Biochemistry **1992**, *31*, 10763–10768

Haloperoxidase Activity of *Phanerochaete chrysosporium* Lignin Peroxidases H2 and H8[†]

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Received May 13, 1992; Revised Manuscript Received August 12, 1992

ABSTRACT: Monochlorodimedone (MCD), commonly used as a halogen acceptor for haloperoxidase assays, was oxidized by hydrogen peroxide in the presence of lignin peroxidase isoenzymes H2 and H8. When oxidized, it produced a weak absorption band with an intensity that varied with pH. This absorbance was used as a simple method for the product analysis because it disappeared when MCD was brominated or chlorinated. We assessed the activity of the lignin peroxidases for oxidation of bromide by measuring the bromination of MCD, the formation of tribromide, the bromide-mediated oxidation of glutathione, and the bromide-mediated catalase-like activity. We analyzed the reaction products of MCD and the halide-mediated oxidation of glutathione when bromide was replaced by chloride. These enzymes demonstrated no significant activity for oxidation of chloride. Unlike other peroxidases, the lignin peroxidases exhibited similar pH–activity curves for the iodide and bromide oxidations. The optimum pH for activity was about 2.5. Surprisingly, this pH dependence of lignin peroxidase activity for the halides was nearly the same in the reactions with hydrogen donors, such as hydroquinone and guaiacol. The results suggested that protonation of the enzymes with $pK_a \approx 3.2$ is necessary for the catalytic function of lignin peroxidases, irrespective of whether the substrates are electron or hydrogen donors. These unique reaction profiles of lignin peroxidases are compared to those of other peroxidases, such as lactoperoxidase, bromoperoxidase, chloroperoxidase, and horseradish peroxidase. Isozyme H2 was more active than isozyme H8, but isozyme H8 was more stable at very acidic pH.

Of numerous electron donors that are oxidized in the presence of peroxidase and H_2O_2, halide ions are oxidized through a unique mechanism (Morrison & Schonbaum, 1976; Griffin, 1991). The two-electron oxidation of halide ions takes place in the reaction with peroxidase compound I (Bjorksten, 1970; Roman & Dunford, 1972; Nakamura et al., 1983), while most other electron or hydrogen donors (AH_2) are oxidized by way of one-electron transfer to their free radical form ($AH^\bullet$) during the reactions with peroxidase compounds I and II (Yamazaki, 1977):

$$\text{peroxidase (or E)} + H_2O_2 \rightarrow \text{compound I (or EO)} + H_2O \quad (1)$$

$$\text{compound I} + AH_2 \rightarrow \text{compound II} + AH^\bullet \quad (2)$$

$$\text{compound II} + AH_2 \rightarrow \text{peroxidase} + AH^\bullet + H_2O \quad (3)$$

In the presence of halide ions (X^-) and halogen acceptor (BH)

$$EO + X^- \rightarrow EOX^- \quad (4)$$

$$EOX^- + BH + H^+ \rightarrow E + BX + H_2O \quad (5)$$

The formation of complexes of peroxidases with oxidized halides, formulated as EOX^-,[1] was suggested in the reactions of halide ions with compound I of chloroperoxidase (Libby et al., 1982; Lambeir & Dunford, 1983) and thyroid peroxidase (Nakamura et al., 1983; Magnusson et al., 1984). In addition to this unique mechanism, the peroxidase–halide reaction provides a rough measure for the oxidizing ability of peroxidase compound I(Neidleman & Geigert, 1986; Taurog & Dorris, 1991). This capability is generally measured as the standard redox potential, but the redox potential for catalytic intermediates of peroxidases has been measured only for horseradish peroxidase (Hayashi & Yamazaki, 1979).

The lignin peroxidase from the white-rot fungus *Phanerochaete chrysosporium* may be characterized by its ability to catalyze the oxidation of many environmentally persistent pollutants which cannot be oxidized by other peroxidase systems (Haemmerli et al., 1986; Hammel et al., 1986; Aust, 1990; Valli & Gold, 1991). The fact that the catalytic intermediates of lignin peroxidases act as stronger oxidants than those of horseradish peroxidase was demonstrated in reactions with a series of methoxybenzenes having different redox potentials (Kersten et al., 1990; Popp & Kirk, 1991). On the other hand, Renganathan et al. (1987) reported that in the presence of H_2O_2, lignin peroxidase oxidized bromide and iodide but not chloride. Since monochlorodimedone (MCD), used for haloperoxidase assays, is itself a peroxidase substrate (Griffin & Ashley, 1984), we reinvestigated the haloperoxidase activity of lignin peroxidases in detail. During our study, we confirmed that, unlike other peroxidases, lignin peroxidase isozymes H2 and H8 exhibited nearly the same pH–activity profile for peroxidations of both bromide and iodide. To characterize the haloperoxidase reactions of lignin peroxidases, we compared the reactions with those of lactoperoxidase, which is known as a typical haloperoxidase (Neidleman & Geigert, 1986), and discussed the difference from the point of enzyme kinetics.

