A study on reducing substrates of manganese-oxidizing peroxidases from *Pleurotus eryngii* and *Bjerkandera adusta*

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Abstract A novel peroxidase, oxidizing Mn^{2+} and different aromatic compounds, was isolated. Hydroquinones, substituted phenols, dyes, other aromatic compounds and Mn^{2+} were compared as reducing substrates, and conclusions presented in the light of a molecular model built by homology modeling. The enzymes showed the fastest reaction rates with Mn^{2+}, but the highest affinity corresponded to hydroquinones and dyes. Oxidation of Reactive Black 5 (an azo-dye not oxidized by Mn^{3+}) was non-competitively inhibited by Mn^{2+}. These findings, together with identification of putative Mn-binding site (involving Glu^{36}, Glu^{40}, Asp^{175} and inner heme propionate) and long-range electron transfer pathways, indicate that different sites are involved in substrate oxidation.

Key words: Peroxidase; Manganese; Hydroquinone; Dye; Substrate binding site; Ligninolytic fungus

1. Introduction

Different peroxidases, classified as manganese peroxidase (MnP; EC 1.11.1.13) or lignin peroxidase (LiP; EC 1.11.1.14), are involved in degradation of lignin by white-rot fungi together with laccases (EC 1.10.3.2) and H_2O_2-producing oxidases (such as EC 1.1.3.7 or EC 1.1.3.10) [1,2]. These organisms have been investigated for environmentally-friendly removal of lignin and lignin-derived compounds in the manufacture of paper pulp [3]. Because of the wide substrate-specificity of the enzymatic mechanism for lignin degradation, which has been thoroughly investigated in *Phanerochaete chrysosporium* and defined as an 'enzymatic combustion' [1], they are also of biotechnological interest for the removal of aromatic contaminants or other recalcitrant compounds from soils and waters [4]. Among white-rot fungi, *Pleurotus eryngii* is being investigated for selective removal of lignin from non-woody materials [5]. The production of ligninolytic peroxidases oxidizing directly both Mn^{2+} and aromatic compounds has been described in this fungus [6,7]. Recently a similar peroxidase was detected in *B. adusta*, a fungus selected for biodegradation of industrial dyes [8], together with LiP already described by Kimura et al. [9]. Despite some reports suggesting LiP production by *Pleurotus* species [10,11], a typical LiP has not been found in this genus. Since the ability to oxidize aromatic compounds directly, i.e. in the absence of redox mediators, is the most distinctive characteristic of the Mn^{2+}-oxidizing peroxidases of *P. eryngii* and *B. adusta*, the substrate specificity of this enzymatic reaction was investigated here using different phenolic and non-phenolic aromatic compounds and dyes as substrates. A molecular model for the *P. eryngii* peroxidase was built, using *P. chrysosporium* LiP and MnP and *Coprinus* peroxidase crystal structures as templates, to provide a structural basis to explain the catalytic properties of the new ligninolytic peroxidases.

2. Materials and methods

2.1. Peroxidase production and purification

Pleurotus eryngii CBS 613.91 (= IJFM A169) was grown in glucose-peptone medium and peroxidases were purified as described elsewhere [6]. *Bjerkandera adusta* DSM 11310 (= IJFM A735) was grown on glucose-ammonia medium [8] at 28°C and 140 rpm. Extracellular proteins from 5-day-old cultures were adsorbed on Q-Sepharose at pH 6, eluted with 500 mM NaCl and dialyzed against 20 mM histidine, pH 6. Then they were fractionated on Mono-Q using a gradient of 0–300 mM NaCl in the same buffer (1 ml/min for 180 min). LiP isoenzymes (LiP1 and LiP2) were purified by Mono-Q chromatography in 20 mM histidine buffer at pH 4.6 (0–200 mM NaCl gradient at a flow rate of 1 ml/min for 80 min). MnP1 and MnP2 were purified by Mono-Q chromatography in 10 mM tartrate at pH 4.6 using 0–0.05 mM NaCl gradient (0.8 ml/min for 35 min). MnP1 from *P. chrysosporium* ATCC 24725 was a gift of C. Palma.

