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Manganese Peroxidase from the Basidiomycete *Phanerochaete chrysosporium*: Spectral Characterization of the Oxidized States and the Catalytic Cycle†

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ABSTRACT: Manganese peroxidase (MnP), an extracellular heme enzyme from the lignin-degrading fungus *Phanerochaete chrysosporium*, catalyzes the Mn(II)-dependent oxidation of a variety of phenols. Herein, we spectroscopically characterize the oxidized states of MnP compounds I, II, and III and clarify the role of Mn in the catalytic cycle of the enzyme. Addition of 1 equiv of H_2O_2 to the native ferric enzyme yields compound I, characterized by absorption maxima at 407, 558, 605, and 650 nm. Addition of 2 or 250 equiv of H_2O_2 to the native enzyme yields compound II or III, respectively, identified by absorption maxima at 420, 528, and 555 nm or at 417, 545, and 579 nm, respectively. These characteristics are very similar to those of horseradish peroxidase (HRP) and lignin peroxidase (LiP) compounds I, II, and III. Addition of 1 equiv of either Mn(II), ferrocyanide, or a variety of phenols to MnP compound I rapidly reduces it to MnP compound II. In contrast, only Mn(II) or ferrocyanide, added at a concentration of 1 equiv, reduces compound II. The Mn(III) produced by the enzymic oxidation of Mn(II) oxidizes the terminal phenolic substrates. This indicates that compounds I and II of MnP contain 2 and 1 oxidizing equiv, respectively, over the native ferric resting enzyme and that the catalytic cycle of the enzyme follows the path native enzyme → compound I → compound II → native enzyme. In addition, these results indicate that Mn(II) serves as an obligatory substrate for MnP compound II, allowing the enzyme to complete its catalytic cycle. Finally, the Mn(II)/Mn(III) redox couple enables the enzyme to rapidly oxidize the terminal phenolic substrates.

Lignin is a complex, optically inactive, and random phenylpropanoid polymer that comprises 20–30% of woody plants (Sarkanen, 1971). Since the biodegradation of cellulose is retarded by the presence of lignin (Crawford, 1981), the catabolism and potential utilization of this polymer are of enormous significance. White rot basidiomycetes are primarily responsible for the initiation of the decomposition of lignin in wood (Crawford, 1981). Recent studies have shown that when cultured under ligninolytic conditions, the white rot basi-

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diomycete *Phanerochaete chrysosporium* produces at least two extracellular heme peroxidases (Gold et al., 1984; Kuwahara et al., 1984; Tien & Kirk, 1984; Glenn & Gold, 1985) that are important components of the ligninolytic system. Lignin peroxidase (LiP)[1] (ligninase) has been studied extensively (Renganathan & Gold, 1986; Kirk & Farrell, 1987; Buswell & Odier, 1987). Recently, a second extracellular enzyme, manganese peroxidase (MnP), has been identified (Kuwahara et al., 1984), purified, and partially characterized (Glenn & Gold, 1985; Pasczynski et al., 1986; Glenn et al., 1986). This peroxidase is an H_2O_2-dependent heme glycoprotein of M_r 46 000, with an iron protoporphyrin IX prosthetic group. The enzyme oxidizes a variety of dyes, phenols, and amines (Kuwahara et al., 1984; Glenn & Gold, 1985; Pasczynski et al., 1986; Glenn et al., 1986). Electronic absorption (Glenn & Gold, 1985; Glenn et al., 1986), EPR, and resonance Raman spectral evidence (Mino et al., 1988) suggest that the heme iron in the native protein is in the high-spin, pentacoordinate, ferric state with histidine coordinated as the fifth ligand.

Most importantly, recent experiments (Glenn & Gold, 1985; Pasczynski et al., 1986; Glenn et al., 1986) have demonstrated that MnP oxidizes Mn(II) to Mn(III) and that the Mn(III) produced, in turn, oxidizes organic substrates (Glenn et al., 1986; Pasczynski et al., 1986). In order to more fully understand the mechanism of MnP, we have prepared all of the oxidation states of the enzyme using H_2O_2 and organic peroxides as oxidants. In addition, we have reduced the oxidized states of the enzyme coupled with the oxidation of selected substrates. These experiments indicate that the oxidized states of MnP are similar to those of HRP (Dunford & Stillman, 1976; Dunford, 1982) and LiP (Renganathan & Gold, 1986). These experiments also indicate that although Mn(II) and a variety of phenols are capable of reducing MnP compound I to compound II, only Mn(II) is capable of efficiently reducing compound II to the native enzyme.

