Lignin Peroxidase Oxidation of Mn^{2+} in the Presence of Veratryl Alcohol, Malonic or Oxalic Acid, and Oxygen†

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ABSTRACT: Veratryl alcohol (3,4-dimethoxybenzyl alcohol) appears to have multiple roles in lignin degradation by *Phanerochaete chrysosporium*. It is synthesized de novo by the fungus. It apparently induces expression of lignin peroxidase (LiP), and it protects LiP from inactivation by H_2O_2. In addition, veratryl alcohol has been shown to potentiate LiP oxidation of compounds that are not good LiP substrates. We have now observed the formation of Mn^{3+} in reaction mixtures containing LiP, Mn^{2+}, veratryl alcohol, malonate buffer, H_2O_2, and O_2. No Mn^{3+} was formed if veratryl alcohol or H_2O_2 was omitted. Mn^{3+} formation also showed an absolute requirement for oxygen, and oxygen consumption was observed in the reactions. This suggests involvement of active oxygen species. In experiments using oxalate (a metabolite of *P. chrysosporium*) instead of malonate, similar results were obtained. However, in this case, we detected (by ESR spin-trapping) the production of carbon dioxide anion radical ($CO_2^{\bullet-}$) and perhydroxyl radical ($^{\bullet}OOH$) in reaction mixtures containing LiP, oxalate, veratryl alcohol, H_2O_2, and O_2. Our data indicate the formation of oxalate radical, which decays to CO_2 and $CO_2^{\bullet-}$. The latter reacts with O_2 to form $O_2^{\bullet-}$, which then oxidizes Mn^{2+} to Mn^{3+}. No radicals were detected in the absence of veratryl alcohol. These results indicate that LiP can indirectly oxidize Mn^{2+} and that veratryl alcohol is probably a radical mediator in this system.

Extracellular enzymes associated with lignin degradation by the basidiomycetous fungus *Phanerochaete chrysosporium* are lignin peroxidase (LiP),[1] manganese peroxidase (MnP), and the H_2O_2-generating enzyme glyoxal oxidase (Kirk & Farrell, 1987). LiP oxidizes aromatic nuclei of lignin to cation radicals that react nonenzymatically, resulting in cleavages in both the aliphatic side chains and the aromatic nuclei. MnP, related to LiP, oxidizes Mn^{2+} to Mn^{3+}. Mn^{3+} can oxidize phenolic units of lignin and has been suggested also to be involved in lignin fragmentation (Wariishi et al., 1989). In addition to these enzymes, an aromatic metabolite, 3,4-dimethoxybenzyl (veratryl) alcohol, seems to be a component of the ligninolytic system of *P. chrysosporium*.

Veratryl alcohol apparently has multiple roles in lignin biodegradation. It stimulates production of LiP (Faison & Kirk, 1985), and it protects LiP from inactivation by H_2O_2 (Tonon & Odier, 1988). It also has been shown to potentiate LiP oxidation of compounds that are not good LiP substrates (Harvey et al., 1986).

We have now found that LiP in the presence of veratryl alcohol, malonic or oxalic acid, and oxygen oxidizes Mn^{2+} to Mn^{3+}. These results provide further evidence that veratryl alcohol functions in part as an electron transfer agent or mediator of oxidations of non-LiP substrates. Because oxalate is a normal metabolite of *P. chrysosporium*, it is possible that LiP, like MnP, oxidizes Mn^{2+} under physiological conditions.

Experimental Procedures

Enzymes. LiP was purified from extracellular fluid of cultures of *P. chrysosporium* BKM-F-1767 (Kirk et al., 1986). The isozyme isolated had a p*I* of 3.7 and was most likely isozyme LiP 4 (Farrell et al., 1989). Enzyme activity was determined by veratryl alcohol oxidation (Kirk et al., 1986).

Spectrophotometric Assays for Oxidation of Mn^{2+}. All experiments were performed with a Philips PU8800 spectrophotometer. The concentration of Mn^{3+} in the reaction mixtures was determined as follows: A standard solution of manganese(III) acetate was made in malonate, pH 3.0, [0.1 mM manganese(III) acetate, 1 mM malonate]. This solution had an absorbance maximum at 266 nm. The manganese(III) acetate used was found to contain 10% (molar ratio) Mn^{2+} (determined by ESR), and the corrected molar extinction coefficient at 266 nm for Mn^{3+}/malonate was determined to be 11 500. For the Mn^{3+}/oxalate complex, the ϵ_{266} was found to be 6600. To determine the amount of Mn^{3+} and veratraldehyde formed in a reaction, the absorbance was monitored at 266 nm for the times indicated and the reaction terminated (when the absorbance leveled off) by addition of 1 mM dithionite. This reduces Mn^{3+}, and the decrease in absorbance at 266 nm was used to calculate the amount of Mn^{3+} formed. Immediately afterward, the spectrophotometer was set to 400 nm and zeroed, and the sample was scanned from 400 to 200 nm. The amount of veratraldehyde formed was calculated from the absorbance at 310 nm.