MATERIALS AND METHODS

Lignin peroxidase isozymes H2 and H8 were prepared according to Tuisel et al. (1990). Lactoperoxidase was

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[†] This work was supported in part by NIEHS Superfund Grant ES04922, by NIH Research Resource Grant 1P41 RR06030-01A1, and by Savannah River Laboratory Grant W89329.

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[1] Abbreviations: E, EO, and EOX^- (or E–HOX), peroxidase, peroxidase compound I, and peroxidase hypohalous ion (or acid) complex, respectively; DTNB, Ellman's reagent or 5,5′-dithiobis(2-nitrobenzoic acid); GSH, glutathione; GSSG, oxidized glutathione, MCD, monochlorodimedone.

0006-2960/92/0431-10763$03.00/0

prepared according to Rombauts et al. (1967). Horseradish peroxidase (isozyme C) and chloroperoxidase were obtained from Toyobo (Osaka, Japan) and Sigma, respectively. The enzyme concentrations were calculated on the basis of ϵ (mM^{-1} cm^{-1}) at Soret peak = 133 for lignin peroxidase isozymes H2 and H8, 114 for lactoperoxidase, and 103 for horseradish peroxidase. Monochlorodimedone (MCD), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), and glutathione (GSH) were obtained from Sigma.

Reactions were carried out at room temperature (25 ± 1 °C) in 20 mM potassium phosphate buffer from pH ranges from 2 to 3.5, in sodium succinate buffer for pH from 3.5 to 6, and in potassium phosphate buffer for pH 6.5–7.5. We measured halogenation of MCD by following the decrease in absorbance at 288 nm. The extinction coefficient for MCD varied with pH, as described by Hewson and Hager (1979). Formation of oxidized halogen molecules was measured by following the increase in absorbance at 353 nm using ϵ (mM^{-1} cm^{-1}) = 25.5 for triiodide, and at 266 nm using ϵ (mM^{-1} cm^{-1}) = 36.4 for tribromide (Libby et al., 1982). Oxidations of hydroquinone and guaiacol were measured by following the decrease in absorbance at 280 nm [ϵ (mM^{-1} cm^{-1}) = 8.28] and the increase at 470 nm, respectively. For the latter, we used a value of ϵ (mM^{-1} cm^{-1}) = 6.65 for oxidized guaiacol formed per mole of H_2O_2 (George, 1953). Oxidation of GSH was measured according to Ellman (1959). We measured optical absorbance with a Shimadzu UV2100U spectrophotometer and O_2 concentration with a YSI Model 5300 biological oxygen monitor.

RESULTS

The haloperoxidase activity is generally measured in the presence of MCD, an acceptor of halogen (Hewson & Hager, 1979; Neidleman & Geigert, 1986). However, care should be taken, particularly when the reaction is carried out at acidic pH. MCD is protonated with $pK_a = 2.99$ (Hewson & Hager, 1979), and its optical absorption spectrum changes drastically with pH (Figure 1). Furthermore, MCD is oxidized directly by peroxidase systems as reported by Griffin and Ashley (1984) and Renganathan et al. (1987). The oxidation of MCD resulted in a decrease of absorbance at 288 nm, as does the halogenation of MCD. The oxidized MCD, however, gave a distinct absorption band at shorter wavelengths, while the band disappeared completely when MCD was brominated (Figure 1). Since the difference in absorbance between oxidized and brominated MCD was relatively large at acidic pH, this result could be used as a simple assay to confirm whether MCD is oxidized or halogenated.

