

Engineering of a Manganese-Binding Site in Lignin Peroxidase Isozyme H8 from *Phanerochaete chrysosporium*

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A Mn²⁺-binding site was created in the recombinant lignin peroxidase isozyme H8 from *Phanerochaete chrysosporium*. In fungal Mn peroxidase, the Mn-binding site is composed of Glu35, Glu39, and Asp179. We generated a similar site in lignin peroxidase by generating an anionic binding site. We generated three mutations: Asn182Asp, Asp183Lys, and Ala36Glu. Its activity, veratryl alcohol, and Mn²⁺ oxidation were compared to those of native recombinant enzyme and to fungal Mn peroxidase isozyme H4, respectively. The mutated enzyme was able to oxidize Mn²⁺ and still retain its ability to oxidize veratryl alcohol. Steady-state results indicate that the enzyme's ability to oxidize veratryl alcohol was lowered slightly. The *K_m* for Mn²⁺ was determined to be 1.57 mM and the *k_{cat}* = 5.45 s⁻¹. These results indicate that the mutated lignin peroxidase is less effective in Mn²⁺ oxidation than the wild type fungal enzyme. The pH optima of veratryl alcohol and Mn oxidation were altered by the mutation. They are one unit of pH value higher than those of recombinant H8 and wild type fungal Mn peroxidase isozyme H4. © 2001 Academic Press

Key Words: site-directed mutagenesis; fungi; lignin degradation.

Most if not all white-rot fungi produce extracellular peroxidases which are thought to be involved in the degradation of lignin (1, 2). These peroxidases fall into two families: the lignin peroxidases (LP) and Mn peroxidases (MnP). Although many fungi produce one or both of these peroxidases, the best characterized are the isozymes from *Phanerochaete chrysosporium*. Both are heme proteins, which share an identical catalytic mechanism. Catalysis is initiated by oxidation of the

heme active site by H₂O₂. The two-electron oxidized heme intermediate (compound I) oxidizes two substrate molecules, each by one electron to complete the catalytic cycle. Compound II is one-electron-oxidized intermediate. These two lignin-degrading peroxidases differ in the nature of their reducing substrate. The LPs catalyze the oxidation of phenolic and nonphenolic aromatic substrates [Kirk, 1986 #1191; Hammel, 1993 #1173; Koduri, 1995 #665], whereas the MnPs catalyze the oxidation of Mn²⁺ to Mn³⁺ (3, 4).

The distinguishing characteristics of LP and MnP are characteristics which define other peroxidase; peroxidases are ubiquitous in nature and differ only in the nature of their reducing substrate. The structural aspects which confer specificity in peroxidases has been extensively studied in cytochrome c peroxidase (5–8). Where peroxidases are thought to share a common compound I and compound II intermediate, specificity of the reducing substrate is thought to be conferred by the substrate binding site. In MnP, the Mn²⁺ binding site was identified by x-ray crystallography (9) and by site-directed mutagenesis [Whitwam, 1997 #1186]. In isozyme H4, the three acidic residues composing of two Glu and one Asp (Glu35, Glu39, Asp179) define the binding site. In LP, the exact binding site(s) has not been unequivocally identified. Several were proposed based on theoretical studies (10) and its crystal structure (11). One of the proposed binding sites for veratryl alcohol is the heme access channel. When amino acids were mutated in the access channel of recombinant H8, the highest decrease was observed in the oxidation rate of the phenolic substrate guaiacol, whereas the oxidation of veratryl alcohol [Ambert-Balay, 1998 #954], and a nonphenolic tetrameric lignin model compound were less affected (Mester *et al.*, in preparation). This would suggest that LP, as suggest for cytochrome c peroxidase (12), has multiple routes for electron transfer. More recently, the involvement of a Trp residue (Trp171) in electron transport from veratryl alcohol was proposed and corroborated by site directed mu-

Abbreviations used: LP, lignin peroxidase; MnP, Mn peroxidase; ABTS, 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonate); rLPH8, recombinant lignin peroxidase isozyme H8.