ESR Measurements. ESR measurements were performed as previously described (Hammel et al., 1985). For quantitation of Mn^{2+}, reaction mixtures contained 50 mM malonate, pH 3.5, 0.2 mM veratryl alcohol, 0.25 mM $MnSO_4$, 0.3 unit/mL LiP, and 0.05 mM H_2O_2. Spectral scans were recorded approximately 10 min after addition of H_2O_2, and controls were as noted in the text. In experiments for detection of radicals, reaction mixtures contained 25 mM oxalate, pH 4.0, 0.2 mM veratryl alcohol, 0.4 unit/mL LiP, 44 mM DMPO or 50 mM PBN (Sigma Chemical Co., St. Louis,

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[1] Abbreviations: LiP, lignin peroxidase; MnP, manganese peroxidase; VA, veratryl alcohol; OA, oxalate dianion; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; PBN, α-phenyl-*N*-*tert*-butylnitrone.

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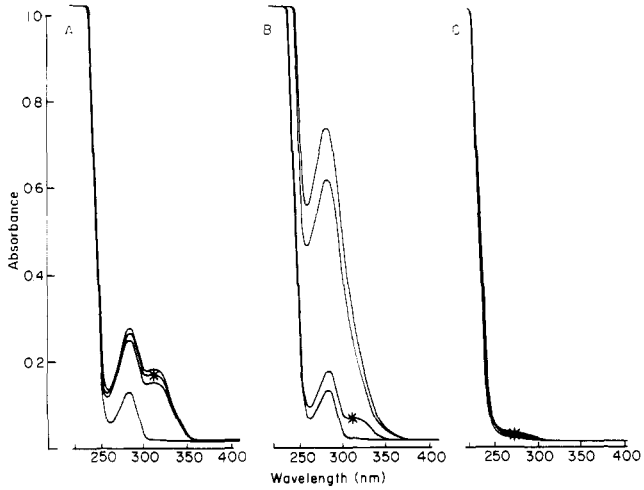

FIGURE 1: UV spectra of reaction mixtures containing 50 mM malonate, pH 3.5, 0.1 unit/mL LiP, and 0.1 mM veratryl alcohol. The lowest spectrum was taken before the addition of 0.1 mM H_2O_2, and the scans were repeated at 2-min intervals thereafter. Asterisks indicate spectra taken immediately after the addition of 1 mM dithionite. Panel A, without $MnSO_4$; panel B, plus 5 mM $MnSO_4$; panel C, plus 5 mM $MnSO_4$ and minus veratryl alcohol.

MO), and 0.1 mM H_2O_2. These reaction mixtures were saturated with air or oxygen as indicated in the text. [^{13}C]-Oxalate (90 atom %) was obtained from Merck, Sharp and Dohme Isotopes (Montreal, Canada).

Oxygen Uptake Experiments. To measure O_2 consumption during LiP reactions, a YSI Model 53 oxygen monitor fitted with a Gilson single-port 1.5-mL reaction chamber was used. Reaction mixtures contained 25 mM malonate or 10 mM oxalate (both at pH 3.0), 0.05 mM veratryl alcohol, and 0.1 unit/mL LiP. Reactions were initiated by addition of 0.05 mM H_2O_2 except where noted. Bovine catalase (Sigma Chemical Co.), when added, was used at a concentration of 9.3 units/mL (at pH 3.0).

Formation of $^{14}CO_2$ from [^{14}C]Oxalate. The complete reaction mixture contained 25 mM 2,2-dimethylsuccinate, pH 4.0, 2 mM veratryl alcohol, 2 mM unlabeled oxalate, 2.8 μCi of [^{14}C]oxalate (8.8 mCi/mmol, Sigma Chemical Co.), and 0.2 unit/mL LiP in a volume of 0.5 mL in a 25-mL flask fitted with a rubber septum. Deviations from these conditions are noted in the text. H_2O_2 (0.2 mM) was added five times at 30-min intervals for a total of 1 mM. Thirty minutes after the final addition, the flasks were flushed with air for 10 min and the $^{14}CO_2$ was trapped in ethanolamine containing scintillation fluid (Kirk et al., 1975). Radioactivity was determined in a Tricarb 4530 scintillation counter (Packard Instruments Co., Downers Grove, IL).

RESULTS

The initial discovery was that, in malonate-buffered reactions, LiP oxidized Mn^{2+} in the presence of veratryl alcohol and H_2O_2. Further examination revealed that O_2 was also required and was consumed during the reaction. Substitution of the malonate with the *P. chrysosporium* metabolite oxalate yielded similar results. In the oxalate reaction, carbon dioxide anion radical and CO_2, as well as $O_2^{\bullet-}$, were formed.