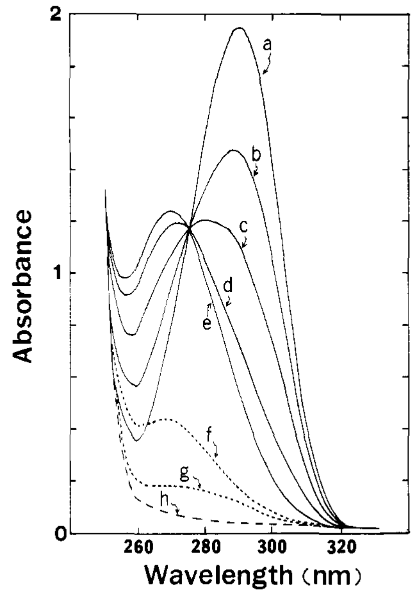

FIGURE 1: pH dependence of optical spectra of MCD and oxidized MCD. 100 μM MCD at pH 5.0 (a), 3.5 (b), 3.0 (c), 2.5 (d), and 2.0 (e). 100 μM oxidized MCD at pH 2.5 (f) and 3.5 (g). The oxidized MCD was obtained by successive additions of lignin peroxidase H2 (0.13 μM for each) or H_2O_2 (100 μM for each) until no further change in the spectrum was observed. Spectrum h was observed 5 min after the reaction started in the presence of 0.13 μM lignin peroxidase H2, 100 μM H_2O_2, 100 μM MCD, and 20 mM NaBr at pH 2.5 or 3.5. The wavelengths of the peaks were 289 nm for (a), 269 nm for (e), and 268 nm for (f).

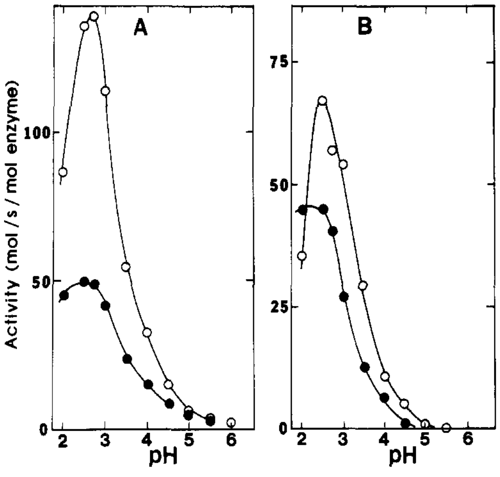

FIGURE 2: Effect of pH on the activity of lignin peroxidases H2 (○) and H8 (●) for the formation of I_3^- (A) and the bromination of MCD (B). The concentrations were 100 μM H_2O_2 and 20 mM NaI for (A) and 100 μM H_2O_2, 100 μM MCD, and 20 mM NaBr for (B).

The iodoperoxidase activity can be measured directly from the formation of triiodide at 353 nm when an excess amount of iodide is present. Figure 2A shows the pH dependence of the initial rate of triiodide formation catalyzed by lignin peroxidases H2 and H8. To measure the rate of bromide oxidation, we used two assay systems: one with MCD and the other without MCD. The rate of MCD bromination was measured from the decrease in absorbance at 288 nm, and the pH dependence of the rate is shown in Figure 2B. Although lignin peroxidases catalyzed the oxidation of MCD by H_2O_2 in the absence of halides, we confirmed from the spectrum of the final reaction product that the formation of oxidized MCD is negligible in the reactions shown in Figure 2B. The oxidation of bromide could also be measured from the increase in absorbance at 266 nm which is due to the formation of tribromide (Figure 3). The stoichiometry of the reaction was calculated on the basis that the equilibrium constant (K) for reaction 6 = 14.4 M^{-1} and ϵ (mM^{-1} cm^{-1}) at 266 nm for Br_3^-

$$Br_2 + Br^- \rightleftharpoons Br_3^- \qquad (6)$$

= 36.4, according to Libby et al. (1982). The amount of oxidized bromine species ($Br_2 + Br_3^-$) formed at pH 3.0 was about 90% of added H_2O_2 on the molar basis. Accumulation of Br_3^- decreased on both sides of pH 3.0. At pH 2.5, the enzyme was inactivated during the reaction, and the further addition of enzyme increased the Br_3^- accumulation. At pH 4, however, Br_3^- disappeared upon the addition of H_2O_2 (Figure 3), probably via reactions 6 and 7. Consistent with the result

$$Br_2 + H_2O_2 \rightarrow 2H^+ + 2Br^- + O_2 \qquad (7)$$

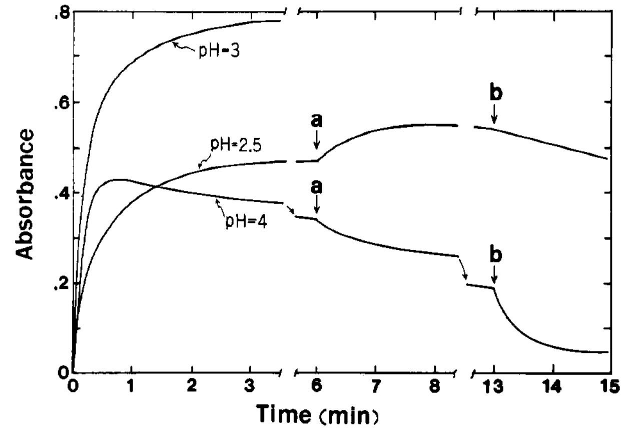

FIGURE 3: Formation of Br_3^-. The absorbance change was observed at 266 nm in the presence of 0.13 μM lignin peroxidase H2, 100 μM H_2O_2, and 20 mM NaBr. At arrows a and b, another 0.13 μM enzyme and 100 μM H_2O_2 were added, respectively.