2.2. Chemicals

The azo dyes Reactive Black 5 ($C_{26}H_{21}N_5O_{19}S_6Na_4$) and Reactive Violet 5 ($C_{20}H_{15}N_3O_{15}S_4CuNa_2$) and the phthalocyanine dye Reactive Blue 38 ($C_{32}N_8NiH_{12}(SO_3H)_n$ (SO_2-NH-C_6H_4-reactive group)$_{4-n}$) were obtained from DyStar (Frankfurt, Germany), ABTS (2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) was purchased from Boehringer, and all other chemicals were from Aldrich.

2.3. Enzymatic activities and steady-state kinetics

In the course of purification, MnP activity was estimated by the formation of Mn^{3+}-tartrate (ε_{238} 6500 M/cm) from 0.1 mM $MnSO_4$ using 100 mM sodium tartrate (pH 5) and 0.1 mM H_2O_2, or as the Mn-dependent oxidation of 1 mM syringol (2,6-dimethoxyphenol) at pH 4.5. Mn^{2+}-independent peroxidase activities on 1 mM syringol and 2 mM veratryl (3,4-dimethoxybenzyl) alcohol were estimated at pH 3 (using 100 mM tartrate and 0.1 mM H_2O_2). The latter conditions were used also for LiP estimation. One activity unit was defined as the amount of enzyme oxidizing one μmol of substrate per min.

Steady-state kinetics constants for different substrates were obtained in 100 mM sodium tartrate, pH 3.5 (except for Mn^{2+} at pH 5, and veratryl alcohol and syringol at pH 3), during the linear phase of reaction. Activities were calculated from the molar absorbances of the reaction products of catechol (1,2-benzenediol) (ε_{238} 6500), hydroquinone (1,4-benzenediol) (ε_{247} 21 000), methoxyhydroquinone (ε_{360} 1252), 2,6-dimethoxyhydroquinone (ε_{397} 562), methylhydroquinone (ε_{250} 21 112), *p*-aminophenol (ε_{246} 15 627), guaiacol (*o*-methoxyphenol) (ε_{456} 12 100), *p*-methoxyphenol (ε_{253} 4990), syringol (ε_{468} 27 500), ferulic (4-hydroxy,3-methoxycinnamic) acid (ε_{310} 8680), α-naphthol (1-hydroxynaphthalene) (ε_{255} 12 800), veratryl alcohol (ε_{310} 9300), ABTS (ε_{420} 36 000) and Mn^{2+} (ε_{238} 6500). In the case of dyes, enzy-

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matic activities were estimated from substrate decolorization, using the molar absorbances of Reactive Black 5 (ε_{558} 50 000), Reactive Violet 5 (ε_{598} 30 000) and Reactive Blue 38 (ε_{620} 75 000). The ε values were obtained from Muñoz et al. [12] or calculated by the authors, excepting that of ferulic acid [13], and given per mole of substrate (and cm^{-1}). Protein concentrations were determined with Bradford reagent. Apparent K_m and V_{max} values were calculated from Lineweaver-Burk plots. Mn^{2+}, methoxyhydroquinone and ferulic acid were assayed as inhibitors of MnP oxidation of Reactive Black 5, and the apparent K_i was obtained for Mn^{2+}.

2.4. Molecular models

The atomic coordinates of the molecular models of *P. chrysosporium* MnP1 [14] and LiPH8 [15,16] were obtained from the Brookhaven Protein Database (entries 1MNP, 1LGA and 1LLG). The gene and predicted protein sequences of *P. eryngii* MnPL1 and MnPL2 are deposited in GenBank/EBI Data Bank (AF007221–AF007224). The model for *P. eryngii* MnPL was built by homology modeling with the ProMod program, using as templates the crystal models 1MNP (*P. chrysosporium* MnP1), 1LGA (*P. chrysosporium* LiPH8), 1QPA (*P. chrysosporium* LiPH2) and 1ARP (peroxidase from a soil fungus, which probably is a *Coprinus* anamorph), and CHARMm refining [17] followed by molecular dynamics refining using X-Plor ver. 3.1. The model was deposited at Brookhaven Protein Database as entry 1A20. Swiss-PdbViewer ver. 2.2 was used for model comparison and identification of a putative Mn-binding site, heme access channel residues, and electron-transfer pathways to heme.

