The Effect of Manganese on the Oxidation of Chemicals by Lignin Peroxidase†

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ABSTRACT: It has recently been discovered that lignin peroxidase isozyme H2 (LiPH2) has the ability to oxidize Mn^{2+} (Khindaria et al., 1995). Furthermore, at pH 4.5, the physiological pH of *Phanerochaete chrysosporium*, LiPH2 oxidizes Mn^{2+} at a much faster rate (25 times) than veratryl alcohol (VA). The ability of Mn^{2+} to act as a redox mediator for indirect oxidations catalyzed by LiPH2 was therefore investigated. In the presence of physiologically relevant levels of oxalate and Mn^{2+}, the rate of LiPH2-catalyzed oxidation of all substrates studied was dramatically increased. Up to 10-fold stimulations were observed compared to the rates of oxidation of substrate in either the presence or absence of VA. We propose that the stimulation is due to the ability of LiPH2 to oxidize Mn^{2+}, producing the strong oxidant Mn^{3+}, at a high rate. The rates of oxidation of the substrates showed a hyperbolic dependence on Mn^{2+} in the presence of oxalate, a chelator which was required for maximal activity. The oxalate dependence of the oxidation rates correlated well with the concentration of the 1:1 complex of Mn^{2+}−oxalate. The relative concentrations of the substrates and H_2O_2 and the rate constants for their reactions with Mn^{3+} determined which chemical was oxidized by the enzymatically produced Mn^{3+}. The importance of the ability of Mn^{2+}−oxalate to stimulate the oxidation of chemicals by LiPH2 is discussed.

The white rot fungus, *Phanerochaete chrysosporium*, secretes many heme-containing isozymes with peroxidase activity which are implicated in the degradation of lignin and organopollutants by this fungus (Tien & Kirk, 1984; Gold et al., 1984; Bumpus et al., 1985; Kirk & Farrell, 1987; Barr & Aust, 1994). Traditionally, these isozymes have been separated into two groups, the lignin peroxidases (LiP)[1] (Kersten et al., 1985; Hammel et al., 1986) and the manganese peroxidases (MnP) (Kirk et al., 1986; Miki et al., 1986). The LiP isozymes (H1, H2, H6, H7, H8, and H10) are able to oxidize a wide variety of organic substrates directly (Paszczynski et al., 1985; Shoemaker et al., 1985; Tien, 1987). The MnP isozymes (H3, H4, H5, and H9) catalyze oxidations which are dependent on manganese as a redox mediator (Glenn & Gold, 1985; Paszczynski et al., 1986; Tien, 1987). Manganese peroxidases oxidize Mn^{2+} to Mn^{3+}, which is then capable of oxidizing a wide variety of organic molecules (Forrester et al., 1988; Tuor et al., 1992; Joshi & Gold, 1993). The oxidation of Mn^{2+} may be an important aspect of the degradative system of *P. chrysosporium*. Manganese is readily accessible to the peroxidases *in vivo* because wood, the natural growth substrate of white rot fungi, has been shown to contain up to 1035 ppm manganese (Young & Guinn, 1966). Lignin peroxidase is also capable of oxidizing molecules indirectly (Shah et al., 1992; Chung & Aust, 1995; Goodwin et al., 1995). Veratryl alcohol (VA), a secondary metabolite of *P. chrysosporium*, has been shown to act as a mediator of oxidations catalyzed by LiP (Shah et al., 1992; Chung & Aust, 1995; Goodwin et al., 1995). It is proposed that VA is oxidized to its cation radical, which acts as an oxidant.

It has recently been demonstrated by our laboratory that the predominant LiP isozyme, H2, has the ability to oxidize Mn^{2+} (Khindaria et al., 1995). The oxidation of Mn^{2+} by LiPH2 is catalyzed by the same mechanism as it is for MnP (Kuan et al., 1993; Khindaria et al., 1995). Compound I, the enzyme intermediate formed by the two electron oxidation of resting ferric peroxidase, can oxidize Mn^{2+} to Mn^{3+}, resulting in the formation of compound II. Compound II is capable of oxidizing another atom of Mn^{2+}, which completes the catalytic cycle of the peroxidase and returns the enzyme to the ferric form. The reduction of the compound II form of LiPH2 and MnP by Mn^{2+} involves a binding step and requires that the Mn^{2+} be chelated (Kuan et al., 1993; Khindaria et al., 1995). Oxalate is capable of chelating Mn^{2+} and is known to be present at millimolar levels in the extracellular environment of *P. chrysosporium* (Popp et al., 1990; Barr et al., 1992; Wariishi et al., 1992; Kuan & Tien, 1993). Oxalate-chelated Mn^{2+} has been shown to be a substrate for both LiPH2 and MnP compound II (Kuan et al., 1993; Khindaria et al., 1995).

