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REVIEW ARTICLE

Substrate oxidation sites in versatile peroxidase and other basidiomycete peroxidases

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Abstract

Versatile peroxidase (VP) is defined by its capabilities to oxidize the typical substrates of other basidiomycete peroxidases: (i) Mn^{2+}, the manganese peroxidase (MnP) substrate (Mn^{3+} being able to oxidize phenols and initiate lipid peroxidation reactions); (ii) veratryl alcohol (VA), the typical lignin peroxidase (LiP) substrate; and (iii) simple phenols, which are the substrates of *Coprinopsis cinerea* peroxidase (CIP). Crystallographic, spectroscopic, directed mutagenesis, and kinetic studies showed that these 'hybrid' properties are due to the coexistence in a single protein of different catalytic sites reminiscent of those present in the other basidiomycete peroxidase families. Crystal structures of wild and recombinant VP, and kinetics of mutated variants, revealed certain differences in its Mn-oxidation site compared with MnP. These result in efficient Mn^{2+} oxidation in the presence of only two of the three acidic residues forming its binding site. On the other hand, a solvent-exposed tryptophan is the catalytically-active residue in VA oxidation, initiating an electron transfer pathway to haem (two other putative pathways were discarded by mutagenesis). Formation of a tryptophanyl radical after VP activation by peroxide was detected using electron paramagnetic resonance. This was the first time that a protein radical was directly demonstrated in a ligninolytic peroxidase. In contrast with LiP, the VP catalytic tryptophan is not β-hydroxylated under hydrogen peroxide excess. It was also shown that the tryptophan environment affected catalysis, its modification introducing some LiP properties in VP. Moreover, some phenols and dyes are oxidized by VP at the edge of the main haem access channel, as found in CIP. Finally, the biotechnological interest of VP is discussed.

Key words: Crystal structures, fungal peroxidases, haem access-channel, lignin biodegradation, manganese-binding site, site-directed mutagenesis, spectroscopic analyses, transient-state kinetics, tryptophanyl radical, versatile peroxidase.

Ligninolytic peroxidases

Degradation of lignin is a key process for carbon recycling in land ecosystems, as well as for utilization of lignocellulosic biomass in paper pulp manufacture and bioethanol production. Degradation of this recalcitrant aromatic polymer is caused in nature by wood-rotting fungi through a process that was defined as an enzymatic combustion (Kirk and Farrell, 1987). Several oxidoreductases (including peroxidases, oxidases, and laccases) secreted by ligninolytic basidiomycetes, the so-called white-rot fungi, are involved in lignin biodegradation (Higuchi, 2004; Martínez *et al*., 2005; Kersten and Cullen, 2007). Ligninolytic haem peroxidases are able to break down model compounds representative of the main linkage types in lignin due to their high redox-potential and specialized catalytic mechanisms (Martínez, 2002, 2007; Hammel and Cullen, 2008). Extracellular oxidases, such as glyoxal oxidase (a copper-radical protein) (Kersten and Kirk, 1987) and aryl-alcohol oxidase (a flavoprotein) (Guillén and Grans, 1994), provide the hydrogen peroxide required by peroxidases. Finally, laccases can directly oxidize only minor phenolic lignin moieties, although their

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Abbreviations: ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonate); CIP, *Coprinopsis cinerea* peroxidase; EPR, electron paramagnetic resonance; MnP, manganese peroxidase; LiP, lignin peroxidase; LRET, long-range electron transfer; PAH, polycyclic aromatic hydrocarbons; RB5, Reactive Black 5; VA, veratryl alcohol; $VA^{\bullet+}$, VA cation radical; VP, versatile peroxidase.

biotechnological interest has increased after discovering that redox mediators (Bourbonnais and Paice, 1990), including natural phenols (Camarero *et al*., 2005), strongly enhance their oxidizing power. Recently, an extracellular haem-thiolate peroxidase, which is also able to degrade lignin model compounds, among other enzymatic reactions, has been found in soil Agaricales, such as *Agrocybe aegerita* (V Briganti) Singer and several *Coprinus* species (Hofrichter and Ullrich, 2006).