Formation of Mn^{3+} in LiP Reactions. Incubation of LiP with veratryl alcohol, $MnSO_4$, malonate buffer, and H_2O_2 led to the formation of a compound absorbing at 266 nm, which is an absorption maximum for the Mn^{3+}/malonate complex (Figure 1). This absorbing compound was sensitive to dithionite, as is Mn^{3+} (Figure 1B). When manganese was omitted from the reaction mixture, absorbance increased at

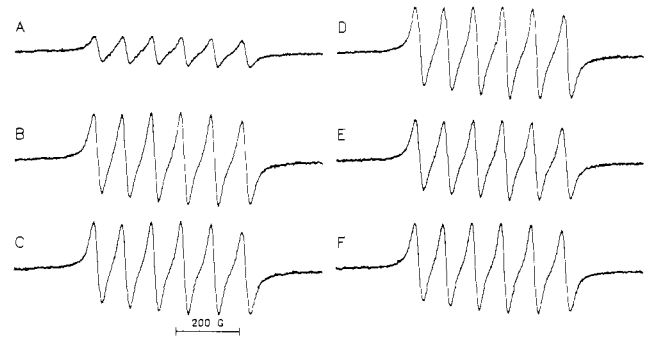

FIGURE 2: ESR spectra of LiP reactions in the presence of $MnSO_4$. The complete reaction mixture contained 50 mM malonate, pH 3.5, 0.2 mM veratryl alcohol, 0.25 mM $MnSO_4$, 0.3 unit/mL LiP, and 50 μM H_2O_2. (A) Complete reaction mixture; (B) complete plus 1 mM dithionite; (C) complete reaction mixture saturated with argon; (D) complete minus H_2O_2; (E) complete minus veratryl alcohol; (F) complete minus LiP.

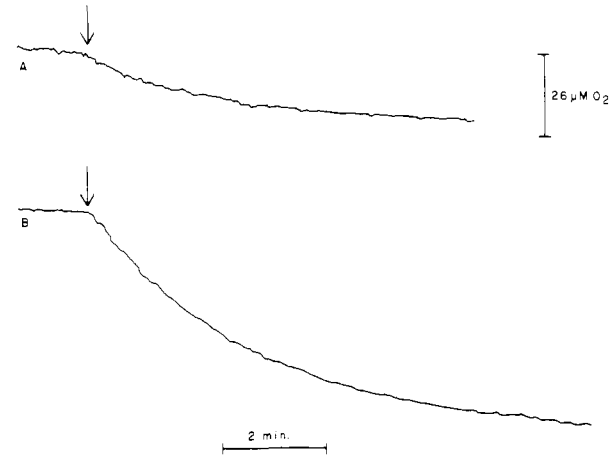

FIGURE 3: Oxygen consumption in LiP reactions in malonate buffer. Reaction mixtures contained 50 mM malonate, pH 3.5, 30 μM veratryl alcohol, and 0.3 unit/mL LiP. Arrows indicate addition of 50 μM H_2O_2. (A) In the absence of 1 mM $MnSO_4$; (B) in the presence of 1 mM $MnSO_4$.

310 nm and at 266 nm but was not sensitive to dithionite (Figure 1A). This is consistent with the formation of veratraldehyde (which absorbs at 310 nm but which also absorbs at 266 nm). The 310-nm absorbance increase was partially suppressed by Mn^{2+} (cf. Figure 1, panels A and B). In the absence of veratryl alcohol, absorbance in this range was negligible (Figure 1C). ESR studies showed a decrease in the concentration of Mn^{2+} in the complete reaction mixture (LiP, malonate, veratryl alcohol, H_2O_2, and O_2) that was associated with the dithionite-sensitive absorbance at 266 nm (Figure 2). The decrease in Mn^{2+} concentration was dependent on the presence of H_2O_2, veratryl alcohol, and LiP and was reversed on addition of dithionite (Figure 2). Together, these results indicate that Mn^{3+} is formed in the complete reaction mixture. We further verified that the decrease in the Mn^{2+} concentration (0.16 mM) was similar to the concentration of Mn^{3+} formed (0.11 mM), as determined by the spectrophotometric assay. The decrease in Mn^{2+} concentration observed by ESR was dependent on the presence of oxygen (Figure 2). This result indicates that, in addition to the components listed above, O_2 is required for the formation of Mn^{3+}. Accordingly, oxygen consumption is stimulated by Mn^{2+} in the reaction mixture (Figure 3).

The amounts of Mn^{3+} and veratraldehyde formed in this reaction were determined spectrophotometrically as described under Experimental Procedures (Table I). We found that the rate of increase in A_{266} as well as the final amounts of Mn^{3+}

Table I: Veratraldehyde and Mn^{3+} Formed in LiP Reactions Containing Malonate[a]

LiP (unit/mL)	initial rate (A_{266}/min)	veratraldehyde (nmol)	Mn^{3+} (nmol)	equiv oxidized[b] (nmol)
0.1	0.39	16	76	108
0.2	0.48	23	98	144
0.3	0.70	25	112	162

[a] Reaction mixtures contained 50 mM malonate, pH 3.5, 30 μM veratryl alcohol, 1 mM $MnSO_4$, 0.1 unit/mL LiP, and 50 μM H_2O_2. [b] The formation of Mn^{3+} requires 1 oxidizing equiv and formation of veratraldehyde requires 2 oxidizing equiv; therefore, total oxidizing equiv = nmol of Mn^{3+} + 2(nmol of veratraldehyde).