Since both Mn^{2+} and VA can be oxidized by LiPH2, there are two potential mediators of indirect oxidations by LiPH2. The pH optimum for the reaction of LiPH2 with Mn^{2+} is 4.5 (Khindaria et al., 1995), which corresponds with the physiological pH of *P. chrysosporium* (Kirk et al., 1978). The pH optimum for the oxidation of VA by LiPH2 is near pH 2 (Tien, 1987), so at pH 4.5 the catalytic turnover of LiPH2 with Mn^{2+}−oxalate is 25 times faster than for the oxidation of VA (Tuisel et al., 1990; Khindaria, et al., 1995). The objective of the present study was to investigate the ramifications of the oxidation of Mn^{2+}−oxalate by LiPH2, an enzyme which is not dependent on manganese for activity. The ability of the two potential redox mediators for LiPH2, Mn^{2+} and VA, are compared. The oxidation of various

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[1] Abbreviations: LiPH2, lignin peroxidase isozyme H2; MnP, manganese peroxidase; VA, veratryl alcohol; ABTS, 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid); TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine.

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substrates by LiPH2 using Mn^{2+}−oxalate was found to be much greater than with VA. The oxidation of guaiacol by LiPH2 in the presence of Mn^{2+}−oxalate was further researched to characterize the reactions involved.

EXPERIMENTAL PROCEDURES

Chemicals. Hydrogen peroxide, succinic acid, malonic acid, chlorpromazine, ABTS [2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid)], and TMPD (*N,N,N′,N′*-tetramethyl-*p*-phenylenediamine) were purchased from Sigma Chemical Co. (St. Louis, MO). Veratryl alcohol (3,4-dimethoxybenzyl alcohol), phenol, guaiacol (2-methoxyphenol), 2,6-dimethoxyphenol, manganese(III) acetate dihydrate, coniferyl alcohol, and vanillylacetone [4-(4-hydroxy-3-methoxyphenyl)-3-buten-2-one] were purchased from Aldrich Chemical Co. (Milwaukee, WI). Manganese sulfate, sodium oxalate, tartaric acid, and sodium succinate were purchased from Mallinckrodt (Paris, NY). The sodium succinate buffer was prepared using purified water (Barnstead NANOpure II system; specific resistance 18.0 MΩ·cm^{-1}).

Enzyme Production and Purification. Lignin peroxidase isozyme H2 (LiPH2) was purified from the extracellular fluid of liquid shaking cultures of *P. chrysosporium* and assayed for activity as described previously (Tuisel et al., 1990). Analytical isoelectric focusing was performed to ensure the purity of the isozyme (Khindaria et al., 1995).

Steady State Kinetics. The initial velocity of guaiacol oxidation by LiPH2 was monitored at 465 nm. The extinction coefficient (6100 M^{-1} cm^{-1}) for oxidized guaiacol was determined by completely oxidizing varying guaiacol concentrations by horseradish peroxidase. A KinTek instruments three syringe stopped-flow apparatus (State College, PA) was used to determine the initial rate of guaiacol oxidation. Each datum point consisted of triplicate assays. The wavelength monitored and the corresponding extinction coefficient for the oxidized product of each substrate were as follows: TMPD, 610 nm, 11 600 M^{-1} cm^{-1} (Paszczynski et al., 1985); chlorpromazine, 525 nm, 12 100 M^{-1} cm^{-1} (Sackett & McCreery, 1979); ABTS, 660 nm, 12 000 M^{-1} cm^{-1} (Barr & Aust, 1993); 2,6-dimethoxyphenol, 468 nm, 10 000 M^{-1} cm^{-1} (Paszczynski et al., 1985); phenol, 398 nm, 6400 M^{-1} cm^{-1} (Chung & Aust, 1995). The oxidation of vanillylacetone and coniferyl alcohol was determined by monitoring the disappearance of these chemicals, which have extinction coefficients of 18 300 M^{-1} cm^{-1} at 336 nm and 13 400 M^{-1} cm^{-1} at 263 nm, respectively (Paszczynski et al., 1985). All enzymatic assays were performed at 25 °C. Spectrophotometric determination of H_2O_2 concentration was performed at 240 nm using an extinction coefficient of 39.4 M^{-1} cm^{-1} (Nelson & Kiesow, 1972).

Calculation of the Fraction of Mn^{2+} Present as a 1:1 Complex with Oxalate. The method outlined by Kuan et al. (1993) was used to determine the concentration of the 1:1 complex of Mn^{2+}−oxalate in the presence of 100 μM Mn^{2+} and various concentrations of oxalate. The binding constants used for the 1:1 and 1:2 complexes of Mn^{2+}−oxalate were $K_1 = 7.9 \times 10^3$ M^{-1} and $K_2 = 79$ M^{-1}, respectively (Meites, 1963). Succinate was used as a buffer because the binding constant for the Mn^{2+}−succinate complex is so low (30 M^{-1}). The same results were observed in acetate buffer.