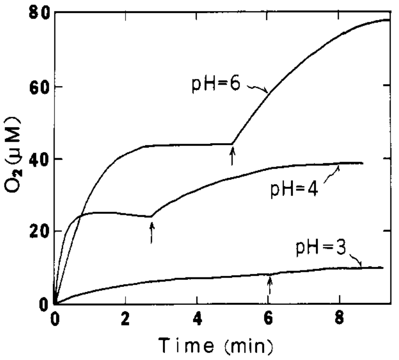

FIGURE 4: Bromide-mediated catalase-like activity of lignin peroxidase H2. The concentrations were 0.13 μM enzyme, 100 μM H_2O_2, and 20 mM NaBr. O_2 evolution was negligible in the absence of NaBr. At the arrows, another 100 μM H_2O_2 was added.

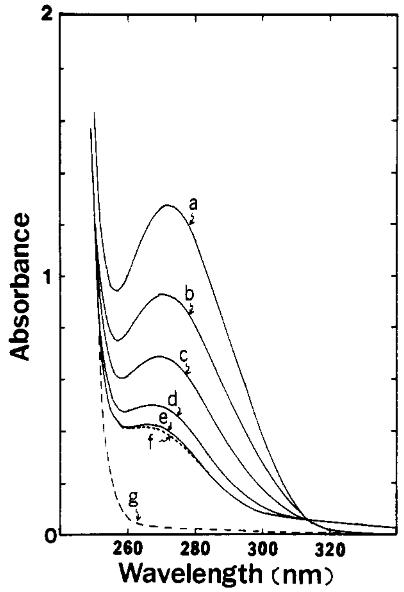

FIGURE 5: Changes in MCD spectra observed upon successive additions of lignin peroxidase H2 and H_2O_2 in the presence of NaCl at pH 2.5. (a) 100 μM MCD; (b and c) the spectra were scanned immediately, and 5 min after 0.13 μM enzyme was added to a solution of 100 μM H_2O_2, 100 μM MCD, and 20 mM NaCl; (d) 5 min after the second addition of 0.13 μM enzyme to (c); (e) 5 min after the third addition of 0.13 μM enzyme; (f) 5 min after the second addition of 100 μM H_2O_2 to (e); (g) same as (c) except 0.13 μM lignin peroxidase H2 was replaced by 5 nM chloroperoxidase.

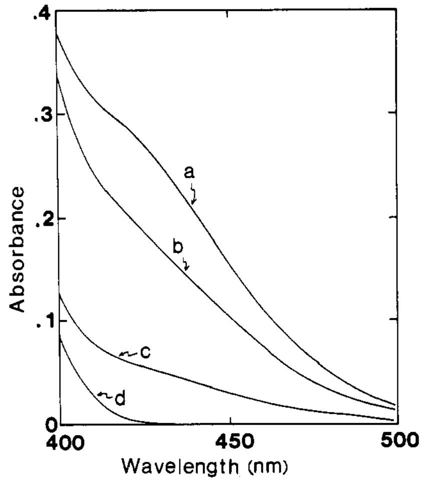

FIGURE 6: Halide-mediated oxidation of GSH catalyzed by peroxidases. The reaction mixture contained 0.13 μM lignin peroxidase H2 or 5 nM chloroperoxidase, 100 μM H_2O_2, 100 μM GSH, and 20 mM NaCl or NaBr. An aliquot (200 μL) of the mixture was added to 200 μM DTNB solution (20 mM phosphate, pH 7.5) 1 min after the reaction, and the spectra were scanned a few minutes after. (a) Lignin peroxidase H2 with or without NaCl; (b) chloroperoxidase + NaCl; (c) lignin peroxidase H2 + NaBr; (d) control (DTNB alone).

shown in Figure 3, the evolution of O_2 became evident as the pH increased (Figure 4). It should be noted that at pH 6, a considerable catalase-like activity was observed without accumulation of Br_3^-. There was no essential difference in the profiles of bromide oxidation between the two isozymes. This apparent bromide-mediated catalase-like reaction as well as the Br_3^- formation was completely inhibited by the presence of 100 μM MCD.