Materials and Methods

Cumene hydroperoxide and H_2O_2 were obtained from Sigma. mCPBA, pNPBA, and all phenolic substrates except guaiacylglycerol β-guaiacyl ether (I) were obtained from Aldrich. I was synthesized as previously described (Goldsby et al., 1980). All other chemicals were of reagent grade. Buffers were prepared in water that had been purified initially by deionization and then by triple glass distillation.

MnP was purified from the extracellular medium of acetate-buffered, agitated cultures of *P. chrysosporium* as previously described (Glenn & Gold, 1985; Renganathan et al., 1985). The purified protein was electrophoretically homogeneous and had an RZ value (A_{407}/A_{280}) of ~6.15 and a millimolar extinction coefficient at 406 nm of 129.3 (Glenn & Gold, 1985). The homogeneous enzyme was dialyzed exhaustively against buffers, prepared from triply distilled water, before use.

MnP-catalyzed oxidation of organic substrates was determined in 1-mL reaction mixtures containing 50 mM sodium lactate, 50 mM sodium succinate, pH 4.5, $MnSO_4$ (0.1 mM), H_2O_2 (0.1 mM), gelatin (3 mg/mL), substrate (0.1 mM), and enzyme (1 μg). Phenolic substrates were dissolved in dimethylformamide and diluted at least 100-fold in triply distilled water to 10 mM. Electronic absorption spectra were recorded on a Shimadzu UV-260 spectrophotometer at room temperature with a spectral bandwidth of 1.0 nm and cuvettes of 1-cm light path. Compounds I, II, and III of MnP were prepared by adding 1, 2, and 250 equiv of H_2O_2, respectively, to the enzyme in 20 mM sodium succinate, pH 4.5. These intermediates were also obtained when the enzyme was oxidized in sodium lactate, pH 4.5.

mCPBA and pNPBA were dissolved in 2-methyl-2-propanol and diluted 100-fold in triply distilled water to a final concentration of 5 mM. One or 25 equiv of these oxidants was then added to the enzyme as described. Phenolic substrates, ferric sulfate, ferrous sulfate, manganese sulfate, and ferrocyanide were each added as aqueous solutions to the oxidized enzymes as described.

The ferrous enzyme was prepared (Gold et al., 1984; Glenn & Gold, 1985) under anaerobic conditions by purging the native enzyme with scrubbed argon in a septum-fitted cuvette, after which $Na_2S_2O_4$ (160 equiv) was added anaerobically. The ferrous–oxy complex (compound III) was generated by purging the ferrous enzyme with O_2 gas above the solution (Renganathan et al., 1985).

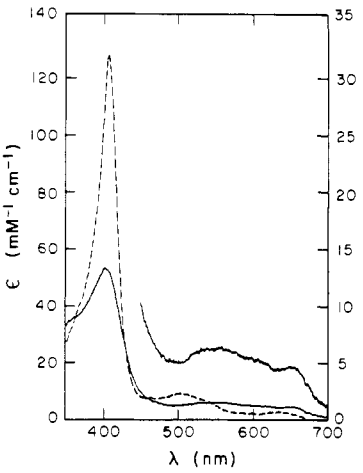

FIGURE 1: Electronic absorption spectrum of the native enzyme (---) and of MnP compound I (—). To produce compound I, 1 equiv of H_2O_2 was added to the native enzyme (1.25 μM) in 1 mL of 20 mM sodium succinate, pH 4.5. Other procedures were as described in the text.

Table I: Oxidation States of Mn Peroxidase

enzyme species	absorption maxima (nm) [ϵ (mM^{-1} cm^{-1})]
ferric[a]	406 [129.3], 502 [9.9], 632 [3.4]
ferrous[a]	435 [108.5], 556 [17.5], 590 [sh][b]
compound I	407 [53.4], 558 [6.4], 617 [sh],[b] 650 [4.7]
compound II[a]	420 [82.6], 528 [9.9], 555 [10.0]
compound III	417 [115.7], 545 [11.6], 579 [9.0]
ferrous + O_2	411 [104.0], 540 [8.9], 580 [6.9]
pNPBA or mCPBA oxidized	407 [54], 558 [6.4], 617 [sh],[b] 650 [4.8]

[a] Glenn and Gold (1985). [b] Sh = shoulder.