Oxygen Production Experiments. Oxygen production was monitored using a Gilson oxygraph equipped with a Clark type oxygen sensitive electrode and a 1.8 mL reaction chamber maintained at 25 °C. The reaction conditions are described in the figure legends.

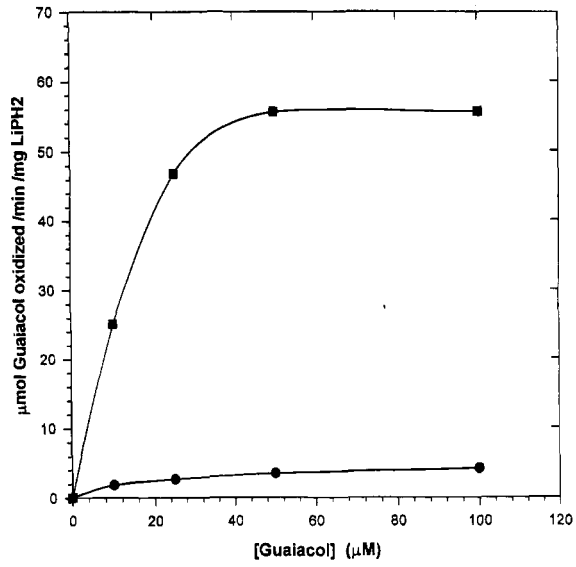

FIGURE 1: Effect of guaiacol on the initial rate of guaiacol oxidation by LiPH2. The reaction mixtures contained 20 mM sodium succinate, pH 4.5, 0.055 μM LiPH2, 100 μM H_2O_2, and the indicated concentration of guaiacol (●); the same as above plus 100 μM Mn^{2+} and 1 mM oxalate (■). The reactions were carried out at 25 °C.

Rate Constants for Reaction with Mn^{3+}. Stock Mn^{3+} solutions were prepared by dissolving 20 mg of manganese(III) acetate dihydrate in 20 mL of MeOH (Aitken & Irvine, 1990). Appropriate aliquots of this solution were mixed with oxalate and succinate, resulting in a relatively stable aqueous mixture. The concentration of Mn^{3+}−oxalate was determined spectrophotometrically at 270 nm using 5500 M^{-1} cm^{-1} as the extinction coefficient (Kuan et al., 1993) immediately prior to the addition of substrate. The oxidation of the substrates was monitored at the appropriate wavelength in the stopped-flow apparatus described above. The oxidation of H_2O_2 was determined by monitoring the disappearance of Mn^{3+}−oxalate at 270 nm using a Shimadzu UV-160 spectrophotometer. Further details are described in Table 2.

RESULTS

The effect of Mn^{2+}, with oxalate present as a chelator, on the initial rate of guaiacol oxidation by LiPH2 is shown in Figure 1. Upon addition of Mn^{2+} and oxalate, there was a dramatic increase in the rate of guaiacol oxidation over the entire range of guaiacol concentrations. At 50 μM guaiacol, the presence of Mn^{2+}−oxalate produced a greater than 16-fold stimulation in the initial rate of guaiacol oxidation. The interaction between LiPH2 and guaiacol appeared to be altered upon addition of Mn^{2+}−oxalate to the system. The rates of oxidation of guaiacol did not increase as the concentration of guaiacol was increased beyond 50 μM.

The dependence of the initial rate of guaiacol oxidation by LiPH2 on Mn^{2+} in the presence of 1 mM oxalate is presented in Figure 2. The oxidation rate had a dependence on Mn^{2+} concentration which followed saturation kinetics, and a K_m of stimulation of 24 μM was calculated.

The ability of Mn^{2+}−oxalate to stimulate the oxidation of a wide variety of subsrates by LiPH2 is presented in Table 1. The effect of veratryl alcohol (VA) on the oxidation of the same substrates is also included. The addition of VA

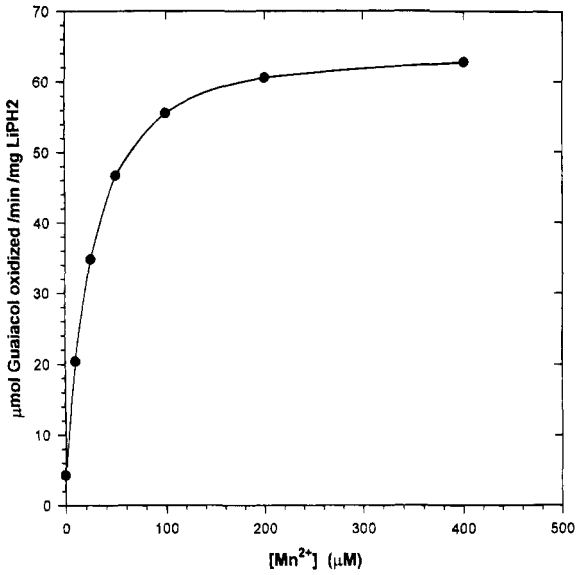

FIGURE 2: Effect of Mn^{2+} on the initial rate of guaiacol oxidation by LiPH2. The reaction mixtures were maintained at 25 °C and contained 20 mM sodium succinate, pH 4.5, 0.055 μM LiPH2, 100 μM H_2O_2, 100 μM guaiacol, 1 mM oxalate, and the indicated amount of Mn^{2+}.