Ligninolytic haem peroxidases from white-rot fungi include lignin peroxidase (LiP), manganese peroxidase (MnP), and versatile peroxidase (VP) (Gold *et al*., 2000; Martínez, 2002; Hammel *et al*., 2008). Due to the high redox-potential required for lignin oxidation, ligninolytic peroxidases have been the object of high interest as industrial biocatalysts (Martínez, 2007). LiP and MnP were first described in *Phanerochaete chrysosporium* Burdsall (order Corticiales) and genes/cDNA encoding isoenzymes LiPH8 and MnP1 cloned in 1987–1988 from the same fungus (Cullen and Kersten, 2004). From this date, related genes have been cloned from other Corticiales, Agaricales, and Polyporales basidiomycetes including: (i) over 20 different *mnp* genes from *Agaricus bisporus* (J.E. Lange) Imbach, *Ceriporiopsis rivulosa* (Berk. & M.A. Curtis) Gilbertson & Ryvarden, *Ceriporiopsis subvermispora* (Pilat) Gilbertson & Ryvarden, *Dichomitus squalens* (P. Karsten) D.A. Reid, *Lentinula edodes* (Berk.) Pegler, *Phlebia radiata* Fries, *P. chrysosporium*, *Phanerochaete sordida* (P. Karsten) J. Eriksson & Ryvarden, *Pleurotus ostreatus* (Jacquin) P. Kummer, and *Trametes versicolor* (L.) Lloyd; and (ii) around 15 *lip* genes from *Bjerkandera adusta* (Willd.) P. Karst., *P. radiata*, *P. chrysosporium*, *Trametes cervina* (Schwein.) Bres., and *T. versicolor* (Ruiz-Dueñas *et al*., 2007*a*). By contrast, VP genes have been cloned only from a few species from the genera *Pleurotus* (order Agaricales), including *Pleurotus eryngii (*DC.) Gillet and *Pleurotus sapidus* (Schulzer) Sacc. among others, and *Bjerkandera* (order Polyporales), including *Bjerkandera adusta* (Willd.) P. Karst. (Camarero *et al*., 1999; Ruiz-Dueñas *et al*., 1999, 2007*a*; Moreira *et al*., 2005). VP could also be produced by other Polyporales basidiomycetes including species from the genera *Panus* (Lisov *et al*., 2007), *Trametes* (Ruiz-Dueñas *et al*., 2007*a*), and *Spongipellis* (GenBank AB244274).

The evolutionary relationships of all the proteins encoded by these genes are shown in the dendrogram of Fig. 1, which also indicates the presence in the different sequences of residues forming the substrate oxidation sites discussed below (involved in the oxidation of Mn^{2+} and high redox-potential aromatic substrates). The dendrogram obtained from comparison of the 56 basidiomycete peroxidases available showed a well-defined cluster corresponding to typical MnP (from *Phanerochaete*, *Ceriporiopsis*, *Phlebia*, and *Dichomitus*). The second cluster is large and includes a small subcluster containing three atypical MnP from *Ganoderma* species (and the so-called Mn-repressed peroxidase from *T. versicolor*), and a large subcluster where all LiP and VP, and some MnP (from *Pleurotus*, *Trametes*, *Phlebia*, *Lentinula*, and *Agaricus*), are located (together with unclassified peroxidases, such as *Trametes* PGV and PGVII). In this heterogenous cluster, typical LiP (from *Phanerochaete*, *Bjerkandera*, *Phlebia*, and *Trametes*) form a relatively homogeneous group, whereas the group of typical VP (from *Pleurotus*) is small, and other VP are related to different MnP. Finally, two peroxidases from the non-lignicolous fungi *Coprinopsis cinerea* (Schaeff.) Redhead, Vilgalys and Moncalvo (synonym *Coprinus cinereus* (Schaeff.) Gray) and *Coprinellus disseminatus* (Pers.) Gray, and the NOPA peroxidase identified in the *P. chrysosporium* genome (Larrondo *et al*., 2005), are not grouped together suggesting that they are not related to ligninolytic peroxidases. Gene fragments from the above peroxidase families have been recently identified in a variety of other basidiomycetes indicating a wider distribution (Morgenstern *et al*., 2008).

Versatile peroxidase

VP oxidizes Mn^{2+}, as MnP (EC 1.11.1.13) does, and also high redox-potential aromatic compounds, as LiP (EC 1.11.1.14) does. Due to their Mn-oxidizing activity, the *Pleurotus* VP isoenzymes were first described as MnP isoenzymes (Martínez *et al*., 1996; Giardina *et al*., 2000), but they were later recognized as representing a new peroxidase type (EC 1.11.1.16). VP is also able to efficiently oxidize phenolic compounds and dyes that are the substrates of generic peroxidases (EC 1.11.1.7), such as the *C. cinerea* peroxidase (CIP) (Baunsgaard *et al*., 1993) and related peroxidases, or the well-known horseradish peroxidase (HRP) (Veitch, 2004). By contrast, LiP is not able to oxidize phenolic compounds efficiently because of inactivation in the absence of veratryl alcohol (VA) or related substrates, and MnP only oxidize phenols in the presence of Mn^{2+}, although a *P. radiata* short MnP seems to be an exception (Hildén *et al*., 2005). Moreover, VP directly oxidizes high redox-potential compounds, for example, the dye Reactive Black 5 (RB5), that LiP can oxidize only in the presence of redox mediators such as VA (Heinfling *et al*., 1998*b*). The Mn^{3+} formed by VP (and MnP) acts as a diffusible oxidizer on phenolic lignin and free phenols, as well as a starter of lipid peroxidation reactions involved in the biodegradation of recalcitrant compounds (Kapich *et al*., 2005). The VP family is included in Class II (fungal peroxidases) of the 'plant, fungal, and bacterial peroxidase' superfamily (Welinder, 1992) together with the MnP, LiP, and CIP families. The interest on VP has increased during the last years, both as

a model enzyme and as a source of industrial/environmental biocatalysts.

