measurements (Figure 5). Both protonated and unprotonated forms of the ferrous enzyme react with O_2 to form the oxycomplex, with the unprotonated form reacting about 5-fold greater than the protonated. An ionizable group with a $pK_a = 6.63$ at 20 °C was found to influence O_2 binding and the ΔH_{ion} for this ionization was determined to be 7.2 kcal/mol. Typically, a carboxylic acid group has a pK_a in the range between 1 and 6 and its ΔH_{ion} values in the range of ±1.5 kcal/mol (Christensen et al., 1976), the pK_a of an imidazole group is 5.6–7.0 and its ΔH_{ion} is 6.9–7.5 kcal/mol (Cohn & Edsall, 1943), and for a thiolate group the pK_a is the range 7.5–10.3 and the ΔH_{ion} values are between 6 and 7 kcal/mol (Cecil, 1963). Taking pK_a and ΔH_{ion} values into consideration, this ionizable group in the ferrous form of isozyme H1 can be assigned to the imidazole of a His residue in the distal pocket. O_2 binding studies using iron porphyrin model compounds indicated that the distal steric hindrance affects mainly the association rate (Ward et al., 1981; Traylor et al., 1981a). It was also demonstrated that the association rate for O_2 (or CO) binding to mutant myoglobin and hemoglobin is increased by about 5–10-fold if the distal His is replaced by Gly (Olsen et al., 1988). Thus, replacement of distal His by Gly possibly removes the steric effect imposed by the distal His. Therefore, the removal of a proton from the distal His may account for the faster association rate of O_2 binding to ferrous isozyme H1 by simply decreasing the steric effect. Such steric effect of distal His has been suggested in CO binding to the ferrous chloroperoxidase (Campbell et al., 1982). Distal steric effects were also suggested to influence the association rates of O_2 and CO binding to model compounds (Traylor, 1981).

A distal ionizable group that affects the redox potential has been indentified in ferrous lignin peroxidase isozyme H1 (Millis et al., 1989). The pK_a determined from the electrochemical and spectroscopic results is about 6.7, very close to the value of 6.63 determined here. Although we do not have a detailed pH study with isozyme H8, a cursory study indicates that pH has a similar effect on O_2 binding to ferrous isozyme H8 (Table I) and the autoxidation of the isozyme H8 oxycomplex. This greatly suggests that the distal His plays the same roles in isozyme H8. These results support the idea that the distal His is responsible for the pH effects on processes involving ferrous lignin peroxidases.

In contrast to lignin peroxidases, there is no pH effect on O_2 binding to hemoglobin (Antonini & Brunori, 1971), myoglobin (Antonini & Brunori, 1971), horseradish peroxidase (Wittenberg et al., 1967), and chloroperoxidase (Lambier & Dunford, 1985). However, a pH effect was observed for CO binding with horseradish peroxidase and chloroperoxidase. The association rate for CO binding to the unprotonated ferrous horseradish peroxidase is twice that to its protonated form (Kertesz et al., 1965), and it is 8 times the rate in the case of ferrous chloroperoxidase (Campbell et al., 1982). These results are consistent with the proposal that the distal His imposes steric hindrance to linear ligand (CO) binding, but favors bent ligand (O_2) binding (Phillip & Schoenborn, 1981). The observation of a pH effect on O_2 binding to ferrous lignin peroxidase, while no effect is observed with myoglobin, horseradish peroxidase, and chloroperoxidase, suggests that the distal His is in closer proximity to the sixth axial position of the heme-iron atom in ferrous lignin peroxidases.

It was demonstrated from studies with iron porphyrin model compounds that O_2 affinity is increased by intramolecular H-bonding to the bound O_2 (Mispelter et al., 1983). Replacement of the distal His by a Gly residue results in an over 100-fold increase in the O_2 dissociation rate from mutant myoglobin and hemoglobin (Olson et al., 1988). This change can be attributed to the ability of the distal His to stabilize the $Fe^{3+}\cdot O_2^-$ moiety through H-bonding to the terminal oxygen atom. This H-bond could be stronger in the oxycomplex of lignin peroxidases than in the oxymyoglobin if indeed the distal His is closer to the O_2 binding site as discussed above. Thus, the higher O_2 affinity found with lignin peroxidases could be the result of a more electron deficient heme environment and a stronger H-bond formed by the distal His to the terminal oxygen atom.

In this paper we have demonstrated the unusual stability of the oxycomplex of two lignin peroxidase isozymes. Despite their physical and kinetic properties being similar to those of other peroxidases and globins, these results provide further evidence that lignin peroxidases contain a heme active site different from other peroxidases that imparts them with unique catalytic activities. Ongoing work in our laboratory involving further characterization of the heme active site of lignin peroxidase may provide additional information on structural aspects which confer on these enzymes their unique catalytic activity.

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