Table 1: Rate of Oxidation of Various Substrates by LiPH2

substrate[a]	rate [μmol/(min·mg of LiPH2)][c] alone	+VA	+Mn^{2+}−oxalate
vanillylacetone[b]	4.0 ± 0.2	5.0 ± 0.2	37 ± 0.3
guaiacol	4.6 ± 0.3	5.6 ± 0.3	51 ± 1
coniferyl alcohol[b]	7.7 ± 0.1		67 ± 2
TMPD	15 ± 1.5	14 ± 0.5	144 ± 5
2,6-dimethoxyphenol	12 ± 0.3	13 ± 0.6	104 ± 2
ABTS	14 ± 0.2	15 ± 0.5	73 ± 1
phenol	17 ± 0.5	17 ± 0.5	56 ± 1
chlorpromazine	12 ± 0.8	12 ± 0.2	48 ± 1

[a] Saturating amounts (approximately 10 times K_m) of each substrate were used: guaiacol 200 μM, TMPD 110 μM, chlorpromazine 100 μM, ABTS 650 μM, 2,6-dimethoxyphenol 330 μM, phenol 8 mM. [b] Only 100 μM vanillylacetone and 120 μM coniferyl alcohol, which corresponds to approximately 3 times K_m, were used because of the high extinction coefficients of the compounds and the fact that the oxidation is measured as the disappearance of substrate. [c] The reaction mixtures contained 20 mM sodium succinate, pH 4.5, 0.055 μM LiPH2, 100 μM H_2O_2, and saturating amounts of each substrate either alone or in the presence of 2.5 mM veratryl alcohol (VA) or 100 μM Mn^{2+} and 1 mM oxalate. Values are averages of 3 determinations with their standard deviations. The reactions were carried out at 25 °C.

stimulated the oxidation of all of the substrates when the substrates were present at low levels (data not shown). The effect of veratryl alcohol on the oxidation of coniferyl alcohol was not determined due to the overlapping absorption spectra of the two species. The data presented in Table 1 were determined at saturating levels of each substrate. There was no oxidation of any of the substrates with just Mn^{2+} and H_2O_2. Veratryl alcohol had essentially no effect on the rates of oxidation in these conditions. However, the addition of Mn^{2+}−oxalate at saturating levels of each substrate resulted in stimulations of the rates of oxidation of every substrate. Significantly greater rates of oxidation were observed upon the addition of Mn^{2+}−oxalate at every concentration of the substrates, and the rates were always greater than those obtained upon the addition of VA (data not shown). Furthermore, the addition of 2.5 mM VA had no effect on the rate of oxidation of guaiacol in the presence of 100 μM Mn^{2+} and 1 mM oxalate (data not shown).

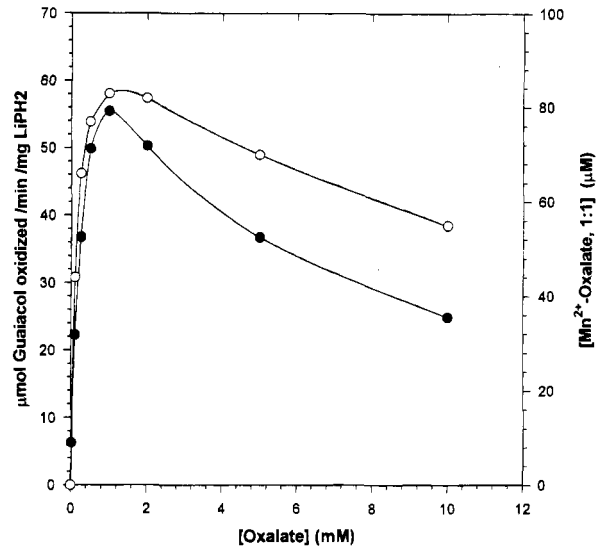

FIGURE 3: Effect of oxalate on the initial rate of guaiacol oxidation by LiPH2 and the concentration of the 1:1 complex of Mn^{2+}−oxalate. Reaction mixtures contained 20 mM sodium succinate, pH 4.5, 0.055 μM LiPH2, 100 μM H_2O_2, 100 μM guaiacol, 100 μM Mn^{2+}, and the indicated concentrations of oxalate. The values for the concentration of the 1:1 complex of Mn^{2+}−oxalate (O) were calculated as outlined in Experimental Procedures. The initial rate of oxidation of guaiacol (●) by LiPH2 was determined by stopped-flow analysis at 25 °C.