3. Results

Two proteins with peroxidase activity on both Mn^{2+}, syringol and veratryl alcohol were isolated from supernatants of *P. eryngii* cultures. They were labeled MnPL1 and MnPL2 because they had higher affinity for Mn^{2+} than for the two other substrates, and were different from Mn^{2+}-oxidizing peroxidases produced under solid-state fermentation conditions [7]. No LiP activity was detected. Opposite to the enzyme pattern found with *P. eryngii*, peroxidases with different activities on the above substrates were identified in *B. adusta* cultures, including: (i) two LiP isoenzymes (LiP1 and LiP2) oxidizing veratryl alcohol, and syringol for a short time period before inactivation, but not Mn^{2+}; (ii) Mn-independent peroxidase [18], acting on syringol but not on Mn^{2+} nor veratryl alcohol; and (iii) two Mn-oxidizing peroxidases (MnP1 and MnP2), similar to those found in *P. eryngii* cultures (i.e. being able to oxidize the three substrates). The latter proteins eluted as a single peak under the conditions used to separate *P. eryngii* MnPL1 and MnPL2, and they were separated only when a slower NaCl gradient was used. Because the peroxidase isoenzymes from each of the fungi showed similar catalytic properties, *P. eryngii* MnPL2 and *B. adusta* MnP1 were used in the studies described below.

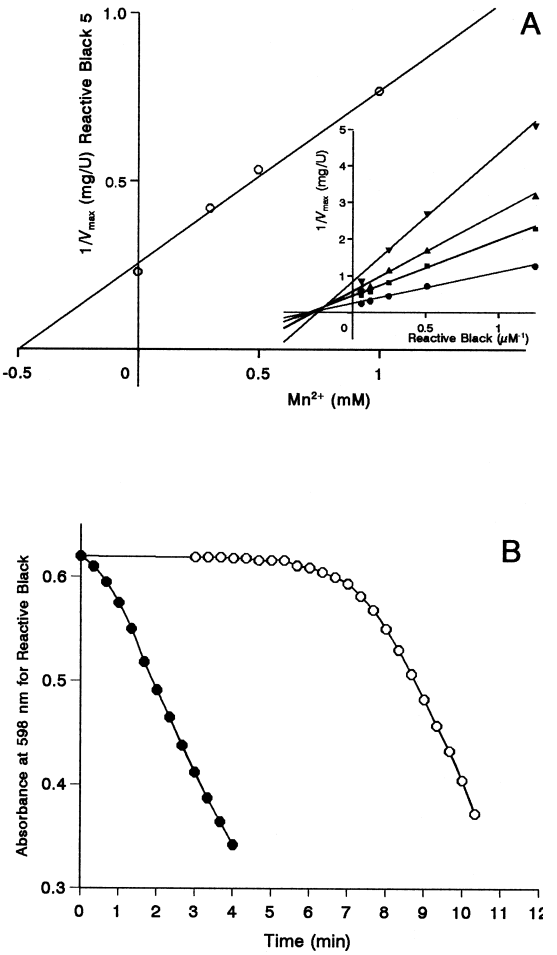

Fig. 1. Substrate inhibition of Reactive Black 5 (12.5 μM) oxidation by *Pleurotus eryngii* MnPL2. **A**. Dixon plot for calculation of K_i of Mn^{2+} (pH 4). The V_{max} values were obtained from Lineweaver-Burk plot (inset) of reaction rates in the presence of 0 (●), 0.3 (■), 0.5 (▲) and 1 mM (▼) Mn^{2+}. **B**. Time-course of dye oxidation in the presence of 0 (●) and 10 μM (○) methoxyhydroquinone (pH 3.5).