Results

The absorption spectrum of the native enzyme (Glenn & Gold, 1985) (Figure 1, Table I) had a Soret maximum at 406 nm with visible bands at 502 and 632 nm. Upon the addition of 1 equiv of H_2O_2, the intensity of the Soret band was reduced to ~40% that of the native enzyme with no apparent shift in the wavelength maximum. The visible region showed a broad absorption with peaks at 558, 608, and 650 nm (Figure 1, Table I). The characteristics of the spectrum in Figure 1 are similar to those of HRP compound I (Dunford & Stillman, 1976; Dunford, 1982) and LiP compound I (Renganathan & Gold, 1986). The addition of 2 equiv of H_2O_2 produced a

[1] Abbreviations: CAT, catalase; CCP, cytochrome *c* peroxidase; CPO, chloroperoxidase; EPR, electron paramagnetic resonance; HRP, horseradish peroxidase; LiP, lignin peroxidase; LPO, lactoperoxidase; mCPBA, *m*-(chloroperoxy)benzoic acid; MnP, manganese peroxidase; pNPBA, *p*-(nitroperoxy)benzoic acid; SA, syringic acid.

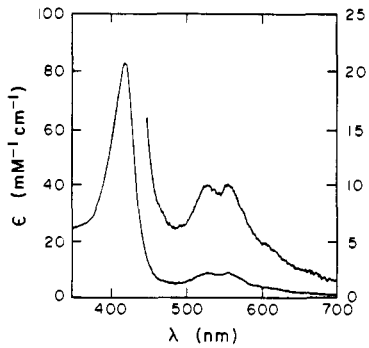

FIGURE 2: Electronic absorption spectrum of MnP compound II. Two equivalents of H_2O_2 were added to the native enzyme (1.25 μM) in 1 mL of 20 mM sodium succinate, pH 4.5. Other procedures were as described in the text.

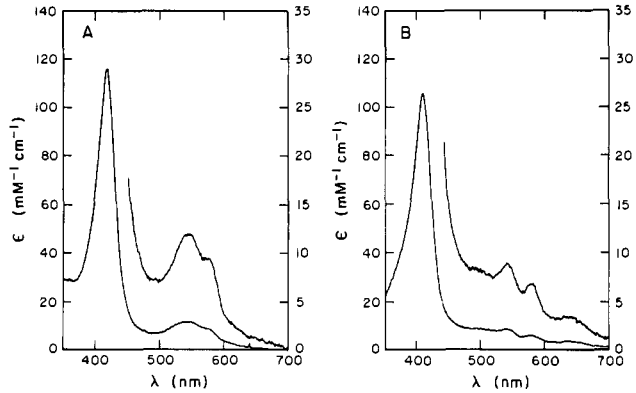

FIGURE 3: Electronic absorption spectrum of MnP compound III: (A) 250 equivalents of H_2O_2 was added to the native enzyme (1.25 μM) in 1 mL of 20 mM sodium succinate, pH 4.5; (B) the ferrous–oxy complex was prepared by adding molecular oxygen to the ferrous enzyme. The latter was prepared by reducing the native enzyme with sodium dithionite as described in the text.

spectrum similar to that of HRP compound II (Dunford & Stillman, 1976; Dunford, 1982) with absorption maxima at 420, 528, and 555 nm (Figure 2, Table I). However, the extinction of the Soret maximum (420 nm) was only ~65% that of the native enzyme.

The oxidation of MnP by several organic peroxides was also investigated. The addition of 1 equiv of mCPBA or pNPBA led to the formation of a MnP compound I intermediate (Table I). However, 25 equiv of these organic peroxides was required to produce a compound II spectrum. Excess cumene hydroperoxide did not result in the oxidation of MnP. MnP compound I spontaneously reverted to compound II with a $t_{1/2}$ of ~60 min at pH 4.5. At this pH, compound II spontaneously reverted to the native enzyme with a $t_{1/2}$ of ~120 min. MnP compounds I and II could also be prepared at pH 3.0 and 6.0. However, compound I was comparatively unstable at pH 3.0, reverting to compound II with a $t_{1/2}$ of ~5 min. Both oxidized intermediates were approximately as stable at pH 6.0 as at pH 4.5.