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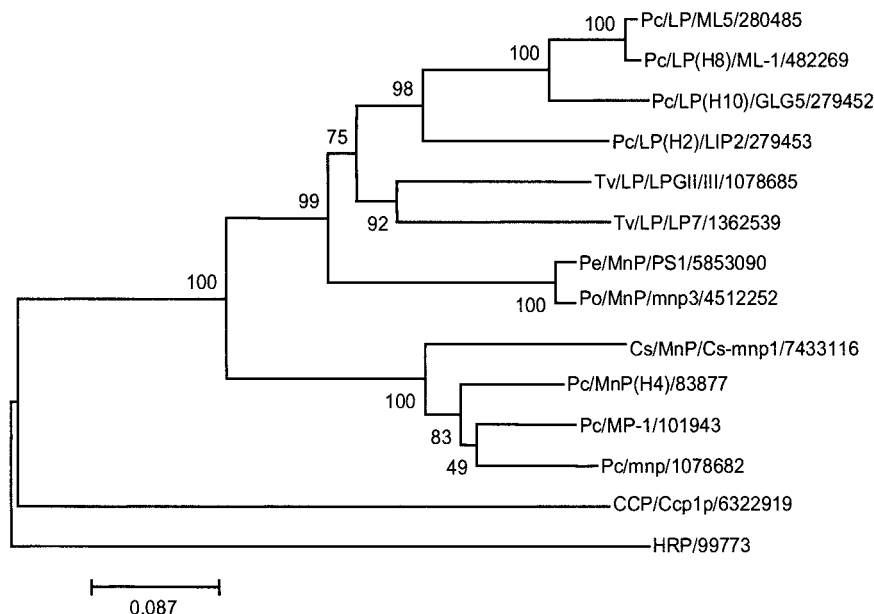


FIG. 1. Phylogenetic comparison of amino acid sequences of fungal peroxidases. The tree was constructed using the neighboring-joining methods as described under Materials and Methods. Abbreviations are: Pc, *P. chrysosporium*; Tv, *T. versicolor*; Pe, *P. eryngii*; Cs, *C. subvermispora*; CCP, cytochrome *c* peroxidase; HRP, horseradish peroxidase. The information provided in the figure is: fungus/LP or MnP (enzyme nomenclature as provided by author)/gene designation as provided by author/gi number. For example, Pc/LP(H8)/ML-1/482269 specifies that the protein sequence is from *P. chrysosporium*; it is a LP, isozyme H8; cDNA ML-1 and the gi number of 482269. Numbers in the phylogenetic tree indicate the percentage of 500 bootstrap replicates in which a group was found.

tagenesis (13). This result suggests another binding site on the surface of LP surrounded by aromatic residues. Here again, the investigators found evidence for multiple site of electron transfer. Mutagenesis of Trp171 to Ser eliminated activity toward veratryl alcohol but not ABTS. They proposed that the heme excess channel permitted direct electron transfer to the heme, as in other peroxidase and that Trp171 allowed for long-range electron transfer.

Evidence for multiple routes for electron transfer also comes from the work of Camarero *et al.* (14). These workers isolated a peroxidase from the ligninolytic fungus *Pleurotus eryngii*, which possessed both LP and MnP activity. A 3-D model was built showing the presence of the heme access channel and Trp170 at the enzyme surface; both are proposed binding site for aromatic substrates. Also found was the putative Mn²⁺ binding site of Glu36, Glu40 and Asp181. Sarkar *et al.* (15) also found evidence for bifunctional peroxidases in *Pleurotus ostreatus*. Phylogenetic analysis (Fig. 1) indicates that these bifunctional peroxidases are evolutionarily distinct from either the LPs or the MnP. In the present study, we investigate whether specificity in these fungal peroxidases is indeed conferred just by the substrate-binding site. Using the model of the enzyme from *P. eryngii*, we created a Mn²⁺-binding site in the rLPH8 from *P. chrysosporium*. We investigated whether this mutant LP, by designing a Mn²⁺-binding site, can oxidize Mn²⁺ in spite of the structural differences between LP and MnP.

MATERIALS AND METHODS

Chemicals. Veratryl alcohol was purchased from Sigma and was vacuum distilled. Oligomers for site directed mutagenesis were purchased from Integrated DNA (Coralville, IA). For the Asn182Asp and Asp183Lys mutations, one single oligonucleotide (and its complement) was used. The underline portion refers to the mutation. 5'-GTCGCAGCGGTGGACAAGGTCGACCCGACC-3'; 5'-GGTCGG-GTCGACCTTGTCACCGCTGCGAC-3'. For the Ala36Glu mutation, the following oligonucleotide (and its complement) was used: 5'-GGCGGCCAGTGC GGCGAGGAGGCGCACGAG-3'; 5'-CTCGTG-CGCCTCCTCGCCGCACTGGCCGCC-3'.