In order to get more information on the biotechnological potential of the *P. eryngii* and *B. adusta* Mn-oxidizing peroxidases, oxidation of a variety of potential substrates, including industrial dyes, was compared. As shown in Table 1, some of

Table 1
Direct oxidation of four representative peroxidase substrates by *Pleurotus eryngii* and *Bjerkandera adusta* Mn-oxidizing peroxidases, compared with typical lignin and Mn-dependent peroxidases (LiP and MnP)[a]

	V_{max} (U/mg)			
	Mn^{2+}	Veratryl alcohol	Reactive Black 5	ABTS
Pleurotus eryngii MnPL2	165	18	5	39
Bjerkandera adusta MnP1	180	19	6	25
LiP (*Bjerkandera adusta*)[b]	0	30	0[c]	28
MnP (*Phanerochaete chrysosporium*)	470	0	0[d]	0

[a]Maximal velocities from Lineweaver-Burk plots (linear regression coefficients being significant for $P > 0.99$) of reactions in 100 mM sodium tartrate pH 5 for Mn^{2+}, pH 3 for veratryl alcohol and pH 3.5 for dyes, containing 0.1 mM H_2O_2 (mean values from triplicate determinations; all standard deviations were below 10% of mean values).
[b]The substrate oxidation pattern by *P. chrysosporium* LiPH8 is similar to that shown for *B. adusta* LiP2.
[c]In the absence of veratryl alcohol an initial velocity of 0.3 U/mg was attained before rapid inactivation. In the presence of 2 mM veratryl alcohol the reaction velocity was 5 U/mg.
[d]No reaction in the presence nor in the absence of Mn^{2+}.

the dyes can be useful to differentiate, together with other substrates, between the above peroxidases and LiP (from *B. adusta* or *P. chrysosporium*) and Mn-dependent peroxidases (MnP from *P. chrysosporium*). Opposite to that found with *P. eryngii* and *B. adusta* Mn-oxidizing peroxidases, the latter enzyme did not exhibit significant Mn-independent activity. Moreover, the two former peroxidases were the only ones oxidizing directly Reactive Black 5, a recalcitrant azo dye which is not decolorized by Mn^{3+}-lactate. The steady-state kinetic constants for the oxidation of syringol, veratryl alcohol, Mn^{2+}, azo (Reactive Black 5 and Reactive Violet 5) and phthalocyanine (Reactive Blue 38) dyes, different hydroquinones, substituted phenols and other aromatic compounds by *P. eryngii* MnPL2 and *B. adusta* MnP1 were calculated, and the apparent K_m and V_{max} values are given in Table 2. With the exception of veratryl alcohol, syringol, catechol, *p*-methoxyphenol and guaiacol, the other 11 reducing substrates showed apparent K_m values similar or even lower than those found for Mn^{2+} oxidation, suggesting high affinity. Both peroxidases also showed high affinity for the oxidizing substrate H_2O_2, with K_m values (4–10 μM) lower than those found for *B. adusta* LiP isoenzymes (40–60 μM). The efficiency (V_{max}/K_m) of the oxidation reactions was the highest for ABTS and Mn^{2+}, followed by hydroquinones. Despite some small differences found (e.g. higher affinity for ferulic acid and lower for hydroquinone oxidation by *P. eryngii* MnPL2), the spectrum of substrates oxidized by both peroxidases was very similar, confirming that they belong to the same group of peroxidases in class II [19].