The effect of the concentrations of oxalate, the Mn^{2+}/Mn^{3+} chelator, on the rate of guaiacol oxidation by LiPH2 in the presence of Mn^{2+} is shown in Figure 3. The addition of Mn^{2+} alone resulted in some increase in the rate of guaiacol oxidation by LiPH2, but the addition of the chelator greatly enhanced the effect. This is consistent with the requirement of a chelator for the reduction of LiPH2−compound II by Mn^{2+} (Khindaria et al., 1995). A similar effect was observed upon addition of either malonate or tartrate, both of which also chelate Mn^{2+} (Martell & Smith, 1977) (data not shown). The addition of only 100 μM oxalate resulted in a 4-fold stimulation of the rate of guaiacol oxidation by LiPH2 in the presence of Mn^{2+}. The oxidation rate increased with increasing oxalate concentration up to 1 mM oxalate, beyond which the rates decreased. The rate of guaiacol oxidation had a dependence on oxalate concentration which corresponded to the concentration of the 1:1 complex of Mn^{2+}−oxalate. As the oxalate concentration increased above 1 mM, the fraction of Mn^{2+} present as the 1:1 Mn^{2+}−oxalate complex decreased, accounting for the decreased activity.

It has been shown that the reaction of Mn^{3+} with H_2O_2 results in the production of molecular oxygen (Archibald & Fridovich, 1982). In the present study, we propose that Mn^{3+}, produced form the oxidation of Mn^{2+} by LiPH2, is the oxidant of guaiacol. Therefore, there should be a competition for Mn^{3+} between H_2O_2 and guaiacol. The effect of increasing concentrations of guaiacol on oxygen production by LiPH2, H_2O_2, and Mn^{2+}−oxalate is shown in Figure 4A. As the amount of guaiacol increased, the rate and extent of O_2 production decreased to nearly complete inhibition at 100 μM guaiacol (Figure 4A). This is consistent with the fact that the rate constant for the reaction of Mn^{3+} with guaiacol is an order of magnitude greater than the reaction of Mn^{3+} with H_2O_2 (Table 2). This was further demonstrated by monitoring both guaiacol oxidation and O_2 production in the presence of 100 μM

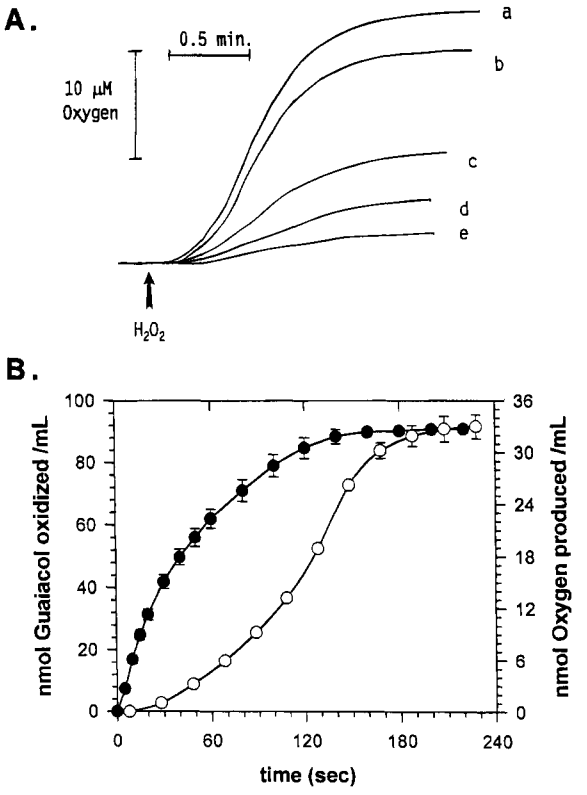

FIGURE 4: Effet of guaiacol on oxygen production by LiPH2 in the presence of Mn^{2+}−oxalate. (A) Reaction mixtures contained 20 mM sodium succinate, pH 4.5, 0.055 μM LiPH2, 100 μM Mn^{2+}, 1 mM oxalate, 100 μM H_2O_2, and varying concentrations of guaiacol: (a) 0 μM; (b) 25 μM; (c) 50 μM; (d) 75 μM; (e) 100 μM guaiacol. (B) Oxidation of guaiacol (●) and oxygen production (○) in the presence of 100 μM guaiacol and 200 μM H_2O_2. All other conditions are the same as in (A). Reactions were initiated by the addition of H_2O_2. All experiments were performed at 25 °C.