To answer whether or not the lignin peroxidases catalyze the chloride oxidation, we compared absorption spectra of the final peroxidase reaction products in the presence and absence of chloride at pH 2.5. The addition of chloride slowed down the rate of spectral change in the reaction of isozyme H2, but not isozyme H8. In both cases, however, the final spectra obtained after successive additions of the enzyme and H_2O_2 were the same as the spectra of oxidized MCD within experimental error (compare curve f in Figures 1 and 5). Figure 5 also shows a spectrum of chlorinated MCD obtained from the reaction of chloroperoxidase. The second experimental evidence for the inability of lignin peroxidases to oxidize chloride is shown in Figure 6. GSH was not oxidized in the presence of lignin peroxidases and H_2O_2 when assayed according to Ellman (1959). GSH oxidation was caused by a combination of lignin peroxidase and bromide, or chloroperoxidase and chloride, but not by lignin peroxidase and chloride.

The pH dependence of the haloperoxidase activity of lignin peroxidases H2 and H8 contrasted to that of algal bromoperoxidase, which exhibits different pH–activity curves for the oxidation of each halide. Manthey and Hager (1989) showed that the optimum pH for bromoperoxidase shifted markedly to the alkaline side in the order of $Cl^- < Br^- < I^-$. They also reported that pyrogallol, hydroquinone, and guaiacol all show similar broad pH optima between pH 4.5 and 8. Therefore, we investigated the pH reaction rate profile of the peroxidation of hydroquinone and guaiacol in the presence of lignin peroxidases and H_2O_2. As shown in Figure 7A, in contrast to bromoperoxidase (Manthey & Hager, 1989), the rate of hydroquinone oxidation decreased markedly as the pH increased. We observed a similar pH–activity curve when hydroquinone was replaced by guaiacol (Figure 7B). These pH–activity curves resembled those for the haloperoxidase

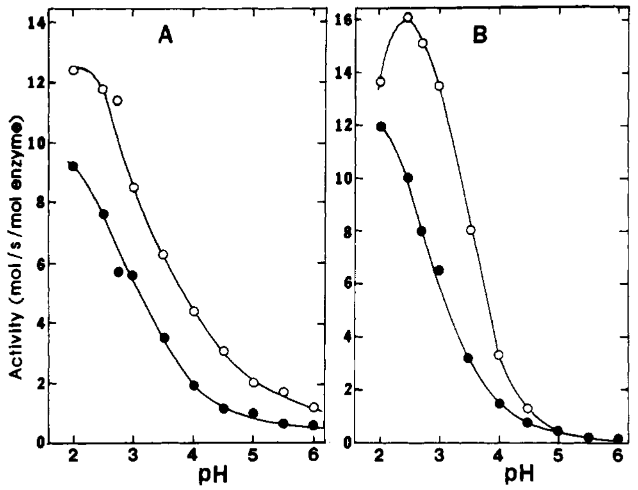

FIGURE 7: Effect of pH on the peroxidation of hydroquinone and guaiacol catalyzed by lignin peroxidases H2 (○) and H8 (●). (A) Hydroquinone. The concentrations were 0.13 μM H2 or 0.12 μM H8, 100 μM hydroquinone, and 200 μM H_2O_2. (B) Guaiacol. The concentrations were 0.13 μM H2 or 0.12 μM H8, 1 mM guaiacol, and 200 μM H_2O_2.

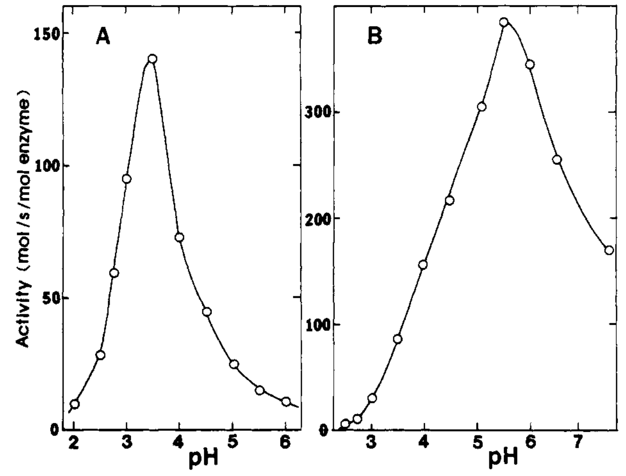

FIGURE 8: Effect of pH on the activity of lactoperoxidase for the bromination of MCD (A) and the formation of I_3^- (B). The concentrations were 100 μM H_2O_2, 100 μM MCD, and 20 mM NaBr for (A) and 100 μM H_2O_2 and 20 mM NaI for (B).

activity (Figure 2), except for those at very acidic pH, where the peroxidase activity might be inhibited by the presence of halides. It should be noted that these pH–activity curves were similar to those of lignin peroxidases for veratryl alcohol oxidation (Tien et al., 1986; Marquez et al., 1988; Tuisel et al., 1990; Wariishi et al., 1991).