Martínez et al. [6] described *P. eryngii* peroxidases as MnP because of low K_m for Mn^{2+}. However, the present results show that these and the related *B. adusta* enzymes could also be considered as hydroquinone-peroxidases or 'dye'-peroxidases. To investigate if their wide range of enzyme-reducing substrates is due to an 'unspecific' substrate binding site or to multiple binding sites, substrate inhibition was investigated. Inhibition by Mn^{2+} was assayed using Reactive Black 5, because this azo dye is not oxidized by Mn^{3+} chelates. As shown in Fig. 1A, Mn^{2+} caused non-competitive inhibition of dye oxidation by *P. eryngii*, and a K_i of 0.5–0.6 mM Mn^{2+} was calculated for both peroxidases. The situation was different in the case of hydroquinones, because complete inhibition (for more than 5 min) of 12.5 μM Reactive Black 5 oxidation by both enzymes was found in the presence of 10 μM methoxyhydroquinone (Fig. 1B). After this lag period, the reaction proceeded at approximately the same rate observed in the absence of inhibitor. Similar results were obtained with ferulic acid, resulting in complete inhibition of Reactive Black 5 oxidation. This suggests chemical reduction of the oxidized dye

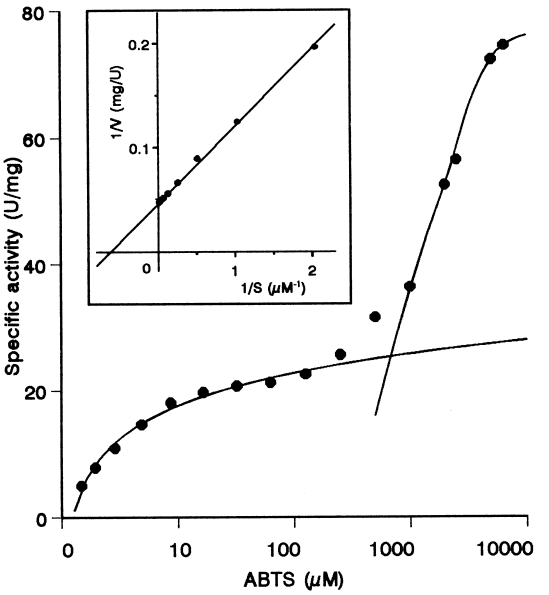

Fig. 2. ABTS oxidation by *Bjerkandera adusta* MnP2: Influence of wide range of substrate concentrations on the velocity of formation of ABTS cation radical (pH 3.5) (semi-logarithmic representation), and Lineweaver-Burk plot for K_m calculation in the μM range (inset).

Table 2
Steady-state kinetics constants (mean values) for oxidation of hydroquinones, substituted phenols, other aromatic compounds, dyes and Mn^{2+} by MnP from *Pleurotus eryngii* and *Bjerkandera adusta*[a]

	P. eryngii MnPL2		*B. adusta* MnP1	
	K_m (μM)	V_{max} (U/mg)	K_m (μM)	V_{max} (U/mg)
Catechol	112	34	64	12
Hydroquinone	22	19	10	16
Methoxyhydroquinone	19	26	10	19
2,6-Dimethoxyhydroquinone	32	33	10	25
Methylhydroquinone	6	17	3	16
p-Aminophenol	21	10	32	11
Guaiacol	92	7	67	5
p-Methoxyphenol	104	33	260	17
Syringol	300	24	170	28
Ferulic acid	10	22	19	22
α-Naphthol	20	31	16	15
Veratryl alcohol	3000	18	4000	19
ABTS	3	39	2	25
Reactive Black 5	4	5	6	6
Reactive Violet 5	12	8	11	7
Reactive Blue 38	7	10	6	7
Mn^{2+}	19	165	17	180

[a]From Lineweaver-Burk plots (linear regression coefficients being significant for $P > 0.99$) of reaction rates in 100 mM sodium tartrate pH 3.5, except for veratryl alcohol and syringol oxidation which were assayed at pH 3 and Mn^{2+} oxidation which was assayed at pH 5, with 0.1 mM H_2O_2 (mean values from triplicate determinations; all standard deviations being below 10% of mean values).