The molecular architecture of VP and other basidiomycete peroxidases includes a haem cofactor located at an internal cavity connected to the solvent by two access channels (Gold *et al*., 2000; Martínez, 2002). The main channel is conserved in all haem peroxidases and is used by hydrogen peroxide to reach and oxidize the haem cofactor forming the two-electron activated Compound I, which is stepwise reduced to Compound II and resting enzyme during two one-electron substrate oxidations. The second channel extends directly to the haem propionates being the site where MnP and VP oxidize Mn^{2+} to Mn^{3+}, as described below. Bifunctional prokaryotic (Class I) ascorbate peroxidase also oxidizes ascorbate near the haem propionates (Sharp *et al*., 2003).

Oxidation of phenolic compounds has been studied in peroxidases from Class III (plant peroxidases) and also from Class II, and the classical hypothesis that the reaction takes place in the vicinity of the haem has been demonstrated for plant HRP and fungal CIP (Smith and Veitch, 1998). However, no crystal structure of a ligninolytic peroxidase complexed with an aromatic substrate or analogue has been reported to date. Moreover, it is generally accepted that the main haem channel, in spite of certain degree of plasticity (Gerini *et al*., 2003), is too narrow for direct contact between the haem cofactor and most aromatic substrates including lignin. Therefore, long-range electron transfer (LRET) from a protein radical at the surface of the enzyme, which would act as the substrate oxidizer, to the haem cofactor has been suggested to explain oxidation of aromatic substrates, redox mediators, and even the lignin polymer. As discussed below, dual oxidation of some aromatic substrates and dyes at the VP surface (by LRET) and at the haem channel is considered in the light of some recent results.

Mn-oxidation site in VP

Enzymatic oxidation of Mn^{2+} to Mn^{3+} is a unique characteristic of basidiomycete MnP and VP, although it has also been reported in prokaryotic catalase-peroxidase (Magliozzo and Marcinkeviciene, 1997). The Mn^{2+}-oxidation site in *P. eryngii* VP is similar to that of *P. chrysosporium* MnP (Gold *et al*., 2000; Sundaramoorthy *et al*., 2005), but it has certain differences as revealed by crystallographic, site-directed mutagenesis, and kinetic studies (Ruiz-Dueñas *et al*., 2007*b*). The Mn^{2+}-binding site in VP is formed by the side-chains of Glu36, Glu40, and Asp175 located in front of the internal (i.e. more distant from the main haem access-channel) propionate of haem (Fig. 2, top), and connected to the solvent by a narrow access-channel that presents a variable geometry during catalysis. The carboxylates of the above amino-acid residues and the haem propionate are responsible of Mn^{2+} binding for subsequent electron transfer to the activated haem of VP Compounds I and II.

The crystal structures (solved up to 1.3 Å resolution) of wild and *Escherichia coli* (Migula) Castellani & Chalmers expressed VP showed a variable orientation of the Glu36 and Glu40 side-chains, whereas that of Asp175 is maintained (Ruiz-Dueñas *et al*., 2007*b*). The position in the *in vitro* activated recombinant VP (Pérez-Boada *et al*., 2002) before its exposure to Mn^{2+} (and in the Mn^{2+} exposed D175A variant, which suffer from a strong decrease in the ability to bind and oxidize the cation) corresponds to an 'open-gate' conformation enabling Mn^{2+} access to the oxidation site. The conformation in which the two glutamate side-chains point toward the Mn^{2+} ion correspond to the 'closed-gate' conformation, and was found in the naturally-folded enzyme from Mn^{2+}-containing *P. eryngii* cultures. In this conformation Glu36, Glu40, Asp175, and propionate carboxylates are at co-ordination distance from Mn^{2+}.