Table 2: Ability of Various Substrates To Inhibit Oxygen Production and React with Mn^{3+} [c]

substrate	K_i for inhibition of O_2 production[a] (μM)	rate constant[b] for reaction with Mn^{3+} (M^{-1} s^{-1})
vanillylacetone	35	5×10^3
guaiacol	40	3×10^3
coniferyl alcohol	28	
TMPD	55	7×10^3
2,6-dimethoxyphenol	60	8×10^3
ABTS	350	1×10^3
phenol	1000	
chlorpromazine	125	
H_2O_2		3×10^2

[a] Reaction mixtures contained 20 mM sodium succinate, pH 4.5, 0.055 μm LiPH2, 100 μM H_2O_2, 100 μM Mn^{2+}, 1 mM oxalate, and varying amounts of each substrate. [b] Reaction mixtures contained 20 mM sodium succinate, pH 4.5, 1 mM oxalate, and varying amounts of Mn^{3+}−acetate and each substrate as outlined in Experimental Procedures. [c] All experiments were performed at 25 °C.

guaiacol and 200 μM H_2O_2 (Figure 4B). Initially, there was a high rate of guaiacol oxidation and a low rate of O_2 production. As the concentration of guaiacol decreased over the course of the reaction, its rate of oxidation decreased, while there was a concomitant increase in the rate of O_2 production.

Table 2 contains rate constants for the reaction of Mn^{3+} with each substrate and K_i values for the inhibition of oxygen production during the oxidation of each substrate by LiPH2.

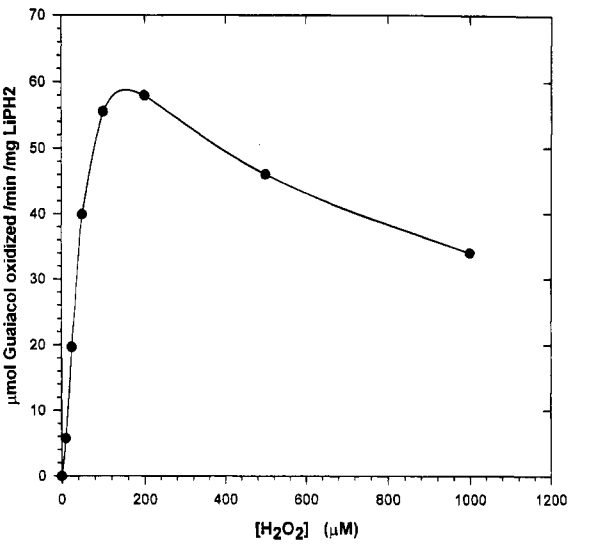

FIGURE 5: Effect of H_2O_2 on the initial rate of guaiacol oxidation by LiPH2. Reaction mixtures contained 20 mM sodium succinate, pH 4.5, 0.055 μM LiPH2, 100 μM Mn^{2+}, 1 mM oxalate, 100 μM guaiacol, and the indicated concentration of H_2O_2. The reactions were carried out at 25 °C.

Vanillylacetone, guaiacol, coniferyl alcohol, TMPD, and 2,6-dimethoxyphenol inhibited O_2 production most efficiently and had the greatest reactivity toward Mn^{3+}. Consistent with these results is the fact that the rate of oxidation of these substrates by LiPH2 was stimulated the most upon addition of Mn^{2+}−oxalate (Table 1).

The effect of H_2O_2 on the rate of guaiacol oxidation by LiPH2 in the presence of Mn^{2+}−oxalate was determined (Figure 5). Consistent with a K_m of 42 μM H_2O_2 (Khindaria et al., 1995), the oxidation rates increased as the concentration of H_2O_2 was initially increased. Increasing the H_2O_2 concentration beyond 200 μM resulted in decreased rates of guaiacol oxidation.

The effect of increasing concentrations of H_2O_2 on the ratio of vanillylacetone oxidized by LiPH2 per equivalent of H_2O_2 added in the presence of Mn^{2+}−oxalate is shown in Figure 6. This experiment was performed to demonstrate the effects of the scavenging of Mn^{3+} by H_2O_2 on the oxidation of vanillylacetone at two different concentrations by LiPH2. The stoichiometry of vanillylacetone oxidized approached 1.0 as the concentrations of H_2O_2 approached zero. As the levels of H_2O_2 increased, the amount of vanillylacetone oxidized per H_2O_2 added decreased. Consistent with a competition for Mn^{3+} between H_2O_2 and vanillylacetone, the increased concentration of H_2O_2 produced less of an effect when a greater amount of vanillylacetone was present. Vanillylacetone was used in place of guaiacol in this experiment because it is difficult to accurately quantitate the stoichiometry of guaiacol oxidation since the product of its oxidation, which is the monitored species, is a polymer (Lindgren, 1960).

DISCUSSION

The results of this investigation demonstrate that the rate of LiPH2-catalyzed oxidation is greatly enhanced in the presence of Mn^{2+} and oxalate. We propose that the increased rate is due to the ability of LiPH2 to rapidly oxidize Mn^{2+} to Mn^{3+}, which is the actual oxidant of the substrates. Evidence for this arises from the fact that the Mn^{2+} dependence of Mn^{2+} oxidation by LiPH2 was the same as