by the inhibitor, more than inhibition of dye oxidation by the enzyme. In the case of methoxyhydroquinone, the semiquinone radical formed after one-electron oxidation could be the dye reductant because of its lower redox potential than that of hydroquinone [20]. We concluded that Mn^{2+} and dyes bind to different sites on the enzyme, and that Mn^{2+} caused non-competitive inhibition of dye oxidation by partial reduction of the oxidized heme. During estimation of catalytic constants for catechol and ABTS, a sigmoidal curve (with two steps in the μM and mM ranges) was obtained for oxidation velocity at different substrate concentrations (Fig. 2). From Lineweaver-Burk plots of the two regions of the curve, K_m values of 2–3 μM (V_{max} 25–40 U/mg) and 1.6 mM (V_{max} 100 U/mg) were obtained for ABTS oxidation by *P. eryngii* MnPL2 or *B. adusta* MnP1. This is congruent with different binding sites involved in oxidation of ABTS (or other substrates). One of them could have higher substrate affinity, and the second one be characterized by high turnover rate resulting in faster substrate oxidation.

4. Discussion

Since their description in 1983–84 [21–23], LiP and MnP of *P. chrysosporium* have been considered as models for all ligninolytic peroxidases. LiP is characterized by high redox potential and efficient oxidation of veratryl alcohol. Nevertheless, it is quickly inactivated by phenolic substrates [24], as formed during lignin degradation. MnP from *P. chrysosporium* differs from LiP by its ability to oxidize Mn^{2+} to Mn^{3+}, which could act as a diffusible oxidant for lignin attack in plant cell wall, and by its incapacity to oxidize aromatic substrates in the absence of the above cation. However, the present study shows that a different type of peroxidase is produced by ligninolytic fungi from the genera *Pleurotus* and *Bjerkandera*. These enzymes oxidize different low redox-potential phenolic compounds, which are typical substrates of plant peroxidases (as horseradish peroxidase, HRP) and fungal laccases (the latter using O_2 as electron acceptor). In addition, they are able to bind and oxidize Mn^{2+} with similar or lower K_m values than *P. chrysosporium* MnP, as well as veratryl alcohol (although with lower affinity for this substrate than LiP). Finally, they are also able to oxidize several high molecular-weight dyes, which are not or very slightly oxidized by LiP in the absence of veratryl alcohol, nor by *P. chrysosporium* MnP. The latter is of biotechnological interest because the azo and phthalocyanine dyes used here are potential contaminants in industrial waste waters.

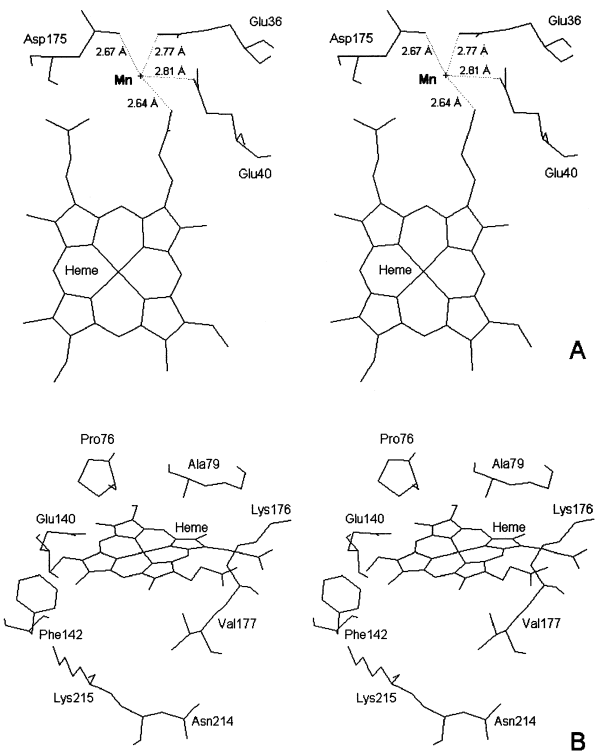

Fig. 3. Stereo view of amino acid residues forming a putative Mn-binding site with the inner heme carboxylate (**A**), and residues at the opening of the main channel of access to heme (**B**) in *Pleurotus eryngii* MnPL.