Since the difference in the pH–activity curves between lignin peroxidases and bromoperoxidase was found to be remarkable, we investigated the pH–activity curves for lactoperoxidase which exhibits typical haloperoxidase activity toward iodide and bromide (Neidleman & Geigert, 1986). The optimum pH for the haloperoxidase activity shifted to higher pH when bromide was replaced by iodide (Figure 8) as reported in the case of bromoperoxidase (Manthey & Hager, 1989). Like many other peroxidases, lactoperoxidase also demonstrated high activity for the peroxidations of hydroquinone and guaiacol above pH 5 (Figure 9). The apparent decrease in the activity for guaiacol peroxidation from pH 3.5 to 4 (triangles in Figure 9) was ascribable to the change of pH buffer from phosphate to succinate. The activity in the acetate buffer was similar to that in the succinate buffer.

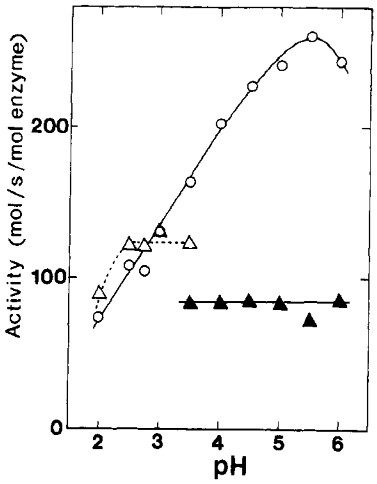

FIGURE 9: Effect of pH on the peroxidation of hydroquinone and guaiacol catalyzed by lactoperoxidase. The concentrations were 200 μM H_2O_2 and 100 μM hydroquinone for (○) and 200 μM H_2O_2 and 1 mM guaiacol for (△) (phosphate buffer) and (▲) (succinate buffer).

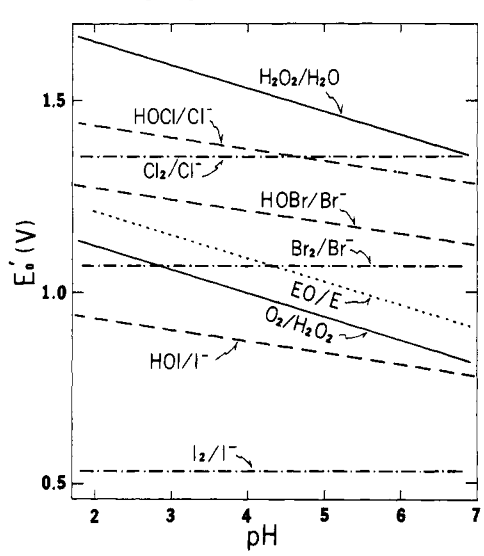

FIGURE 10: pH dependence of reduction potentials (E_0') for redox couples related to haloperoxidase reactions. The values of E_0 at 25 °C in volts (E_0' at pH 0) are 1.77 for H_2O_2/H_2O, 0.682 for O_2/H_2O_2, 0.5345 for I_2/I^-, 1.0652 for Br_2/Br^-, 1.3583 for Cl_2/Cl^-, 0.99 for HOI/I^-, 1.33 for $HOBr/Br^-$, and 1.45 for $HOCl/Cl^-$ (Moeller, 1952).

DISCUSSION

Thermodynamic consideration of haloperoxidase reactions is an interesting subject (Neidleman & Geigert, 1986; Taurog & Dorris, 1991) because the oxidation of halides by peroxidase compound I is assumed to occur without steric hindrance and consequently reflects directly the thermodynamic features of the reaction. Standard reduction potentials (E_0) for redox couples of H_2O_2/H_2O, XOH/X^-, and $X_2/2X^-$, which are related to haloperoxidase reactions, are known. The E_0 values for the above couples, shown in the legend of Figure 10, are obtained at pH 0 (Moeller, 1952), and the pH dependence of E_0' (V) at room temperature is expressed as

$$E_0' = E_0 - 0.06\text{pH}, \quad \text{for the } H_2O_2/H_2O \text{ and } O_2/H_2O_2 \text{ couples} \qquad (8)$$

$$E_0' = E_0 - 0.03\text{pH}, \text{ for the HOX/X}^- \text{ couple} \qquad (9)$$

Equations 8 and 9 are applied when the two-electron reduction is accompanied by uptake of two or one proton, respectively. Since HCl, HBr, and HI are strong acids, the halides will exist in the dissociated forms above pH 2. The pH dependence of these E_0' values is shown in Figure 10. The E_0' value for the compound I/peroxidase (EO/E) couple can be obtained only for horseradish peroxidase, being calculated to be 0.97 V at pH 6 from the data of Hayashi and Yamazaki (1979) according to an equation of Dunford (1991). At this pH, two protons are needed to reduce EO to E (Yamada & Yamazaki, 1974):

$$\mathrm{EO} + 2\mathrm{e}^- + 2\mathrm{H}^+ \rightarrow \mathrm{E} + \mathrm{H_2O} \tag{10}$$