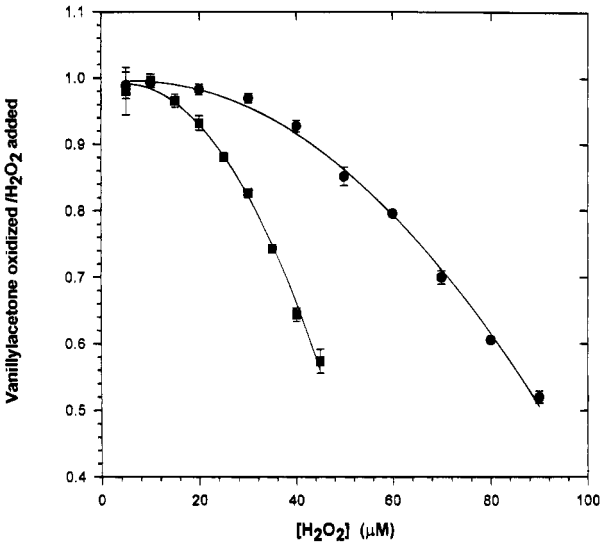

FIGURE 6: Stoichiometry of vanillylacetone oxidized per equivalent of H_2O_2 added. Reaction mixtures were maintained at 25 °C and contained 20 mM sodium succinate, pH 4.5, 0.055 μM LiPH2, 100 μM Mn^{2+}, 1 mM oxalate, the indicated amount of H_2O_2 and either 50 μM (■) or 100 μM (●) vanillylacetone. The curves shown represent second order mathematical fits. The values represent the mean of triplicate measurements. The error bars indicate standard deviation.

that for guaiacol oxidation by LiPH2. The K_m for stimulation of guaiacol oxidation was 24 μM Mn^{2+}, which agrees well with the published K_m value of 19 μM Mn^{2+} for Mn^{2+} oxidation by LiPH2 (Khindaria et al., 1995). The slightly higher K_m found in this study may be due to the greater concentration of H_2O_2 at which it was determined, since the oxidation of Mn^{2+} by LiPH2 is a ping pong bi bi mechanism (Segel, 1975). The guaiacol dependence of the rate of guaiacol oxidation by LiPH2 in the presence of Mn^{2+}–oxalate represents the rate of the reaction of guaiacol with Mn^{3+}. Thus, in the presence of Mn^{2+}–oxalate, the oxidation rates did not obey typical saturation kinetics with respect to the concentration of guaiacol. The oxidation rate of guaiacol should increase with increasing concentrations of guaiacol until the rate of production of Mn^{3+} by the enzyme becomes limiting. Once this occurs, further increases in the concentration of guaiacol should have no effect. Indeed, this was observed. In the presence of Mn^{2+}–oxalate the rate of guaiacol oxidation did not increase as the concentration of guaiacol was increased beyond 50 μM. However, increasing the concentration of Mn^{2+}, until the enzyme is saturated with respect to Mn^{2+}, should increase the rate of guaiacol oxidation. This is consistent with the results of this study.

The role of VA in LiPH2-catalyzed oxidation has been the focus of many investigations (Harvey et al., 1986; Valli et al., 1990; Koduri & Tien, 1994; Chung & Aust, 1995; Goodwin et al., 1995). Our laboratory has demonstrated the ability of VA to act as a mediator of indirect oxidation by LiP (Chung & Aust, 1995; Goodwin et al., 1995). The rates of oxidation of the substrates studied were determined to be much greater in the presence of Mn^{2+}–oxalate than VA over all substrate concentrations. This is attributed to the ability of LiPH2 to oxidize Mn^{2+} at a much higher rate than VA, especially at pH 4.5, the physiological pH of the fungus. A recent study of the ability of VA to act as a mediator of LiPH2-catalyzed oxidations found that the rate constants for the reactions of LiP with VA and chlorpromazine were comparable (Goodwin et al., 1995). The addition of saturating levels of VA to low levels of chlorpormazine during LiP catalysis resulted in the greatest stimulation of the oxidation rate of chlorpromazine (Goodwin et al., 1995). It was proposed that because the amount of VA was saturating, LiP was operating at V_{max} and the product of VA oxidation, the VA cation radical, oxidized chlorpromazine. At saturating levels of chlorpromazine, the addition of VA had essentially no effect on chlorpromazine oxidation by LiP. LiP was already operating at V_{max}, which, due to the comparable rate constants, was the same in the presence of either saturating amounts of VA or chlorpromazine. This relationship with VA appeared to be consistent with the substrates used in this study. However, the presence of Mn^{2+} resulted in increased rate of turnover of LiPH2, and Mn^{3+} is capable of oxidizing many organic substrates. Therefore, Mn^{2+}–oxalate stimulated the rate of oxidation by LiPH2 even at saturating levels of each substrate. In accord with the ability of Mn^{2+} to effectively inhibit VA oxidation (Khindaria et al., 1995), the addition of VA had no effect on the ability of Mn^{2+}–oxalate to stimulate LiPH2-catalyzed oxidation. It is proposed that Mn^{2+}–oxalate is a more physiologically significant mediator than VA for long range electron transfer reactions catalyzed by LiPH2.