Table II: Veratraldehyde and Mn^{3+} Formed in LiP Reactions Containing Oxalate[a]

oxalate (mM)	veratraldehyde (nmol)	Mn^{3+} (nmol)
25	0	6.1
10	0	91
1	14	119

[a] Reaction mixtures contained oxalate, pH 3.0, 50 μM veratryl alcohol, 1 mM $MnSO_4$, 0.1 unit/mL LiP, and 50 μM H_2O_2.

Table III: Formation of Veratraldehyde by LiP in Various Buffers[a]

buffer (pH)	rel rate of veratraldehyde formation
tartrate (3.0)	100
malonate (3.0)	85
2,2-dimethylsuccinate (3.0)	106
oxalate (3.0)	0
2,2-dimethylsuccinate (4.0)	76
2,2-dimethylsuccinate (4.0) + 2 mM oxalate	13

[a] Assay mixtures contained 25 mM buffer, 2 mM veratryl alcohol, 0.1 unit/mL LiP, and 0.4 mM H_2O_2.

and veratraldehyde formed were dependent on the concentration of LiP in the reaction mixture. At the higher concentrations of LiP, it was apparent that the equivalents of oxidized products (veratraldehyde plus Mn^{3+}, 144 and 162 nmol) were greater than the equivalents of added H_2O_2 (100 nmol).

Lignin Peroxidase Reactions in the Presence of Oxalate. When oxalate was used in place of malonate in these reactions, Mn^{3+} was also formed. However, the amount of Mn^{3+} increased when the initial oxalate concentration was decreased (Table II). This may be due to inhibition of veratryl alcohol oxidation by LiP in the presence of oxalate (Table III). At pH 3, solutions of tartrate, malonate, and 2,2-dimethylsuccinate (25 mM) all supported significant veratraldehyde formation by LiP and H_2O_2. In contrast, 25 mM oxalate at pH 3 allowed no veratraldehyde formation. In fact, addition of a lower concentration of oxalate (2 mM) to a 2,2-dimethylsuccinate-buffered system (pH 4) caused a 6-fold reduction in the rate of veratraldehyde formation. We examined the kinetics of oxalate inhibition of veratryl alcohol oxidation in 25 mM tartrate buffer, pH 3.0. It appears to be a noncompetitive inhibition with apparent K_i = 790 μM.

We monitored oxygen concentration in this reaction and observed that, in the absence of Mn^{2+}, oxygen consumption stopped after 4–5 min (as was the case with malonate buffer, Figure 4A). In the presence of Mn^{2+}, O_2 concentration decreased until it was depleted; addition of catalase at this time caused a transient rise in O_2 concentration (Figure 4, panels B and D), indicating that H_2O_2 had been formed. In accord, oxygen consumption in the presence of Mn^{2+} could be initiated by a catalytic amount of H_2O_2 (5 μM, Figure 4C). In this case there was a lag in the rate of O_2 consumption (compared

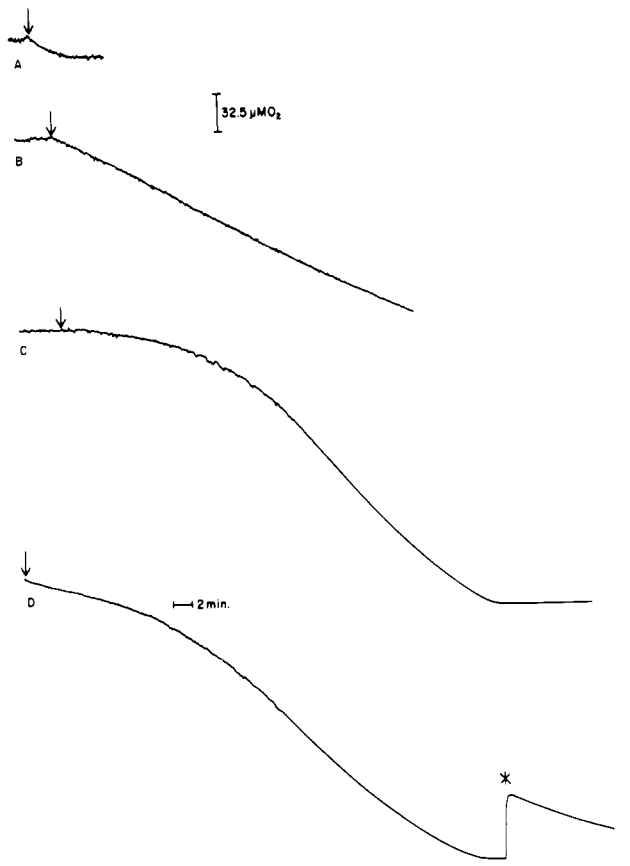

FIGURE 4: Oxygen consumption in LiP reactions in oxalate buffer. Reaction mixtures contained 10 mM oxalate, pH 3.0, 0.1 unit/mL LiP, and 50 μM veratryl alcohol. Arrows indicate addition of H_2O_2. (A) Standard reaction mixture, 50 μM H_2O_2; (B) plus 1 mM $MnSO_4$, 50 μM H_2O_2; (C) same conditions as in (B) but with 5 μM H_2O_2; (D) same conditions as in (C), asterisk indicates addition of 9.3 units/mL catalase.

to addition of 50 μM H_2O_2), but it eventually increased and all the oxygen was consumed.