In contrast to that reported for MnP (Sundaramoorthy *et al*., 1994, 1997), the recombinant VP crystals grown in the presence of Mn^{2+} were unstable, and the addition of Zn^{2+} to grow crystals after Mn^{2+} exposure resulted in an intermediate conformation of the metal binding site, with the Glu40 side-chain at a position different from that found in wild VP crystals. The 'closed-gate' conformation described above coincides with that observed in *P. chrysosporium* MnP crystallized in the presence of Mn^{2+}, where three homologous residues (Glu35, Glu39, and Asp179) participate in metal co-ordination (Sundaramoorthy *et al*., 1994) (Fig. 2, centre). Opening of the glutamate side-chains was not observed in MnP crystals grown in the absence of Mn^{2+} (Sundaramoorthy *et al*., 1997). However, displacement of Glu35 and Glu39 side-chains was found in crystals of a mutated MnP variant that does not bind Mn^{2+} (Sundaramoorthy *et al*., 1997) and in MnP crystals where the inhibitor Sm^{3+} was removed from the metal binding site (Sundaramoorthy *et al*., 2005). Additional amino acid residues, which are absent in VP, contribute to orientation of the Mn^{2+} ligands in MnP affecting their mobility. This is the case of Arg177 forming a salt bridge with Glu35 necessary for the optimal Mn^{2+} co-ordination and ligation geometry in MnP (Gelpke *et al*., 2000)

Site-directed mutagenesis was performed to evaluate the involvement of the three above acidic residues in VP catalysis (Ruiz-Dueñas *et al*., 2007*b*). The E36A, E40A, and D175A mutations caused 4–20-fold decrease in Mn^{2+} oxidation activity (k_{cat}) under steady-state conditions (characterized by VP saturating concentrations of both oxidizing and reducing substrates). Moreover, the selectivity constant (k_{cat}/K_m, also known as catalytic efficiency) was 250–1250-fold decreased, indicating that the above residues are responsible for selective binding of Mn^{2+} near