The addition of excess H_2O_2 (250 equiv) to the native enzyme or to MnP compound II led to the formation of MnP compound III. The absorption maxima of this intermediate were at 417, 545, and 579 nm (Figure 3A, Table I). Reduction of the native ferric enzyme to the ferrous form followed by purging with O_2 led to the formation of the equivalent ferrous–oxy form of MnP. The absorption spectrum of this intermediate had maxima at 411, 540, and 580 nm (Figure 3B, Table I).

The extinction coefficients at 430 nm for the native enzyme, compound I, and compound II were 25.0, 27.5, and 64.5 mM^{-1} cm^{-1}, respectively; therefore, the reduction of MnP compounds

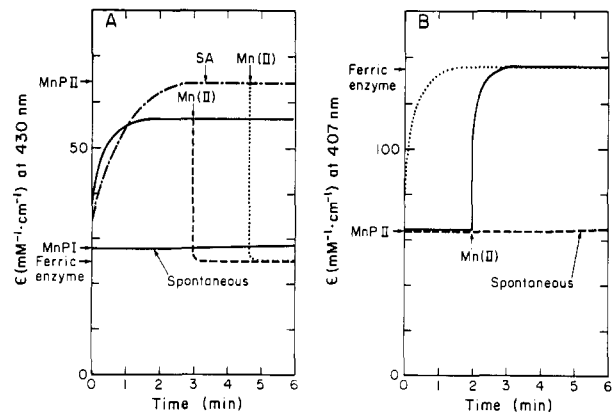

FIGURE 4: Reduction of MnP compounds I and II: (A) MnP compound I was prepared by adding 1 equiv of pNPBA to the native enzyme as described in the text. Reduction of MnP compounds I and II was followed at 430 nm. Addition of 1 equiv of syringic acid (SA) (–·–) or of Mn(II) (—) led to the conversion of compound I to compound II. Addition of a second equivalent of SA did not result in the reduction of compound II. In contrast, the addition of a second equivalent of Mn(II) (---, ···) resulted in the reduction of compound II. (B) MnP compound II was prepared directly by adding 2 equiv of H_2O_2 to the native enzyme. Conversion of compound II to the native enzyme was followed at 407 nm. Addition of 1 equiv of Mn(II) (···) resulted in the reduction of compound II. Addition of SA (—) had no effect.

I and II could be followed at this wavelength. The addition of 1 equiv of either syringic acid (SA) or Mn(II) to compound I resulted in its rapid reduction to MnP compound II. A variety of other phenols and amines including *p*-cresol, guaiacol, vanillyl alcohol, 4-hydroxy-3-methoxycinnamic acid, isoeugenol, I, ascorbic acid, and *o*-dianisidine were also able to reduce MnP compound I to MnP compound II. Subsequent addition of a second equivalent of SA or the phenols listed above to MnP compound II produced by the reduction of MnP compound I did not reduce compound II to the native enzyme (Figure 4A). In contrast, the addition of a second equivalent of Mn(II) to compound II produced via the reduction of compound I resulted in the rapid reduction of MnP compound II to the native enzyme (Figure 4A).

To clarify the role of Mn(II) in the reduction of MnP compound II, this enzyme intermediate was obtained directly by oxidizing the native enzyme with 2 equiv of H_2O_2. The reduction of MnP compound II to the native enzyme was then followed by monitoring the increase in absorbance at 407 nm. The addition of 1 equiv of SA to MnP compound II generated directly did not result in any spectral shift (Figure 4B), indicating that MnP compound II is not readily reduced by this substrate. Likewise, addition of 1 equiv of the other organic substrates listed above had no effect on MnP compound II. In contrast, the addition of Mn(II) resulted in the rapid reduction of MnP compound II to the native enzyme (Figure 4B).

Figure 5A shows that the one-electron substrate ferrocyanide was able to reduce MnP compound I to MnP compound II. Subsequent addition of a second equivalent of ferrocyanide generated the native enzyme (Figure 5A). Ferrous sulfate gave the same result as ferrocyanide. However, Fe(II) could not replace Mn(II) as a redox coupler during the oxidation of phenols by MnP (Glenn & Gold, 1985; Glenn et al., 1986). Figure 5B shows that in the presence of Fe(II) the enzyme had little effect on guaiacol, whereas the subsequent addition of Mn(II) to the system resulted in the immediate oxidation of guaiacol as monitored at 420 nm.