ESR Spin-Trapping Studies. ESR studies provided insight into the reactions involving oxalate. Figure 5A shows the ESR spectrum obtained from an aerobic reaction mixture containing veratryl alcohol, H_2O_2, LiP, and the spin-trapping agent DMPO in oxalate buffer. If any one of the above components was omitted, no signal was obtained. When purged with 100% oxygen, the reaction mixture gave a six-line spectrum that was very much reduced in intensity and superimposed by a second signal (Figure 5C). On the basis of the literature data (Connor et al., 1986), the six-line spectrum was assigned to a DMPO–CO_2^- adduct (a^N = 15.7 G, a_β^H = 18.7 G), formed by trapping $CO_2^{\bullet-}$ by DMPO. The new signal appearing in the oxygen-saturated reaction mixture was assigned to a DMPO–OOH adduct (a^N = 14.0 G, a_β^H = 11.5 G), formed by trapping $^\bullet$OOH by DMPO. Because of oxygen broadening, the γ-hydrogen couplings in DMPO–OOH could not be resolved.

To confirm that the DMPO–CO_2^- species originated from oxalate, the [^{12}C]oxalate buffer was replaced by [^{13}C]oxalate (90 atom %) and the ESR spectrum again recorded. The rationale for this experiment is as follows: The nuclear magnetic moment of carbon-12 is zero, so there is no detectable hyperfine coupling from the attached ^{12}C nucleus in the DMPO–$^{12}CO_2^-$ adduct. However, since the magnetic moment of ^{13}C is $^1/_2$, one should observe a detectable coupling from the ^{13}C nucleus in the DMPO–$^{13}CO_2^-$ adduct. In accord, the spectrum shown in Figure 5B was obtained in the presence of [^{13}C]oxalate buffer, all other conditions being the same.

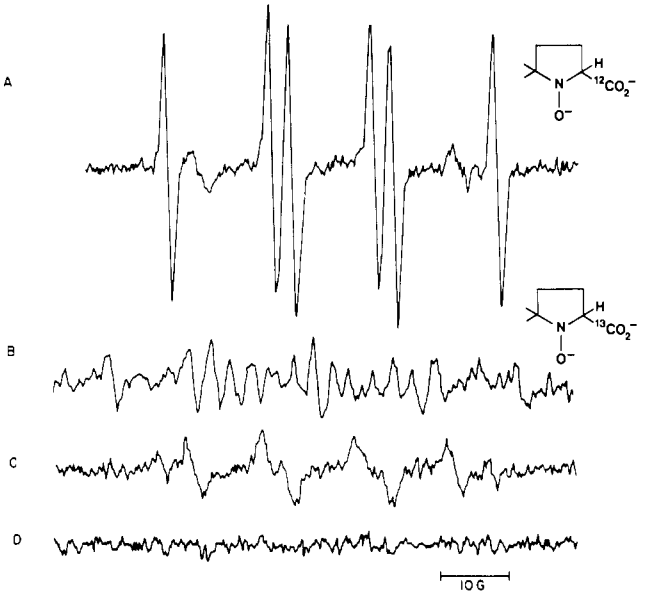

FIGURE 5: ESR spectra of the spin adducts obtained during the oxidation of veratryl alcohol by LiP in the presence of oxalate and DMPO. Reaction conditions are described under Experimental Procedures. (A) Air-saturated reaction mixture containing [^{12}C]-oxalate; (B) air-saturated reaction mixture containing [^{13}C]oxalate; (C) oxygen-saturated reaction mixture containing [^{12}C]oxalate; (D) air-saturated reaction mixture containing [^{12}C]oxalate and minus veratryl alcohol.

This spectrum was assigned to a mixture of DMPO–$^{13}CO_2^-$ adduct (a^N = 15.7 G, a_β^H = 18.7 G, a_β^{13C} = 12.0 G) and DMPO–$^{12}CO_2^-$ adduct.

A similar set of experiments was carried out with the spin trap PBN. Figure 6A shows the ESR spectrum from an aerobic incubation mixture containing veratryl alcohol, H_2O_2, PBN, and LiP in [^{12}C]oxalate buffer. The spectrum comprises a mixture of spin adducts, PBN–$^{12}CO_2^-$ adduct (a^N = 15.9 G, a^H = 4.6 G), and an unknown adduct (a^N = 14.6 G, a^H = 2.5 G). When the reaction was carried out after the solution was purged with 100% oxygen, the intensity of the PBN–$^{12}CO_2^-$ adduct decreased and that of the unknown adduct increased slightly (Figure 6C). Replacement of [^{12}C]oxalate with [^{13}C]oxalate in the reaction mixture in air yielded a spectrum composed of PBN–$^{13}CO_2^-$ adduct (a^N = 15.9 G, a_β^H = 4.6 G, and a_β^{13C} = 11.7 G) and the same unknown species (Figure 6B). In the presence of 100% oxygen, the spectrum due to PBN–$^{13}CO_2^-$ was absent, whereas the spectrum of the unknown adduct remained unchanged (Figure 6, panels C and D). This indicates that the unknown species is not derived from oxalate. The presence of veratryl alcohol was necessary to give the ESR signals in all of these incubations.