Malonate, tartrate, and oxalate were all found to effectively stimulate the rate of guaiacol oxidation in the presence of Mn^{2+}. The effect of oxalate was studied since it is proposed to be the physiological chelator of Mn^{2+}/Mn^{3+} in the extracellular environment of *P. chrysosporium* (Kuan & Tien, 1993). The reduction of the compound II form of both LiPH2 and MnP to ferric enzyme by Mn^{2+} has been shown to require chelated Mn^{2+} (Kuan et al., 1993; Khindaria et al., 1995). Furthermore, this reaction with MnP has been shown to be specific for the 1:1 complex of Mn^{2+}–oxalate (Kuan et al., 1993). Accordingly, it was found that, in the presence of Mn^{2+}, the oxalate dependence of the rate of guaiacol oxidation by LiPH2 corresponded with the concentration of the 1:1 complex of Mn^{2+}–oxalate. This indicates that the 1:1 complex of Mn^{2+}–oxalate is also the preferred substrate of LiPH2–compound II and provides further evidence that the redox couple, Mn^{2+}/Mn^{3+}, is acting as a mediator of guaicol oxidation by LiPH2. Although the rate of guaiacol oxidation decreased more with increasing oxalate concentration than the concentration of the 1:1 complex, this phenomenon was also observed during the reduction of MnP compound II by Mn^{2+}–oxalate (Kuan et al., 1993).

The proposed role of LiPH2 in these reactions is to oxidize Mn^{2+} to Mn^{3+}, which oxidizes the substrates of interest. Therefore, the reactions of Mn^{3+} were further investigated. It has been shown that Mn^{3+} can also oxidize H_2O_2, resulting in O_2 production (Archibald & Fridovich, 1982). It was proposed by Khindaria and co-workers that this reaction is responsible for the O_2 production which occurs in reaction mixtures containing LiPH2, H_2O_2, and Mn^{2+}–oxalate. Therefore, the enzymatically produced Mn^{3+} may oxidize either H_2O_2 or an organic substrate. The competition for Mn^{3+} between H_2O_2 and organic substrate was demonstrated in this study. The rate and extent of O_2 production by LiPH2, H_2O_2, and Mn^{2+}–oxalate were effectively inhibited by guaiacol, which appeared to be preferentially oxidized by Mn^{3+}. This is consistent with the rate constants for the reactions of Mn^{3+}, which were approximately an order of magnitude greater for guaiacol than for H_2O_2. This was true for almost all of the substrates studied. It was also shown

that the ratio of vanillylacetone oxidized per equivalent of H_2O_2 added decreased with increasing concentrations of H_2O_2. As the H_2O_2 levels increased, two Mn^{3+} would still be produced per turnover of the enzyme, but the amount of Mn^{3+} which reacted with vanillylacetone would be reduced. In addition, the effect of the increasing H_2O_2 levels was less pronounced in the presence of more substrate, which further indicates competing reactions. The ability of each substrate to inhibit O_2 production, which is a reflection of its reactivity with Mn^{3+}, correlated well with the amount that the rate of oxidation of each substrate was stimulated upon addition of Mn^{2+}−oxalate. This is in agreement with the proposed mechanism of oxidation by LiPH2 in the presence of Mn^{2+}−oxalate. The overall effect of the reactivity of H_2O_2 in this system is exemplified by the dependence of the rate of guaiacol oxidation on the concentration of H_2O_2. The rate increased with initial increases in H_2O_2 concentration, consistent with a K_m of 42 μM for H_2O_2 reduction by ferric LiPH2 in the first step of the peroxidase catalytic cycle (Khindaria et al., 1995). With continued increases in the concentration of H_2O_2, the increasing effects of the scavenging of Mn^{3+} by H_2O_2 are evidenced by the decreased rate of guaiacol oxidation. While the rate of guaiacol oxidation by LiPH2 did decrease at very high levels of H_2O_2, even at 1 mM H_2O_2 the rate of guaiacol oxidation is still stimulated tremendously by the addition of Mn^{2+}−oxalate.

In conclusion, Mn^{2+} in the presence of oxalate has been shown to dramatically stimulate the rate of LiPH2-catalyzed oxidations. Furthermore, Mn^{2+}−oxalate was demonstrated to be a much more effective mediator than VA. This is proposed to be due to the high rate of LiPH2-catalyzed production of Mn^{3+}, a diffusible oxidant which can perform mediation through electron transfer reactions. The physiological relevance of this study is stressed. The experiments were performed at pH 4.5, the physiological pH of the extracellular environment of *P. chrysosporium*, and levels of Mn^{2+} and oxalate which could exist *in vivo*. This effect has been demonstrated for several substrates which represent monomeric components of lignin, in particular, coniferyl alcohol, which is the most common natural lignin precursor (Sarkanen & Ludwig, 1971). Because LiPH2 is the major peroxidase isozyme produced under our culture conditions, a greater importance of Mn^{2+} and oxalate is implicated in both lignin and pollutant degradation by *P. chrysosporium*.

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