To further confirm the role of Mn(III) as a redox coupler in this system, we examined the direct effect of Mn(III) and

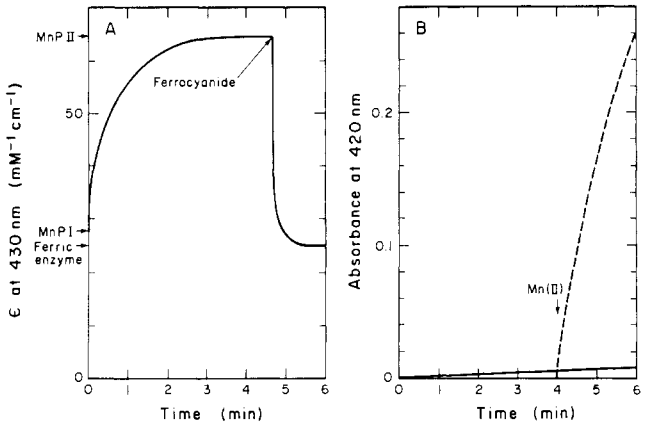

FIGURE 5: Reduction of MnP compounds I and II by ferrocyanide: (A) Reduction of MnP compounds I and II was followed at 430 nm. The addition of 1 equiv of ferrocyanide reduced compound I to compound II. The addition of a second equivalent of ferrocyanide reduced compound II to the native enzyme. (B) Oxidation of guaiacol by MnP was followed at 420 nm. Initial reaction mixtures (—) (1 mL) contained enzyme (1 μg), guaiacol (0.1 mM), H_2O_2 (0.1 mM), and ferrocyanide (0.1 mM). After 4 min, Mn(II) (0.1 mM) was added to the reaction and the oxidation of guaiacol was monitored (- - -).

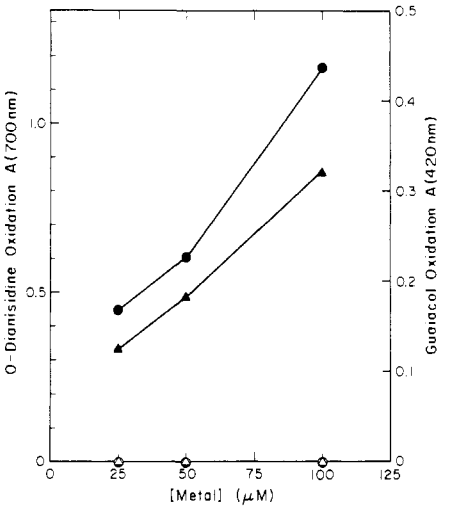

FIGURE 6: Oxidation of *o*-dianisidine and guaiacol by manganese(III) lactate. *o*-Dianisidine oxidation ΔA/min in the presence of Mn(III) (●) or Fe(III) (○) and guaiacol oxidation ΔA/min in the presence of Mn(III) (▲) or Fe(III) (△) were followed at 700 and 420 nm, respectively. Reaction mixtures contained substrate (0.1 mM) and either manganese(III) acetate or ferric sulfate at the concentrations indicated in 50 mM sodium lactate, pH 4.5.

Fe(III) on the oxidation of the aromatic amine *o*-dianisidine and the phenol guaiacol. Figure 6 shows that Mn(III) oxidized both *o*-dianisidine and guaiacol and that the rate of oxidation was dependent on the concentration of the metal ion. In contrast, Fe(III) was not able to oxidize these substrates.

DISCUSSION

Manganese peroxidase is a heme-containing enzyme isolated from the extracellular medium of ligninolytic cultures of the white rot basidiomycete *P. chrysosporium* (Kuwahara et. al., 1984; Glenn & Gold, 1985; Pasczynski et al., 1986; Glenn et al. 1986). The enzyme requires H_2O_2 as a cosubstrate and oxidizes a variety of phenols, amines, and organic dyes (Glenn & Gold, 1985; Pasczynski et al., 1986; Glenn et al., 1986). While the enzyme was originally isolated as a Mn(II)-dependent peroxidase (Kuwahara et al., 1984), recent studies (Glenn & Gold, 1985; Pasczynski et. al., 1986; Glenn et al., 1986) have demonstrated that the enzyme oxidizes Mn(II) to Mn(III) and that the Mn(III) then acts as an obligatory redox coupler, oxidizing various organic substrates. The prosthetic group of MnP is iron protoporphyrin IX (Glenn & Gold, 1986). In this respect, MnP resembles other fungal and plant peroxidases (Dunford & Stillman, 1976; Hewson & Hager, 1979). Previous studies using electronic absorption (Glenn & Gold, 1985), EPR, and resonance Raman spectroscopies (Mino et al., 1988) indicate that the iron in the native protein is in the high-spin, ferric, pentacoordinate state with histidine coordinated as the fifth ligand.