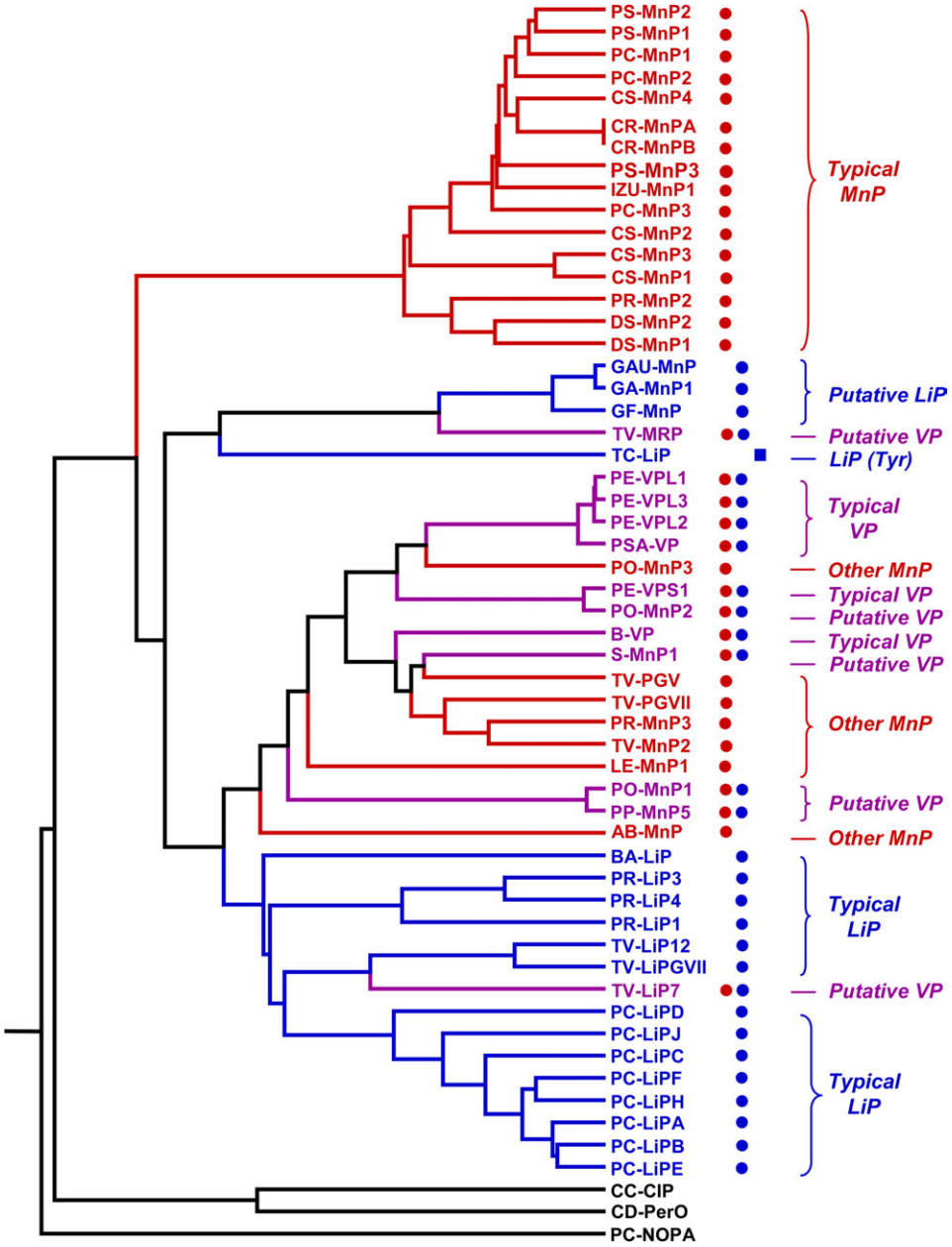

Fig. 1. Evolutionary relationships and structural–functional classification of basidiomycete peroxidases (mature proteins) including 53 ligninolytic enzymes belonging to the MnP, LiP, and VP families (and some related unclassified peroxidases) and three low-redox potential peroxidases. The phylogenetic analysis was performed using MEGA4 (Tamura *et al*., 2007) with Poisson-corrected evolutionary distances, complete deletion option (269 positions in the final dataset), and UPGMA clustering method. The structural–functional classification was based on the presence in the protein sequence or molecular structure of the structural motifs characteristic of the above families, namely: (i) Mn^{2+}-oxidation site formed by three acidic residues (red circles); and (ii) exposed catalytic tryptophan oxidizing high redox-potential substrates (blue circles). As a result of the structural analysis, three putative LiP (previously described as MnP) and six putative VP (unclassified or described as LiP or MnP) were identified. The presence of a catalytic tyrosine in the LiP from *T. cervina*, playing the same function of the above catalytic tryptophan, is also indicated (blue square). Species abbreviations: AB, *A. bisporus;* B, *Bjerkandera* sp; BA, *B. adusta*; CC, *C. cinerea*; CD, *C. disseminatus*; CR, *C. rivulosa*; CS, *C. subvermispora*; DS, *D. squalens*; GA, *G. applanatum*; GAU, *G. australe*; GF, *G. formosanum*; IZU, unidentified basidiomycete IZU-154; LE, *L. edodes*; PC, *P. chrysosporium*; PE, *P. eryngii*; PO, *P. ostreatus*; PP, *P. pulmonarius*; PR, *P. radiata*; PS, *P. sordida*; PSA, *P. sapidus*; S, *Spongipellis* sp; TC, *T. cervina*; and TV, *T. versicolor*. Protein sequence entries: AB-MnP, CAG27835; B-VP, AAO47909; BA-LiP, 1906181A; CD-PerO, AAZ14938; CC-CIP, CAA50060; CR-MnPA, ABB83812; CR-MnPB, ABB83813; CS-MnP1, AAB03480; CS-MnP2, AAD43581; CS-MnP3, AAD45725; CS-MnP4, AAO61784; DS-MnP1, AAF31329; DS-MnP2, AAF31330; GA-MnP1, BAA88392; GAU-MnP, ABB77244; GF-MnP, ABB77243; IZU-MnP1, no entry available but sequence taken from the literature (Matsubara *et al*., 1996); LE-MnP1, BAE79199; PC-LiPA (isoenzyme H8), AAA53109; PC-LiPB (isoenzyme H8), AAA33741; PCLiPC (isoenzyme H10), AAA33739; PC-LiPD (isoenzyme H2), CAA33621; PC-LiPE (isoenzyme H8), AAA33738; PC-LiPF, AAA33736; PC-LiPH (isoenzyme H8), AAA56852; PC-LiPJ, AAD46494; PC-MnP1, AAA33744; PCMnP2, AAA33745; PC-MnP3, AAB39652; PC-NOPA, AAU82081; PE-VPL1, AAD01401; PE-VPL2, AAD01404; PE-VPL3, CAD56164 (and DQ056374); PE-VPS1, AAD54310; PO-MnP1, AAA84396; PO-MnP2, CAB51617; PO-MnP3, BAA33449; PP-MnP5, AAX40734; PR-LiP,

the haem. The decreases in VP selectivity constant were much lower than found in similar MnP variants (E40A and D175A) (Kishi *et al*., 1996; Whitwam *et al*., 1997; Youngs *et al*., 2001). This is due to differences in k_{cat}, which decreased from similar values in both native enzymes (around 300 s^{-1}) to much lower values in the MnP variants (1–3 s^{-1}) than in the VP variants (15–85 s^{-1}). In fact, removal of the three carboxylates in a triple variant (E36A/E40A/D175A) was required to lower the VP activity to levels (<1% k_{cat}) similar to those found in MnP single variants.