Hydrogen peroxide was purchased from Fisher Scientific. The concentration of H₂O₂ was determined spectrophotometrically at 240 nm using an extinction coefficient of 39.4 M⁻¹ cm⁻¹ (16). All other chemicals were commercially available and used without further purification.

Site-directed mutagenesis. rLPH8 was expressed from its cDNA using cDNA λ ML-1 as previously described (17). LP isozyme H8 mutations were performed using the pET21a + LPH8 plasmid as the template [Ambert-Balay, 1998 #954]. All manipulations were as described in Quick-Change site-directed mutagenesis kit (Stratagene, La Jolla, CA). The mutations were performed in two steps. We first mutated either Ala36Glu with the oligonucleotide listed above or Asn182Asp and Asp183Lys with the corresponding oligonucleotide listed above. The Asn182Asp and Asp183Lys mutations were both generated with one oligonucleotide. After confirming the mutation(s) by sequencing, the other oligonucleotides were used to generate the corresponding mutation.

Enzyme preparation. The recombinant isozyme H8 and the mutated isozyme H8 were expressed in *E. coli* strain BL21 and refolded as described previously [Ambert-Balay, 1998 #954]. The fungal MnP H4 was prepared and purified according to Pease *et al.* [Pease, 1989 #721]. The concentration of recombinant and fungal MnP enzyme

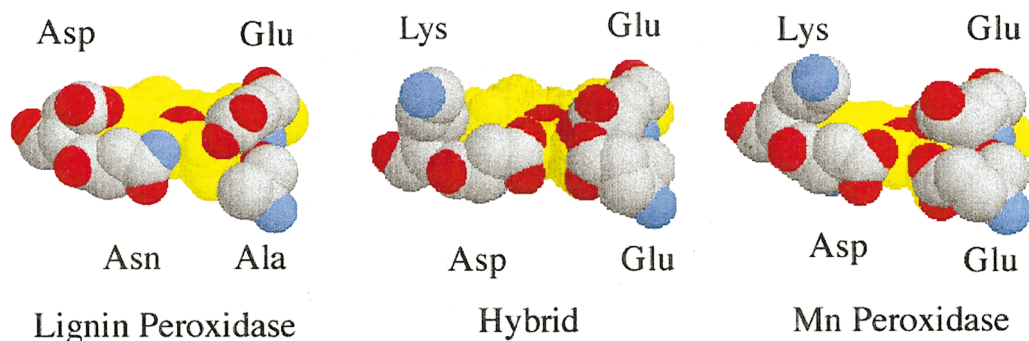


FIG. 2. Amino acid residues at the Mn^{2+} -binding site in the wild-type rLPH8 (1LGA), the triple mutated rLPH8 and the MnP isozyme H4 (1MNP). The figure was drawn with Swiss-PdbViewer (29). The “hybrid” structure is not experimental but was made from the mutation options of the Swiss-PdbViewer.

were determined spectrophotometrically at 408 nm using the extinction coefficients of 168 and 127 $\text{mM}^{-1} \text{cm}^{-1}$, respectively (18, 19).

Enzyme reactions. All reactions were performed at 28°C. Steady-state kinetic experiments for veratryl alcohol oxidation were performed at pH 3.5 using a sodium tartrate buffer, while for Mn^{2+} oxidation a malonate buffer was used (pH 4.5). The rate of veratryl alcohol oxidation to veratraldehyde was measured at 310 nm using an extinction coefficient of 9300 $\text{M}^{-1} \text{cm}^{-1}$ (20). The formation of Mn^{3+} -malonate complex was detected at 270 nm using an extinction coefficient of 8500 $\text{M}^{-1} \text{cm}^{-1}$ [Kuan, 1993 #1189].

Analysis of protein sequences. Sequence alignments were performed with CLUSTAL (21). The phylogenetic tree was constructed using the MEGA program (22).

RESULTS

Design of the Mn-binding site. A Mn^{2+} -binding site was designed in LP isozyme H8 by modeling the Mn^{2+} -binding site of MnP (Fig. 2). The binding site in MnP is located in an anionic pocket containing Asp179, Glu35 and Glu39. The corresponding site in LP isozyme H8 is occupied by Asn182 for Asp179 and Ala36 for Glu35. The corresponding site for Glu39 of MnP in LP is Glu40, thus no changes were made. The residue adjacent to Asp179 in MnP is Lys180. The corresponding residue in LP is Asn182. Although this residue is not proposed to be involved in substrate binding, due to the charge difference of the two amino acids, we decided to mutate this residue to Lys.