Table II: Absorption Maxima (nm) of Oxidized Intermediates of Several Peroxidases

peroxidase[a]	compound I	compound II	compound III	ref
MnP	407, 558, 605, 650	420, 528, 555	417, 545, 579	this work
LiP	408, 550, 608, 650	420, 525, 556	419, 543, 578	*b*
HRP	400, 557, 622, 650	420, 527, 554	413, 546, 583	*c*
CPO	367, 545, 610, 688	438, 542, 571	432, 555, 586	*d*
LPO	410, 562, 600, 662	433, 537, 568	428, 551, 590	*e*

[a] Abbreviations: MnP, manganese peroxidase; LiP, lignin peroxidase; HRP, horseradish peroxidase; CPO, chloroperoxidase; LPO, bovine lactoperoxidase. [b] Renganathan and Gold (1986). [c] Dunford and Stillman (1976). [d] Palcic et al. (1980); Nakajima et al. (1985). [e] Kimura and Yamazaki (1979).

The primary reaction product of peroxidases with H_2O_2 is the oxidized intermediate compound I. This intermediate accepts both oxidizing equivalents of H_2O_2 and is thus in the formal Fe(V) oxidation state (Dunford, 1982; Hewson & Hager, 1979; Rutter et al., 1983); i.e., it contains two additional oxidizing equivalents over the native ferric enzyme (Theorell, 1941). Mössbauer (Schultz et al., 1984) and ENDOR (Roberts et al., 1983) studies indicate an [Fe^{IV}=O] state of the heme iron for HRP compound I, and thus only one oxidizing equivalent is present at the iron center. In HRP, the second oxidizing equivalent is stored as a porphyrin π-cation radical (Schultz et al., 1984; Dolphin et al., 1971; Roberts et al., 1981).

MnP Compound I. The electronic absorption spectrum obtained on the addition of 1 equiv of H_2O_2 to native MnP has a Soret maximum at 407 nm with reduced intensity relative to the native enzyme and additional maxima at 558 and 650 nm (Figure 1, Table I), and thus this appears to be a compound I species. The absorption maxima for several peroxidase compound I species are compared in Table II. The spectral characteristics of MnP compound I are most similar to those of HRP compound I and LiP compound I. The reduced Soret intensity suggests the π-cation radical nature of the MnP compound I porphyrin ring (Dunford, 1982). Furthermore, the absence of absorbance in the 680-nm region suggests that this Fe^{IV}=O porphyrin π-cation radical has an A_{2u}-type or HRP-type electronic ground state (Dolphin et al., 1971; Dolphin & Felton, 1974; Palcic et al., 1980).

MnP Compound II. One-electron reduction of HRP compound I by a peroxidase substrate results in the formation of compound II (Dunford & Stillman, 1976; Dunford, 1982). This intermediate has a formal oxidation state of IV. Mössbauer (Schultz et al., 1984) and resonance Raman (Rakhit et al., 1976; Felton et al., 1976; Terner & Reed, 1984) spectroscopies indicate an Fe^{IV}=O structure for HRP compound II. Recent resonance Raman evidence also suggests an Fe^{IV}=O structure for LiP compound II (Andersson et al., 1987). As with HRP compound II and LiP compound II, the

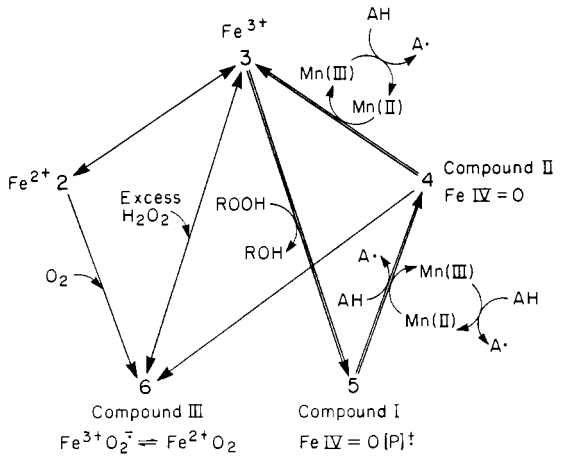

FIGURE 7: Interrelationship between the five oxidation states of MnP. Reaction paths 3 → 5 → 4 → 3 indicate one catalytic cycle of the enzyme. AH = organic substrate.