Transient-state kinetic constants showed that reduction of both VP Compounds I and II by Mn^{2+} (estimated from changes of their characteristic spectra using stopped-flow rapid spectrophotometry) was modified by the E36A, E40A, and D175A mutations. The apparent second-order rate constants revealed that reduction of Compound II was much more affected (10^3–10^4-fold lower k_{3app}) than Compound I reduction (80–325-fold lower k_{2app}), the former being a rate-limiting step in VP catalysis. Site-directed mutagenesis of *P. chrysosporium* MnP also showed that reduction of both Compound II (Kusters-van Someren *et al*., 1995; Kishi *et al*., 1996) and Compound I (Whitwam *et al*., 1997; Youngs *et al*., 2001) by Mn^{2+} was lowered by mutations at the binding site. However, the decreases of apparent second-order rate constants were lower in the VP than in the homologous MnP variants. Moreover, the first-order constants for Compound II reduction (Compound I reduction is too fast to be measured) showed a strong increase of the dissociation constant (300–1000-fold), but only slightly affected k_3 (2–30-fold decrease) in the case of VP mutants, whereas a strong decrease in k_3 (100–1200-fold) was also observed in the corresponding MnP variants.

The selectivity constant for Mn^{2+} also decreased (50–75-fold) when the acidic side-chains were shortened in the E36D and E40D variants that, nevertheless, retained 30–50% of their steady-state activity (k_{cat}). This strongly contrasts with the behaviour of *P. chrysosporium* MnP, where similar mutations (E35D and E39D) caused 50–100 fold decrease of k_{cat} (Kishi *et al*., 1996; Youngs *et al*., 2001). The same tendency was observed in the first-order rate constant for Compound II reduction (k_3) that showed 40–120-fold lower values in the MnP variants but only a 1–4-fold decrease in the VP variants.

The kinetic constants of VP mutated variants at Glu36 and Glu40 positions showed that the Mn^{2+}-oxidation site of VP, although having the same Mn-co-ordinating residues as found in MnP, operates in a slightly different way, in the sense that Mn^{2+} is still efficiently oxidized when one of the three amino acid carboxylates is removed (Glu/Asp → Ala mutations) or when the length of the acidic side-chains is reduced (Glu → Asp mutations) resulting in much higher k_{cat} and k_3 values than found for homologous MnP variants. A putative MnP has been reported in *Ganoderma applanatum* (Pers.) Pat., *Ganoderma australe* (Fr.) Pat., and *Ganoderma formosanum* T.T. Chang & T. Chen (Maeda *et al*., 2001) with only two acidic residues at the putative Mn^{2+}-oxidation site. However, the catalytic properties of these peroxidases should be re-examined, since sequence alignment showed a tryptophan residue homologous to the VP Trp164 (or LiP Trp171) discussed below (Fig. 1).

Mn-oxidation sites (red circles in Fig. 1) have been identified in the sequences (and molecular models when available) of 23 MnP and six VP, as well as in five putative VP that were initially described as MnP, including MnP1 from *P. ostreatus* and MnP5 from *Pleurotus pulmonarius* (Fr.) Quel., or LiP (and one of them as an unclassified Mn-repressed peroxidase). The kinetic constants of these putative VP on representative substrates should be re-examined.

VP oxidation of high redox-potential substrates: LRET mechanism

LRET occurs in different redox proteins, like prokaryotic cytochrome-*c* peroxidase that oxidizes cytochrome *c* at the protein surface, and transfers electrons to a stable tryptophan radical near the haem cofactor via three amino acid residues (Pelletier and Kraut, 1992). LRET has been suggested to be involved in lignin biodegradation by ligninolytic haem peroxidases for electron transfer both: (i) in the peroxidase molecule, to overcome the steric hindrances preventing direct interaction between the haem group and the lignin polymer; and (ii) in the lignin macromolecule itself resulting in breakdown of the most labile inter-unit linkages (Schoemaker *et al*., 1994*a*, *b*).

Crystal structures and homology models of two *P. eryngii* VP isoenzymes revealed three possible LRET pathways for the oxidation of high redox-potential aromatic compounds (Camarero *et al*., 1999; Ruiz-Dueñas *et al*., 1999; Pérez-Boada *et al*., 2005). They would start either at Trp164 or His232 of isoenzyme VPL, and at His82 or Trp170 (homologous to VPL Trp164) of isoenzyme VPS1. These residues are exposed, and less than 11 Å apart from the haem. The corresponding pathways would be similar to those proposed for *P. chrysosporium* LiP that would start at His82, Trp171, or

P20010; PR-LiP1, AAW59419; PR-LiP4, AAW66483; PR-MnP2, CAC85963; PR-MnP3, CAC84573; PS-MnP1, BAC06185; PS-MnP2, BAC06186; PS-MnP3, BAC06187; PSA-VP, CAJ01576; S-MnP1, BAE79812; TC-LiP, AB191466; TV-LiP12, AAA34049; TV-LiP7, CAA83147; TV-LiPGII, CAA53333; TV-MnP2, CAA83148; TV-MRP, AAB63460; TV-PGV, CAA54398; and TV-PGVII, CAA91043.