Evolution of $^{14}CO_2$ from [^{14}C]Oxalate. The oxidation of oxalate by the LiP/veratryl alcohol/H_2O_2/O_2 system should produce CO_2. This was confirmed with ^{14}C-labeled oxalate (Table IV). In these experiments, 2,2-dimethylsuccinate at pH 4 was the buffer. In the complete reaction, most of the added ^{14}C label was recovered as $^{14}CO_2$. In the presence of the spin-trapping agents DMPO or PBN, the amount of $^{14}CO_2$ recovered was decreased by approximately 50%; in the absence of LiP, veratryl alcohol, or H_2O_2, no $^{14}CO_2$ was formed.

Discussion

Our findings allow the formulation of an overall reaction scheme in which LiP, in the presence of H_2O_2, veratryl alcohol, O_2, and oxalate, oxidizes Mn^{2+} to Mn^{3+} (Figure 7). We propose that LiP oxidizes veratryl alcohol and this radical oxidizes oxalate, regenerating veratryl alcohol. The breakdown

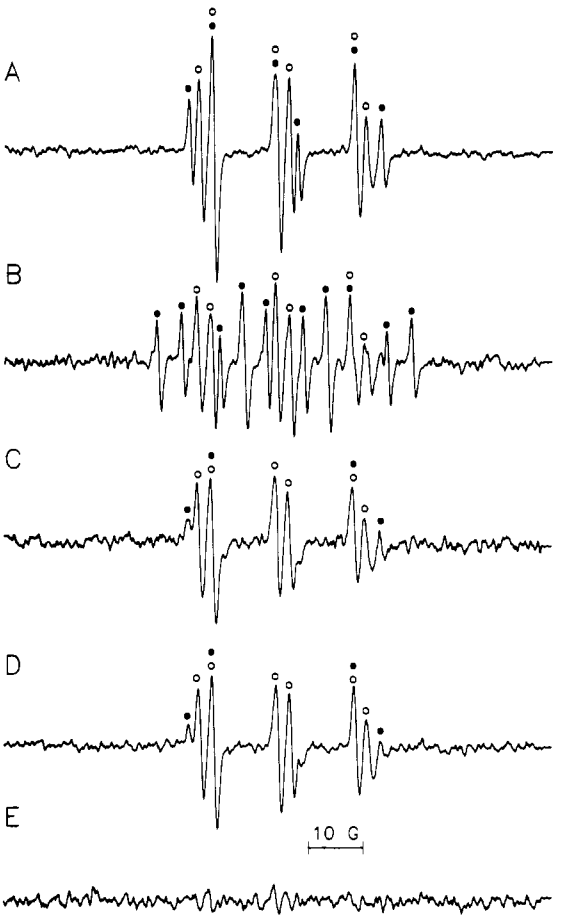

FIGURE 6: ESR spectra of the spin adducts obtained during the oxidation of veratryl alcohol by LiP in the presence of oxalate and PBN. Reaction conditions are described under Experimental Procedures. (A) Air-saturated reaction mixture containing [^{12}C]oxalate; (B) air-saturated reaction mixture containing [^{13}C]oxalate; (C) oxygen-saturated reaction mixture containing [^{12}C]oxalate; (D) oxygen-saturated reaction mixture containing [^{13}C]oxalate; (E) air-saturated reaction mixture containing [^{12}C]oxalate and minus veratryl alcohol. Filled circles designate the lines assigned to the PBN–CO_2^- adducts, and open circles indicate the lines assigned to the unidentified species.

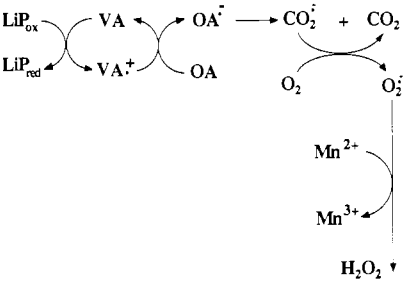

FIGURE 7: Proposed mechanism for the LiP oxidation of Mn^{2+} in the presence of veratryl alcohol (VA), oxalate (OA), and oxygen.

products of the oxalate radical, CO_2 and $CO_2^{\cdot-}$, have been identified by ESR spin-trapping studies (Figures 5 and 6) and ^{14}C-labeling experiments (Table IV). The 50% inhibition of $^{14}CO_2$ evolution from [^{14}C]oxalate in the presence of spin-trapping agents is consistent with the breakdown of the oxalate radical to CO_2 and the formate radical. Malonate can also function in the LiP oxidation of Mn^{2+}, but we did not identify the putative intermediate radical species.