Soret maximum of MnP compound II is red-shifted to 420 nm and the visible maxima are at 528 and 555 nm (Figure 2, Table II). In contrast to HRP compound II, the intensity of the Soret of MnP compound II is reduced to ~65% that of the native enzyme. Under the conditions used for the production of MnP compound II (Figure 2), with no substrate other than H_2O_2 present, the second equivalent of H_2O_2 probably reduces compound I to compound II and presumably is oxidized in turn to $HO_2^{\bullet}/O_2^{\bullet-}$.

Effect of Other Oxidants. HRP is oxidized to compound I by such organic peroxides as methyl and ethyl hydroperoxide and pNPBA (Schonbaum & Lo, 1972). Lignin peroxidase (Renganathan & Gold, 1986) is also oxidized to compound I by mCPBA. The addition of 1 equiv of either pNPBA or mCPBA oxidizes MnP to compound I (Table I). However, it requires ~25 equiv of either mCPBA or pNPBA to oxidize native MnP to MnP compound II, suggesting that neither organic peroxide is particularly effective at reducing MnP compound I to compound II.

MnP Compound III. The addition of excess H_2O_2 converts HRP to compound III (Dunford & Stillman, 1976; Yamazaki, 1974). The absorption maxima of HRP compound III are at 413, 546, and 583 nm (Table II). The absorption spectrum of the ferrous–oxy species of HRP, produced by the addition of oxygen to ferrous HRP (Dunford & Stillman, 1976; Yamazaki & Yokota, 1965), is very similar to that of HRP compound III. HRP compound III contains 4 oxidizing equiv over the ferrous state of the enzyme (Dunford & Stillman, 1976; Wittenberg et al., 1967). These studies suggest that HRP compound III is either a ferric superoxide complex $[Fe(III)O_2^{\bullet-}]$ or a ferrous–oxy complex $[Fe(II)O_2]$ (Dunford & Stillman, 1976; Hewson & Hager, 1979) (Figure 7.) The addition of excess H_2O_2 to native MnP yields an intermediate with absorption maxima at 417, 545, and 579 nm (Figure 3A, Table II). MnP compound III may also be formed by the addition of O_2 to the ferrous enzyme (Figure 3B, Table I). The amount of H_2O_2 required to produce MnP compound III (~250 equiv) is similar to that required to produce HRP compound III but considerably more than that required to produce LiP compound III (Renganathan & Gold, 1986).

Catalytic Cycle of MnP. The oxidation of phenols and other organic substrates by MnP is dependent on Mn(II) (Kuwahara et al., 1984; Glenn & Gold, 1985). Apparently the enzyme first oxidizes Mn(II) to Mn(III), which subsequently oxidizes the organic substrates (Glenn & Gold, 1985; Pasczynski et al., 1986; Glenn et al., 1986). In order to elucidate the role of Mn(II) in the mechanism of the enzyme, we examined the ability of Mn(II) and various phenols to reduce the oxidized states, compounds I and II of MnP. The catalytic cycles of HRP and LiP follow the path native enzyme → compound I → compound II → native enzyme. In the process, compound I is reduced to the native enzyme via two one-electron steps (Renganathan & Gold, 1986; Dunford, 1982). A similar pathway has been found for MnP. Addition of 1 equiv of Mn(II) rapidly reduces MnP compound I to compound II. A second equivalent of Mn(II) rapidly reduces MnP compound II to the native ferric form (Figure 4A) with the oxidation of Mn(II) to Mn(III) as previously shown (Glenn & Gold, 1985; Glenn et al., 1986). Similarly, MnP compound I is reducible by phenolic substrates. The addition of 1 equiv of syringic acid or *p*-cresol reduces MnP compound I to MnP compound II, albeit at a slower rate (Figure 4A). However, the addition of a second equivalent of either of these phenols to the MnP compound II so formed does not lead to its reduction to the native enzyme (Figure 4A). In contrast, the addition of 1 equiv of Mn(II) to MnP compound II formed by the reduction of MnP compound I rapidly reduces MnP compound II to the native enzyme (Figure 4A).