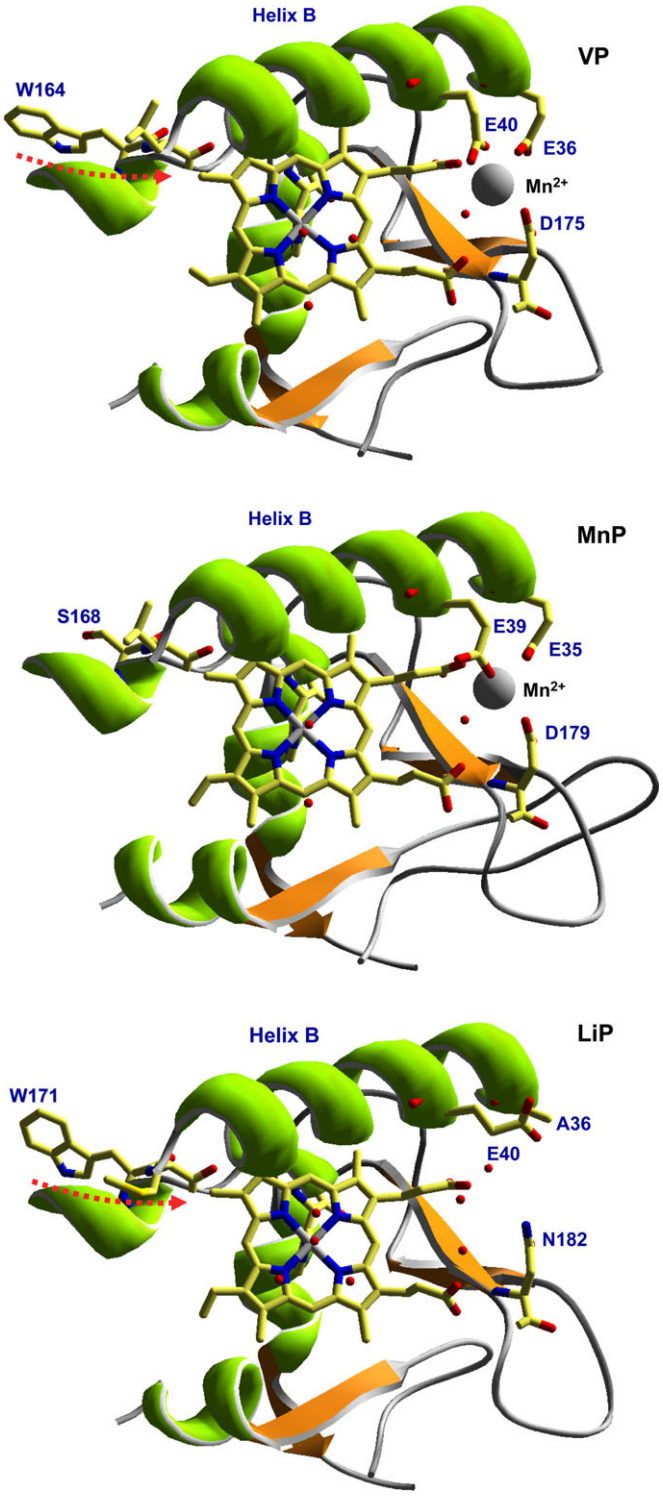

Fig. 2. Axial views of the haem region showing the exposed catalytic tryptophan in VP (Trp164) and LiP (Trp171) responsible for oxidation of high redox-potential substrates by LRET to haem (red arrow), and the residues forming the Mn^{2+}-binding site near the internal propionate of haem in VP (Glu36, Glu40, and Asp175) and MnP (Glu35, Glu39, and Asp179). Mn^{2+} is shown as a van der Waals sphere, and several water molecules are included (red spheres). From PDB entries, 2BOQ, 1YYD, and 1LLP for VP, MnP, and LiP, respectively.

His239 (Du *et al*., 1992; Schoemaker *et al*., 1994*a*; English *et al*., 1995; Johjima *et al*., 1999). With the purpose of investigating the functionality of these three putative pathways, two single mutations (W164S and H232F) and one double mutation (W164S/P76H) were introduced in VPL that: (i) removed the two pathways present in this isoenzyme; and (ii) incorporated the absent pathway (Pérez-Boada *et al*., 2005). The steady-state kinetic constants of the variants obtained definitively showed that Trp164 (see Fig. 2, top) is required for the oxidation of the two high redox-potential model substrates investigated, VA and RB5, whereas the two other putative pathways (starting at His232 and His82) did not seem to be involved in catalysis. Transient-state kinetics showed that the W164S mutation completely blocked the reduction of Compound II by both substrates, whereas the apparent second-order rate constant for Compound I reduction was only partially decreased (15–50-fold lower k_{2app} values) (Ruiz-Dueñas *et al*., 2008*a*).