As long as this stoichiometry is maintained, the pH dependence of E_0' for the EO/E couple obeys eq 8 and is shown as a dotted line in Figure 10. The difference in the E_0' values between the H_2O_2/H_2O and EO/E couples is regarded as a loss of free energy in reaction 1. If one assumes that the reaction of EO with halides (reaction 4) is rate-limiting both in the halogenation of MCD and in the formation of trihalide (Libby et al., 1982), the halide oxidation will be controlled by the E_0' value for the $\mathrm{EOX}^-/(\mathrm{X}^- + \mathrm{E})$ couple instead of for the $\mathrm{X_2}/2\mathrm{X}^-$ couple which has been adopted for thermodynamic consideration in the halogenation reaction (Neidleman & Geigert, 1986; Taurog & Dorris, 1991).

Here, a question arises as to whether or not a proton is involved in the reaction of EO with X^-. HOX is a very weak acid, and its $\mathrm{p}K_\mathrm{a}$ varies with halogen species [7.43 for HOCl and 11.00 for HOI (Moeller, 1954)]. According to the analysis of acid–base properties of peroxidases (Yamazaki et al., 1978), it is reasonable to express the uptake of a proton by the enzyme in reaction 4 as

$$\mathrm{EO} + \mathrm{X}^- + \mathrm{H}^+ \rightarrow \mathrm{E{-}\overset{H}{O}X}\ (\text{or E–HOX}) \tag{4'}$$

Then, the reaction with a halogen acceptor will be

$$\mathrm{E{-}HOX} + \mathrm{BH} \rightarrow \mathrm{E} + \mathrm{BX} + \mathrm{H_2O} \tag{5'}$$

One can assume that the ratio of [E–HOX] to [EO] at a transition state determines the rate of halide oxidation reactions:

$$\log\left([\mathrm{E{-}HOX}]/[\mathrm{EO}]\right) = \Delta E_0'/0.03 - \log K + \log [\mathrm{X}^-] \tag{11}$$

where $\Delta E_0' = E_0'(\mathrm{EO/E}) - E_0'(\mathrm{HOX/X^-})$ and $K = [\mathrm{E}]\cdot[\mathrm{HOX}]/[\mathrm{E{-}HOX}]$. The pH dependence of the rate of reaction of horseradish peroxidase compound I with iodide has been analyzed kinetically (Roman & Dunford, 1972), but the result might also be interpreted in terms of this thermodynamic consideration. In eq 11, the $\Delta E_0'$ value can be obtained from Figure 10, but unfortunately, the value of constant K is not yet known. However, as long as the K value does not change significantly, we can discuss the ability of compound I to oxidize halide directly on the basis of $\Delta E_0'$.

Equation 11 shows that the [E–HOX]/[EO] ratio increases with an increase in halide concentration and a decrease in pH. We assume that the thermodynamic barrier in the halide oxidation by peroxidases disappears when this ratio reaches about 10. Although this assumption may not be applied at very low pH because of inhibitory effects of halides (Kimura & Yamazaki, 1978; Andrews & Krinsky, 1982; Lambier & Dunford, 1983; Renganathan et al., 1987), in general the haloperoxidase activity of a peroxidase reaches a maximum level at higher pH in the following order of reactions with $\mathrm{Cl}^- < \mathrm{Br}^- < \mathrm{I}^-$ (Manthey & Hager, 1989) and with an increase in halide concentrations (Thomas et al., 1970). Similar shifts of pH optima are also observed in the haloperoxidase reaction of lactoperoxidase (Figure 8). It is therefore remarkable that the pH–activity curves for oxidations of iodide and bromide are nearly the same for lignin peroxidases. The result leads us to conclude that under these experimental conditions, the haloperoxidase activity of lignin peroxidases is controlled not by a thermodynamic factor but by a kinetic factor such as protonation of the enzyme at a catalytic site with $\mathrm{p}K_\mathrm{a} \approx 3.2$. A similar $\mathrm{p}K_\mathrm{a}$ value was reported in the reaction of lignin peroxidase compound II with veratryl alcohol (Wariishi et al., 1991). The interesting observation in the present paper is that the peroxidations of hydroquinone and guaiacol are also controlled by the same protonation. In general, the oxidation of hydrogen donors such as hydroquinone and phenols by peroxidase systems is favorable near neutral pH, while that of electron donors such as ascorbate, halides, and veratryl alcohol is at acidic pH. This is a common feature in reactions of most peroxidases. In this sense, lignin peroxidases H2 and H8 belong to a rather atypical group of peroxidases, which might be called acid peroxidases. On the other hand, like other peroxidases, the rate constant for EO formation (reaction 1) is pH-independent, being $(4.2\text{–}6.5) \times 10^5\ \mathrm{M^{-1}\,s^{-1}}$ over the pH range 3–7 for lignin peroxidases (Andrawis et al., 1988; Marquez et al., 1988; Harvey et al., 1989). Therefore, it seems likely that in the reactions of lignin peroxidases, the rate-limiting step is reaction 5′ in the haloperoxidase reactions and reaction 3 in the classical peroxidase reaction. In both reactions, a proton may have to be supplied from a catalytic site of the enzyme, but not from $\mathrm{AH_2}$ in reaction 3 or BH in reaction 5′.