MnP compound II was prepared directly by the addition of 2 equiv of H_2O_2 to the native enzyme. As shown in Figure 4B, the addition of 1 equiv of Mn(II) to MnP compound II rapidly reduces this intermediate to the native enzyme. In contrast, the addition of 1 equiv of SA to MnP compound II has no effect. Multiple equivalents of phenols do reduce MnP compound II; however, the conversion is very slow. For example, the $t_{1/2}$ for conversion of MnP compound II to the native enzyme by 1 equiv of Mn(II) is <10 s, while the $t_{1/2}$ for the conversion of MnP compound II to the native enzyme by 20 equiv of SA or guaiacol is 72 or 160 s, respectively. Thus, it appears that the enzyme cannot efficiently complete its catalytic cycle in the absence of Mn(II). This explains the absolute Mn(II) requirement for enzymic activity.

The one-electron reductant, ferrocyanide, is also capable of reducing the oxidized states of MnP. Addition of 1 equiv of ferrocyanide reduces MnP compound I to MnP compound II. Subsequent addition of a second equivalent of ferrocyanide reduces MnP compound II to the native enzyme (Figure 5A). This titration confirms that MnP compound I is reduced to the native enzyme via two single-electron steps. Although ferrocyanide is able to reduce the oxidized states of MnP, the Fe(III) formed in the reaction cannot oxidize the phenolic substrates, as shown in Figures 5B and 6. This is predicted by the relative redox potentials of Fe(III) and the phenolic substrates (see below).

All of these results indicate the interrelationship between the redox states of the enzyme as depicted in Figure 7. Formally, peroxidases can exist in five redox states from 2+ to 6+ (Yamazaki, 1974). Earlier, we observed the native ferric and ferrous forms of the enzyme (Glenn & Gold, 1985). Together with the spectral characterization of compounds I, II, and III reported here, we have now demonstrated all five oxidation states of the enzyme. In addition, we have shown that both Mn(II) and phenols are able to reduce MnP compound I to MnP compound II, although the rate of reduction with Mn(II) is faster; however, only Mn(II) can efficiently reduce MnP compound II to the native enzyme.

In previous work, we demonstrated that MnP cannot oxidize Co(II) or Ni(II) (Glenn et al., 1986). The oxidation/reduction potentials for Co(III)/Co(II), Mn(III)/Mn(II), and $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$ are approximately 1.8, 1.5, and 0.36 V, respectively (Latimer, 1952), suggesting that, solely on the basis of thermodynamics, the oxidation/reduction potentials

for MnP compounds I and II are sufficient to allow both oxidized states to oxidize the terminal substrate, guaiacol, with an oxidation reduction potential of 0.75 V (Ogawa, 1985). Thus, other factors must hamper the oxidation of guaiacol by MnP compound II. Guaiacol and the other organic substrates may not have ready access to the catalytic center of MnP compound II. As described above, the two oxidizing equivalents carried by HRP compound I and presumably by MnP compound I reside in an Fe^{IV}=O center and a porphyrin π-cation radical. In the conversion of compound I to compound II, the porphyrin π-cation radical is reduced to a normal porphyrin. This suggests that the porphyrin radical is exposed as a peripheral site as recently suggested (Ortiz de Montellano, 1987), and this peripheral site may be sterically available to both larger phenols and amines as well as to Mn(II) and Fe(II). In contrast, the Fe^{IV}=O center in MnP compound II may be partially buried and only fully available to the Fe(II) and Mn(II) ions.

To our knowledge, this fungal peroxidase is the only known enzyme system that utilizes freely diffusible Mn(II)/Mn(III) as an obligatory redox couple. This arrangement has several advantages. Most importantly, the redox potential of Mn(II)/Mn(III) falls between those of the enzyme's oxidized states and the terminal phenolic substrates. In addition, the complexes formed by Mn(III) and α-hydroxy acids such as lactate (Glenn & Gold, 1985) are stable, yet have a high redox potential (Waters & Littler, 1965; Archibald & Fridovich, 1983). Finally, both Mn(II) and Mn(III) are soluble and relatively stable oxidation states in aqueous medium. Our present results appear to account for the manganese dependence of this unique manganese peroxidase. Further studies exploring the interaction between the Mn ion, the phenolic substrate, and the enzyme are in progress.

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