The proper pH of the oxalate buffer was critical to the detection of radical adducts of the carbon dioxide anion radical. Neither PBN–CO_2^- nor DMPO–CO_2^- could be detected from incubations carried out in oxalate buffers at pH < 4. This

Table IV: $^{14}CO_2$ Evolution from [^{14}C]Oxalate

reaction mixture[a]	dpm	μmol of $^{14}CO_2$
complete	3.8×10^6	1.40
+DMPO	1.6×10^6	0.68
+PBN	1.6×10^6	0.68
−LiP	2.5×10^3	<0.01
$-H_2O_2$	6.8×10^3	<0.01
−veratryl alcohol	2.9×10^3	<0.01

[a] Complete reaction mixture (0.5 mL) contained 25 mM 2,2-dimethylsuccinate, pH 4.0, 2 mM veratryl alcohol, 2.66 mM oxalate (6.2×10^6 dpm), 0.2 unit/mL LiP, and a total of 1 mM H_2O_2 added in five equal aliquots over 2.5 h. The concentration of DMPO and PBN was 20 mM.

can be explained, in part, in terms of the pH dependency of the radical adducts. For example, the pK_a of the PBN–CO_2^- adduct is approximately 2.8. Since the ionized form of the adduct (PBN–CO_2^-) probably decays more slowly than the nonionized form (PBN–CO_2H), it is clearly advantageous to use higher pHs (Connor et al., 1986).

In order to determine whether both PBN–CO_2^- and DMPO–CO_2^- were formed by trapping of the carbon dioxide anion radical, it was essential to demonstrate that these adducts were sensitive to molecular oxygen. These adducts have previously been shown to be formed via a nonradical mediated mechanism. The present data demonstrating the effect of oxygen on the formation of these adducts (Figures 5 and 6) are, therefore, highly significant. To our knowledge, this is the first demonstration of the production of superoxide adducts from the reaction between $CO_2^{\bullet-}$ and O_2 (Wong et al., 1988). Past difficulty has been attributed to the low reaction rate constant between $O_2^{\bullet-}$ and the spin trap (Wong et al., 1988). In the present work, the superoxide radical formed in the oxalate buffer should be significantly protonated (perhydroxyl radical) since the pK_a of $O_2^{\bullet-}$ is 4.8. The reason why we could observe the DMPO–OOH adduct in our experiments is probably due to the relatively higher rate constant (10^3 M^{-1} s^{-1}) for the reaction between $^{\bullet}$OOH and DMPO (Finkelstein et al., 1986). Unfortunately, we could not verify the production of perhydroxyl radical by using PBN due to spectral complexity. The observed inhibition of $^{14}CO_2$ evolution in the presence of spin traps also implicates the intermediacy of the carbon dioxide anion radical.

LiP does not oxidize Mn^{2+} or oxalate directly. Because O_2 is required, it follows that the carbon dioxide anion radical does not oxidize Mn^{2+} directly. The carbon dioxide anion radical is known to reduce oxygen to superoxide ($k = 10^9$ M^{-1} s^{-1}), forming carbon dioxide (Adams & Wilson, 1969). Perhydroxyl radical (the protonated form of $O_2^{\bullet-}$) was also identified in ESR spin-trapping studies (Figure 5). $O_2^{\bullet-}$/$^{\bullet}$OOH is a well-known Mn^{2+} oxidant (Kono et al., 1976; Archibald & Fridovich, 1982) and is most likely the species responsible for Mn^{2+} oxidation in this reaction. We observed O_2 consumption in these reactions and H_2O_2 functioned catalytically, initiating O_2-consuming reactions (Figure 4). H_2O_2 is also produced in these reactions, as addition of catalase causes an increase in O_2 concentration greater than can be derived from the amount of H_2O_2 added initially. These findings, therefore, support the sequence shown in Figure 7. (Incidentally, our results provide a biochemical method for producing hydroperoxyl radical that might be useful).

The inhibition of LiP-catalyzed oxidation of veratryl alcohol by oxalate appears to be noncompetitive. This is consistent with the proposed mechanism of oxalate oxidation, which includes oxalate binding to the proposed veratryl alcohol radical. These results do not distinguish between an enzyme-bound and a free veratryl alcohol radical. Another possible explanation for oxalate inhibition is that the superoxide formed from oxalate oxidation inactivates LiP (Wariishi & Gold, 1989) and the role of veratryl alcohol is to protect the enzyme from inactivation. However, since the inhibition by oxalate is not competitive, a simple model of veratryl alcohol oxidation competing with oxalate oxidation (which leads to enzyme inactivation and subsequent regeneration by veratryl alcohol) is not correct. More work is needed to elucidate the exact mechanism for this reaction.

When malonate was used in the reaction in place of oxalate, the intermediates involved were not identified. Its involvement might be different from that of oxalate. One possible mechanism is addition of oxygen to a malonate C-centered radical, forming a peroxyl radical. A malondialdehyde peroxyl radical has been proposed by MacDonald and Dunford (1989) to oxidize Mn^{2+}, generating Mn^{3+} and a hydroperoxide. A similar mechanism could be involved in our system.

The scheme shown in Figure 7 might have physiological significance. LiP, veratryl alcohol, and H_2O_2-generating enzymes are secreted by the fungus, and Mn^{2+} is prevalent in wood (Young & Guinn, 1966). Oxalate has been detected in cultures of white-rot fungi (Takao, 1965), and we have found oxalate production by *P. chrysosporium* BKM-F-1767 (data not shown), consistent with that report. Thus, all components necessary for LiP oxidation of Mn^{2+} are present in fungus-colonized wood.