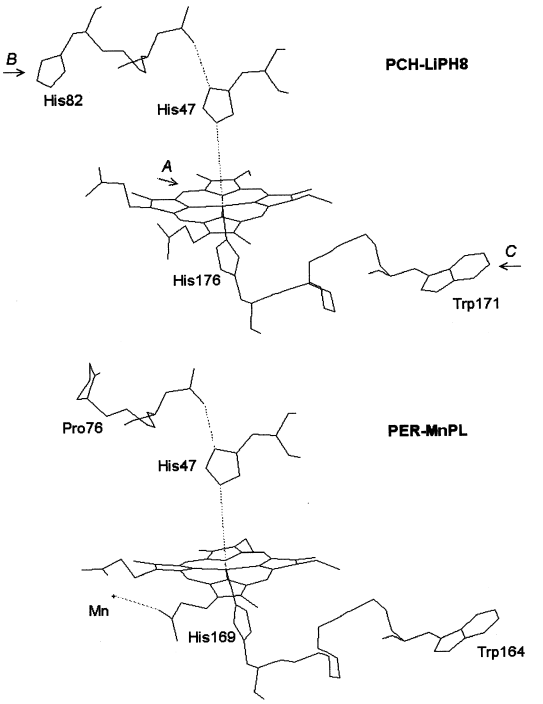

Fig. 4. Electron transfer pathways proposed for substrate oxidation by *Phanerochaete chrysosporium* LiP (A, heme edge; B $His^{82} \rightarrow His^{47}$; and C, $Trp^{171} \rightarrow His^{176}$), and position of heme and corresponding amino-acid residues in the molecular model of *Pleurotus eryngii* MnPL.

The *P. eryngii* and *B. adusta* peroxidases investigated here only catalyze Mn-independent reactions at acidic pH, as found for LiP oxidation of veratryl alcohol. Some oxidation of veratryl alcohol by plant peroxidases has also been reported to occur only at very low pH [25,26], although oxidation of phenols by HRP has an optimum around pH 5. It has been suggested that LiP dependence of acidic pH could be related to some acidic amino-acid residues involved in veratryl alcohol binding (only in their protonated form) [15]. However, the similar pH effect on catalytic properties of different peroxidases could be more likely due to increased redox potential of oxidized heme at low pH. As already mentioned, the capacity of direct oxidation of phenols, as found in *P. eryngii* and *B. adusta* peroxidases, is a common characteristic of many peroxidases. Why LiP and MnP from *P. chrysosporium*, and some other ligninolytic fungi, lost the ability to oxidize

phenols is not well understood. In the case of *P. chrysosporium* MnP, the enzyme can oxidize phenols and dyes via Mn^{3+} [27]. In the case of LiP from *B. adusta* it was found that the enzyme is quickly inactivated during oxidation of phenols or dyes but, as already reported for LiP [28], the reaction can proceed in the presence of veratryl alcohol. It is interesting to mention that the above *P. eryngii* and *B. adusta* enzymes are much more efficient oxidizing hydroquinones than fungal phenoloxidases, the K_m values for hydroquinone oxidation being 100–250-fold higher than found for *P. eryngii* laccases [12]. This is of interest for lignin biodegradation because hydroquinone, methoxy-hydroquinone and 2,6-dimethoxyhydroquinone are quantitatively important products from H (*p*-hydroxyphenyl), G (guaiacyl) and S (syringyl) lignin units [29].