Once halides are oxidized by EO to E–HOX (reaction 4′), there are a variety of secondary reactions which can occur depending on the kind of halogen acceptors:

$$\mathrm{E{-}HOX} \rightarrow \mathrm{E} + \mathrm{HOX} \tag{12}$$

$$\mathrm{E{-}HOX} + \mathrm{MCD}\ (\mathrm{MCD'{-}H}) + \mathrm{H}^+ \rightarrow \mathrm{E} + \mathrm{H_2O} + \mathrm{MCD'{-}X} \tag{13}$$

$$\mathrm{E{-}HOX} + \mathrm{GSH} \rightarrow \mathrm{E} + \mathrm{H_2O} + \mathrm{GS{-}X} \tag{14}$$

$$\mathrm{GS{-}X} + \mathrm{GSH} \rightarrow \mathrm{GS{-}SG} + \mathrm{H}^+ + \mathrm{X}^- \tag{14'}$$

$$\mathrm{E{-}HOX} + \mathrm{X}^- + \mathrm{H}^+ \rightarrow \mathrm{E} + \mathrm{H_2O} + \mathrm{X_2} \tag{15}$$

$$\mathrm{E{-}HOX} + \mathrm{H_2O_2} \rightarrow \mathrm{E} + \mathrm{H_2O} + \mathrm{H}^+ + \mathrm{O_2} \tag{16}$$

The chemical entity of the halogenating intermediate in haloperoxidase reactions still remains to be solved. By examining competitive inhibition of halogenation of MCD by sulfur compounds, Libby et al. (1982) concluded that unlike the iodide-dependent reactions, the bromide- and chloride-dependent reactions do not involve the formation of free molecular halogen intermediates in the case of chloroperoxidase. From kinetic analysis of iodination by thyroid peroxidase, however, Nakamura et al. (1984) concluded that iodination of tyrosine by E–HOI is rate-limiting at low concentrations of iodide, suggesting E–HOI as an iodinating intermediate. A horseradish peroxidase–chlorine complex, possibly EOCl^- obtained from the reaction of E with sodium chlorite at alkaline pH, exhibited an optical spectrum similar to compound II (Shahangian & Hager, 1982).

As seen in Figure 10, the formation of free molecular halogen is easier in the order of $\mathrm{Cl}^- < \mathrm{Br}^- < \mathrm{I}^-$, and the value of $E_0'(\mathrm{HOX/X^-}) - E_0'(\mathrm{X_2/X^-})$ becomes greater in the same order. These thermodynamic features are reflected on the manifold reaction profiles depending on the kind of halides and halogen acceptors. Although the reduction potential of the ferric/ferrous couple is higher for lignin peroxidases than for horseradish peroxidase (Millis et al., 1989; Banci et al., 1991), this may not necessarily be true for that of the catalytic intermediates. Incidentally, in contrast to a general concept reviewed by Neidleman and Geigert (1986), horseradish peroxidase also catalyzes the oxidation of bromide to form Br_3^- (Ashley & Griffin, 1981), and the bromide-mediated catalase-like reaction (Griffin, 1983). From the present result, therefore, we cannot conclude whether or not the reduction potential of EO of lignin peroxidase is higher than that of horseradish peroxidase. Lignin peroxidase and horseradish peroxidase as well as lactoperoxidase all can catalyze the oxidation of iodide and bromide, but not chloride.

ACKNOWLEDGMENT

We thank Ms. Aida Albo (Biotechnology Center, Utah State University) for purification of lignin peroxidase isozymes H2 and H8 from *P. chrysosporium* and Professor Shingo Nakamura, Hirosaki University, Japan, for donation of lactoperoxidase. We also thank professor Michael Gold for helpful discussions during the course of this work.

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