It is not yet clear how important Mn^{3+} is in lignin degradation. Because it oxidizes phenolic units to phenoxy radical species, which have been shown to lead to interunit cleavages in certain lignin model compounds, it might play that role. Indirect evidence also points to a role for Mn^{3+}. It has been shown recently that, when grown with high Mn concentrations, cultures of *P. chrysosporium* produce no detectable LiP but high levels of MnP; those cultures still degrade lignin, albeit at a reduced rate (Bonnarme & Jeffries, 1990). Also, certain lignin-degrading fungi create deposits of MnO_2 in lignin degradation zones of wood (Blanchette, 1984); these deposits could well reflect MnO_2 formation via disproportionation of Mn^{3+}. Despite these observations, however, further work is required to define the role of Mn^{3+}, if any.

Perhaps more significantly than demonstrating that LiP can oxidize Mn^{2+}, our results provide support for the previous evidence (Harvey, 1986) that veratryl alcohol participates in LiP-catalyzed oxidation of non-LiP substrates, possibly as an enzyme-associated mediator or electron transfer agent (Harvey et al., 1989). Our results show that veratryl alcohol enables LiP to oxidize oxalate and Mn^{2+}, suggesting that radical transfer from veratryl alcohol to oxalate occurs. Similar radical transfer reactions could also be involved in LiP oxidation of lignin. However, despite the evidence for a veratryl alcohol cation radical, the radical has not been observed and there is no evidence to suggest that this species is stable enough to dissociate from the enzyme and function as a diffusible oxidant. In addition to the identified radicals formed in our system, an unknown species was detected in ESR experiments using PBN as the spin-trapping agent. This signal may be due to a PBN–veratryl alcohol radical adduct, but further work is needed to confirm this. It could also be a PBN-derived radical.

Added in Proof

A paper by Akamatsu et al. (1990), published while the present paper was in press, describes the decarboxylation of oxalate by LiP + veratryl alcohol and H_2O_2. Those results and ours are in accord. The essence of the present paper was

described at the 199th National Meeting of the American Chemical Society in Boston, MA, in April 1990.

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Registry No. LiP, 42613-30-9; VA, 93-03-8; OA, 144-62-7; Mn, 7439-96-5; O_2, 7782-44-7; $CO_2^{\bullet-}$, 14485-07-5; $^{\bullet}OOH$, 3170-83-0; malonate, 141-82-2; veratrylaldehyde, 120-14-9.

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Kinetic Mechanism of Orotate Phosphoribosyltransferase from *Salmonella typhimurium*†

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ABSTRACT: The chemical mechanism of the phosphoribosyltransferases (PRTases), although largely unknown, may proceed either via a concerted direct-transfer mechanism or with a two-step mechanism involving a carboxonium-like intermediate. To study this question, we have cloned the *Salmonella typhimurium pyrE* gene, coding for the enzyme orotate phosphoribosyltransferase (EC 2.2.4.10, OPRTase), and developed a bacterial strain that overproduces the enzyme, which we have purified to homogeneity. Initial velocity and product inhibition studies indicated that *S. typhimurium* OPRTase follows a random sequential kinetic mechanism. This result was further confirmed by equilibrium isotope exchange studies on two substrate–product pairs, PRPP–PP_i and OMP–orotate. In addition, the rates of the individual equilibrium isotope exchanges allowed us to conclude that PP_i release and PRPP release were the rate-determining steps in the forward and reverse reactions, respectively. Although partial reactions between the two substrate–product pairs, PRPP–PP_i and OMP–orotate, were observed, further studies revealed that these exchanges were a result of contaminations. Our results are significant in that *S. typhimurium* OPRTase, like most PRTases but in contrast to its yeast homologue, follows sequential kinetics. The artifactual partial isotope exchanges found in this work may have implications for similar prior work on the yeast enzyme. In view of the careful isotope effect studies of Parsons and co-workers [Goitein, R. K., Chelsky, D., & Parsons, S. M. (1978) *J. Biol. Chem. 253*, 2963-2971] and the results obtained by us, we propose that PRTases may involve a direct-transfer mechanism but with low bond order to the leaving pyrophosphate moiety and attacking base.

Orotate phosphoribosyltransferase (EC 2.2.4.10, OPRTase)[1] catalyzes the formation of an N-glycosidic bond between α-D-5-phosphoribosyl pyrophosphate (PRPP) and orotate to form orotidine 5-phosphate (OMP, see Scheme I), a step in the de novo biosynthesis of the pyrimidine nucleotides (Neuhard & Nygaard, 1987). The transferase reaction is accompanied by anomeric inversion at the C1 of the ribosyl group (Chelsky & Parsons, 1975). OPRTase is one of a family of

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[1] Abbreviations: PRPP, α-D-5-phosphoribosyl pyrophosphate; HGPRTase, hypoxanthine–guanine phosphoribosyltransferase; OPRTase, orotate phosphoribosyltransferase; UPRTase, uracil phosphoribosyltransferase; IPTG, isopropyl β-D-thiogalactopyranoside; TEA, triethylammonium; TSK-DEAE, diethylaminoethyl-substituted Toyo-Pearl resin.

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