The ability of *P. eryngii* peroxidases to oxidize Mn^{2+} can be explained by the identification of a putative Mn-binding site in the molecular model obtained for the enzyme (Fig. 3A). As described for *P. chrysosporium* MnP [14], Mn^{2+} will be bound by carboxylic oxygens of three acidic amino-acid residues (Glu^{36}, Glu^{40} and Asp^{175} of *P. eryngii* MnPL) and the inner heme propionate. The residues involved in Mn^{2+} binding by *P. chrysosporium* MnP1 have been proved by site-directed mutagenesis [30,31]. However, identification of the aromatic substrate binding sites of peroxidases appears as an elusive matter. In the case of LiP a binding site for veratryl alcohol has been suggested at the heme access channel, including Phe^{148} [15]. Aromatic residues also appear to be involved in aromatic substrate binding by HRP [32,33]. It is interesting to mention that the above Phe residue is substituted by a polar residue (Gln^{145}) in the Mn-dependent peroxidase of *P. chrysosporium* (MnP1) whereas Phe^{142} occupies this position in *P. eryngii* MnPL (Fig. 3B).

Little is known about the molecular mechanisms of oxidation of high molecular-weight compounds, including dyes, by peroxidases. In the case of lignin it is generally accepted that the polymer does not attain the heme and that a veratryl alcohol cation radical acts as redox mediator [34]. However, *P. eryngii* and *B. adusta* MnP can oxidize directly high molecular-weight dyes, some of them with a relatively high redox potential (as the Reactive Black 5 which is not oxidized by Mn^{3+}-lactate). For the direct oxidation of these dyes, a long-range electron transfer from protein surface to heme is strongly suggested. This had also been postulated for veratryl alcohol oxidation by LiP, although veratryl alcohol at the above binding site could transfer electrons directly to the heme edge (pathway A in Fig. 4, top) [15]. In this way, Schoemaker et al. [34] suggested electron transfer from His^{82} at the heme channel binding site via distal His (pathway B in Fig. 4, top). Recently, the observation of hydroxylation of Trp^{171} in LiP crystals (PDB entries 1QPA and 1LGA) led Choinowski et al.[(1)] to suggest electron transfer from this superficial aromatic residue to heme, in this case via proximal His (pathway C in Fig. 4, top). Trp^{171} is near a second potential aromatic substrate binding site in LiP [35], which includes three additional aromatic residues. By comparing the heme-surrounding region of LiPH8 and MnP1, it is possible to deduce that the two above pathways do not exist in *P. chrysosporium* MnP1 (Ser^{78} and Ser^{168} occupying the positions of LiP His^{82} and Trp^{171}). However, Trp^{164} in the molecular model of *P. eryngii* MnPL occupies the same position as Trp^{171} in the LiPH8 model and an electron transfer pathway can be envisaged from this residue in the MnPL surface (Fig. 4, bottom), which could be involved in oxidation of high molecular-weight dyes and other aromatic substrates.

The non-competitive inhibition of dye oxidation by Mn^{2+} confirmed that Mn^{2+} and dyes (or other peroxidase substrates) bind to different sites of *P. eryngii* and *B. adusta* MnP. Moreover, results from oxidation of ABTS and other substrates suggest that several binding sites (in addition to the Mn-binding site) could exist at the protein surface, being involved in substrate oxidation via long-range electron transfer. Their redox potentials will be inversely proportional to the number of covalent or H bonds between the heme and the amino-acid residue abstracting the electron from the substrate [36]. Veratryl alcohol should probably be oxidized in the proximity of heme, its high K_m revealing a binding site with lower substrate affinity than those involved in oxidation of Mn^{2+}, phenolic substrates or dyes. By contrast, ABTS and other substrates with lower redox potentials have more possibilities to find a high affinity binding site at the protein surface and to be efficiently oxidized by a longer range electron transfer reaction even at low substrate concentrations (the K_m values obtained being 2–3 μM). Moreover, if their concentration increases they could be oxidized with a higher reaction velocity at a lower affinity site. A similar situation, including two binding sites with different substrate affinities, has been reported for cytochrome-*c* oxidation by cytochrome-*c* peroxidase [37].

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(1) From Brookhaven Protein Database entry 1QPA: T.H. Choinowski, K.H. Winterhalter and K. Piontek, Refinement of lignin peroxidase isozyme H2 at 1.7 Å resolution reveals a hydroxyl group on the C_β of tryptophan 171. Does tryptophan 171 harbour the free radical? (unpublished).

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