We first made the Asn182Asp and the Asp183Lys change in one step (one oligonucleotide containing both mutations). This was followed by the mutation of Ala36Glu. This mutagenesis scheme was also carried out in the reverse order where the Ala36Glu was first generated followed by the double mutation. This strategy yielded three different recombinant LP enzymes. One contained the Ala36Glu mutation. Another contained the Asn182Asp and Asp183Lys mutation. The third contained all three mutations (Ala36Glu–Asn182Asp–Asp183Lys).

Steady-state kinetic parameters for veratryl alcohol and H_2O_2 . After generating the putative Mn^{2+} -binding site in recombinant LP, we analyzed the mutant enzymes with steady-state methods. First we determined whether the mutation altered the reactivity of enzyme towards veratryl alcohol and H_2O_2 . Table 1 summarizes the steady-state kinetic parameters for veratryl alcohol oxidation by the mutated enzymes compared to wild type recombinant LP. The mutation did alter, but not significantly, the kinetic parameters for veratryl alcohol. The highest K_m was observed for the single mutant Ala36Glu; however, the increase was less than threefold. The double and triple mutant had no effect on the K_m . In all cases the K_m remained in the same order of magnitude (0.2–0.5 mM). More signifi-

TABLE 1
Steady-State Kinetics of Veratryl Alcohol Oxidation^a

Peroxidase	K_m (VA) (mM)	K_m (H_2O_2) (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (VA) ($\text{M}^{-1} \text{s}^{-1}$)	k_{cat}/K_m (H_2O_2) ($\text{M}^{-1} \text{s}^{-1}$)
rLPH8	0.19	0.021	2.2	1.2×10^4	1.0×10^5
A36E	0.56	0.027	1.5	2.7×10^3	6.3×10^4
N182D, D183K	0.20	0.007	0.16	8.0×10^2	2.3×10^4
Triple mutant	0.29	0.006	1.1	3.8×10^3	1.8×10^5

^a Reaction mixtures contained 50 mM sodium tartrate buffer (pH 3.5), 0.1 μM of enzyme, H_2O_2 and veratryl alcohol in various concentrations. For K_m determination of H_2O_2 the final concentration of veratryl alcohol was 2 mM. When the K_m for veratryl alcohol was determined, H_2O_2 was added at the final concentration of 0.1 mM.

TABLE 2
Steady-State Kinetics of Mn Oxidation^a

Peroxidase	K_m (Mn ²⁺) (mM)	k_{cat} (s ⁻¹)	k_{cat}/K_m (Mn ²⁺) (M ⁻¹ s ⁻¹)
Fungal MnP (H4)	0.062	530	8.4×10^6
Triple mutant	1.6	5.4	3.4×10^3
N182D, D183K	n.a. ^b	n.a.	—
A36E	n.a.	n.a.	—
rH8	n.a.	n.a.	—

^a Reaction mixtures contained 50 mM sodium malonate buffer (pH 4.5), 0.1 mM H₂O₂, 0.1 μ M enzyme, and MnSO₄ in various concentrations.

^b n.a., no activity.

cant changes were observed for the K_m for H₂O₂. The K_m remained unchanged in the case of the Ala36Glu mutation while the K_m of the double (Asn182Asp–Asp183Lys) and triple mutants were approximately three-fold lower than that of the wild type rLPH8.

The k_{cat} was slightly altered in the case of Ala36Glu mutant and the triple mutant, while the double mutant showed very low rate of veratryl alcohol oxidation (Table 1). The specificity constant, k_{cat}/K_m is a more accurate indicator than K_m for changes in reactivity with the substrate of interest. Accordingly, although the K_m for H₂O₂ was lowered for the triple mutant, the k_{cat}/K_m remained relatively unchanged thus indicating a lack of change in reactivity. The most dramatic change in k_{cat}/K_m for H₂O₂ was observed for the double mutant Asn182Asp/Asp183Lys. When compared to the k_{cat}/K_m values for veratryl alcohol, the changes in value for H₂O₂ are comparatively small. Thus, the mutations generated have minimal effect on the reactivity toward H₂O₂ when compared to veratryl alcohol.

Steady-state kinetic parameters for Mn²⁺. Neither the recombinant wild type nor the Ala36Glu and the double mutant (Asn182Asp/Asp183Lys) were able to oxidize Mn²⁺ (Table 2). However, the triple mutant did exhibit activity toward Mn²⁺. The K_m for Mn²⁺ is much higher and the k_{cat} value is much lower than that of wild type fungal MnP. Nonetheless the triple mutant showed measurable ability to oxidize Mn²⁺, while the wild type recombinant enzyme and the other mutants clearly showed no activity.

Effect of pH on activity. The generation of the triple mutant altered the pH optima for oxidation of veratryl alcohol. Whereas the wild type fungal and the recombinant wild type both oxidized veratryl alcohol maximally at pH 2.5, the triple mutant exhibited a pH optimum at 3.5 (Fig. 3). When the Mn²⁺-oxidizing activity of the triple mutant was compared to that of the fungal wild type MnP, the same trend was observed. The maximal pH for the mutant recombinant LP was one pH value higher than that of the fungal MnP (Fig. 4).

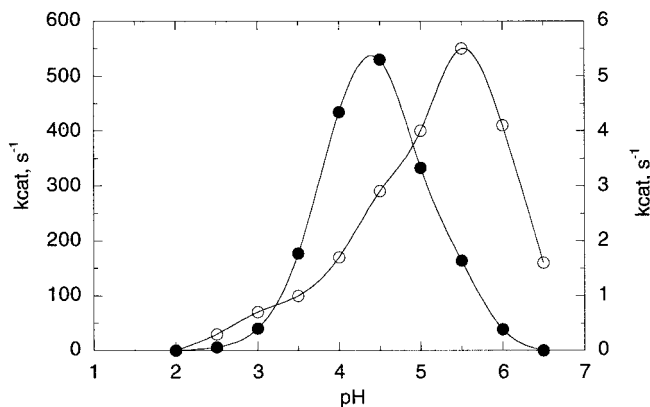


FIG. 3. Effect of pH on the veratryl alcohol oxidation by the wild type rLPH8 (closed circle) and by the triple mutant rLPH8 (open circle). Reaction mixtures are as described in the legend to Table 1.

DISCUSSION

Khindaria *et al.* (23) reported that LP isozyme H2 from *P. chrysosporium* possessed the ability to oxidize both veratryl alcohol and Mn²⁺. They reported that compound I, but not compound II of LP is capable of oxidizing Mn²⁺. This finding has yet to be substantiated by sequencing (24) or crystallographic (11) data showing a Mn²⁺ binding site in isozyme H2. The existence of a bifunctional enzyme, however, has been recently reported by Camarero *et al.* (14). These workers clearly demonstrated both MnP and LP activity with an enzyme from *P. eryngii*. Although the structure of the enzyme has yet to be determined, these workers constructed a 3-D model of the enzyme, based on LP coordinates. A putative manganese-binding site was found consisting of Glu36, Glu40 and Asp181. A bifunctional enzyme has also been reported by Sarkar *et al.* (15). These workers isolated an enzyme with LP and

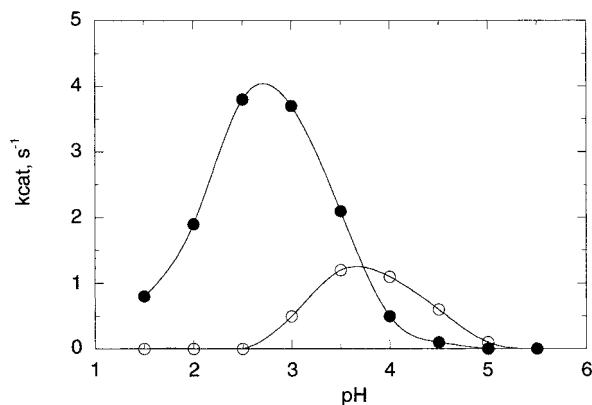


FIG. 4. Effect of pH on the Mn²⁺ oxidation by the fungal MnP isozyme H4 (closed circle) and the triple mutant rLPH8 (open circle). Reaction mixtures are as described in the legend to Table 2.

	20	48	171	186
PcLPH8	IQQNLFHGGQCGAEAEH	SIRLVFHD-----	WMLSAHSVAAVNDVDP--	
PcLPH2	IQQNLFHGGQCGAEAEH	ALRMVFHD-----	WLLSAHSIAAANDVDP--	
TvLPGI/IV	LQQNLFHGGGLCTAEAEH	SLRLTFHD-----	WLLTAHTVAAANDVDP--	
PcMnP	LQKNLFDDGACGE	DAH	ALRLTFHD-----	WLLSAHSVAAADHVDE--
PePs1	IQTNLFDAQCQGE	EVH	SLRLTFHD-----	WLLASHTIAAADHVDP--
PcMnPH4	LQETLFQG-DCGE	DAH	EVRLTFHD-----	SLLASHTVARADKVDE--
PcMnP-1	LQETIFQN-ECGE	DAH	EVRLTFHD-----	SLLASHSVARADKVDQ--

FIG. 5. Multiple alignment of the amino acid sequences of LP, bifunctional peroxidases and MnP at regions of the Mn^{2+} -binding site and aromatic substrate binding site. Key residues are highlighted. Note that Trp171 (aromatic substrates, LP activity), Glu36, and Asp182 (Mn^{2+} binding, MnP activity) are conserved only in the bifunctional peroxidases. Refer to the legend to Fig. 1 for abbreviations and to find gi numbers.

MnP activity from *P. ostreatus*. The enzymological data is actually supported phylogenetic analysis. The tree shown in Fig. 1 was constructed with protein sequences and clearly shows a clustering of the bifunctional enzymes, separate from the MnPs and the LPs. The existence of a distinct class of bifunctional enzymes is also supported by alignment analysis of the amino acids. The putative Mn^{2+} -binding site of Glu 35 or 36, Glu 39 or 40 and Asp 179 or 181 is only found in the MnPs and the bifunctional enzymes, not in the LPs (Fig. 5). Figure 5 also shows the conservation of Trp171 in the LPs or in the bifunctional enzymes. Thus, only the bifunctional enzymes have binding sites for both aromatic substrates and Mn^{2+} . Similar bifunctional enzymes have also been discovered in *Bjerkandera* sp. BOS55 (25), however, no sequence information is available for this enzyme.

The existence of bifunctional enzymes is actually predicted from a model of divergent evolution (26). In regards to how MnPs and LPs evolved, the traditional model is that gene duplication alleviates selective pressure on the duplicate gene thereby allowing it to undergo non-synonymous nucleotide substitution. Being freed from purifying selection, the duplicate gene is now permit to evolve randomly such that it will eventually emerge as a gene encoding a protein with altered function. Hughes (26) proposed an alternative model where the evolution of functionally distinct new enzymes is preceded by a period of gene sharing where the shared gene encodes a bifunctional enzyme. Gene duplication then allows each daughter gene to specialize for one of the functions of the ancestral gene.

Our work has further supported the structural basis for the bifunctional enzyme exhibiting both MnP and LP activity. When compared to MnPs from *P. chrysosporium*, the mutated recombinant LP was less efficient in the oxidation of Mn^{2+} as indicated by a higher K_m and a lower k_{cat} and k_{cat}/K_m values. Thus our work may have defined the minimal structural requirements for a Mn^{2+} -binding site and thus MnP activity. A possible explanation for the high K_m is a lack of an Arg, which was showed to be important in the Mn^{2+} binding (Arg177 in *mnp1* gene). When Arg177 was mutated to Lys or Ala (the corresponding amino acid in LP) the

binding constant (K_d) and K_m increased while k_{cat} remained unchanged (27). Nevertheless, the present study shows that the major structural difference between LPs and MnPs is the lack of a manganese-binding site in LPs. A bifunctional peroxidase could be constructed simply by designing a manganese-binding site in recombinant LP.

Our work here shows that an alternate route of long-range electron transfer can be created in peroxidases simply by generating an alternate substrate binding site. This was also shown by the work of Timofeevski *et al.* (28). These workers showed that the ability to oxidize veratryl alcohol oxidation by *P. chrysosporium* recombinant MnP could be generated by mutating Ser168 to Trp. This Trp is present in LPs as well as in the bifunctional peroxidases. Trp171 has been suggested to be involved in the electron transport from a surface bound veratryl alcohol or tetrameric nonphenolic model compound to the heme (13). These bifunctional peroxidases may prove to be valuable in providing information on the structural basis for catalysis and on role of LP and MnP in lignin degradation. The triple mutant exhibited an increase in pH optimum for veratryl alcohol oxidation. Thus, they may also provide information on the structural basis for the factors influencing the pH properties of catalysis.

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