The above results were in agreement with those obtained by mutagenesis of homologous Trp171 (Fig. 2, bottom) and His82 in *P. chrysosporium* LiP (mutagenesis of His239 has not been reported) (Doyle *et al*., 1998; Mester *et al*., 2001*a*; Gelpke *et al*., 2002) and contrasted with some unconfirmed results suggesting that VA could be oxidized at the haem channel (Ambert-Balay *et al*., 1998). Involvement of a tryptophan residue in VP catalysis had been suggested by *N*-bromosuccinimide bromination of *Pleurotus* and *Bjerkandera* VP (Ayala-Aceves *et al*., 2001; Ruiz-Dueñas *et al*., 2001; Kamitsuji *et al*., 2005; Pogni *et al*., 2005). In addition to the site-directed mutagenesis of *P. eryngii* VP described above, mutagenesis of the catalytic tryptophan of *P. ostreatus* VP has recently been reported using a homologous expression system (Tsukihara *et al*., 2008). It has also been shown that a tetrameric lignin model compound is degraded by LiP but it is not oxidized by its W171S variant (Mester *et al*., 2001*a*). This suggests that polymeric lignin would be oxidized at the same exposed tryptophan involved in VA oxidation. However, this conclusion requires additional investigation, since it has also been reported that a lignin dehydrogenation polymer would bind LiP at His239 (Johjima *et al*., 1999).

Concerning the aromatic substrate oxidation mechanism, Pérez-Boada *et al*. (2005) provided the first direct evidence on the formation of a protein (Trp164) radical in a ligninolytic peroxidase using low-temperature electron paramagnetic resonance (EPR) of H_2O_2-activated *P. eryngii* VP. However, indirect evidence on the formation of a Trp171 radical had already been reported in LiP (Blodig *et al*., 1999). This is in agreement with EPR results obtained with *B. adusta* VP (Pogni *et al*., 2005), but contrasted with unconfirmed LiP studies that assigned the Compound I EPR signal to a porphyrin π cation radical (Khindaria and Aust, 1996). Additional

spectroscopic and density functional theory studies confirmed the location of the tryptophan radical in VP, and showed that it exists in the neutral form (Pogni *et al*., 2006, 2007).

Substitution of Trp164 either by serine or histidine resulted in similar incapability of both variants to oxidize high redox-potential compounds, indicating that the imidazole side-chain is not playing a similar role as the indole side-chain. Interestingly, a LiP-type peroxidase has been reported in *T. cervina* that has an exposed tyrosine residue involved in catalysis, although located at a different position than the LiP and VP catalytic tryptophan (Miki *et al*., 2006). However, the W164Y variant of VP was not catalytically-active on VA and RB5 under steady-state conditions due to the blocked reduction of Compound II, although the corresponding tyrosinyl radical was clearly detected by EPR (Ruiz-Dueñas *et al*., 2008*c*).

A significant difference between VP and *P. chrysosporium* LiP concerns autocatalytic modification of LiP Trp171. It has been reported that Trp171 in *P. chrysosporium* LiP is naturally β-hydroxylated, most probably due to peroxide excess (with respect to reducing substrate) in fungal cultures (Blodig *et al*., 1998, 2001). However, this is not the case in VP as shown by X-ray diffraction of crystals from both wild and peroxide-treated recombinant VP (Pérez-Boada *et al*., 2005). The origin and physiological significance of this difference is to be determined.

Exposed tryptophan residues homologous to VP Trp164 (blue circles in Fig. 1) have been identified in the sequences (and molecular models when available) of 14 LiP and six VP, as well as in three putative LiP and five putative VP, the latter already mentioned above. The kinetic constants of these putative LiP (and VP) on veratryl alcohol and other substrates should be re-examined.

VP oxidation of high redox-potential substrates: catalytic Trp164 environment

Some characteristics of the surface environment of the catalytic tryptophan (VP Trp164 and LiP Trp171) could also be at the origin of differences in the oxidation of high redox-potential aromatic substrates, such as the need for redox mediators in LiP oxidation of some compounds that are directly oxidized by VP (e.g. RB5), and the lower VP efficiency oxidizing VA. With the purpose of investigating this hypothesis, different residues near the Trp164 of VP were modified by site-directed mutagenesis (Ruiz-Dueñas *et al*., 2008*a*). Some of the mutations strongly modified the kinetics of VA and RB5 oxidation by VP, demonstrating that other residues at the catalytic tryptophan environment contribute to substrate oxidation by ligninolytic peroxidases.

The most outstanding characteristic of the LiP surface around the catalytic Trp171 is the protruding side-chain of Phe267 in an environment with a partial negative charge, whereas VP has several basic residues in the Trp164 environment, and Ala260 at the position of LiP Phe267 (Ruiz-Dueñas *et al*., 2008*a*). It was first considered by the authors that the lack of direct activity of LiP on RB5 could be due to the presence of Phe267 interrupting the furrow where this large substrate would be accommodated in VP. However, this did not seem to be the case since introduction of a phenylalanine residue (A260F variant) did not modify RB5 oxidation by VP. By contrast, VP activity on RB5 was eliminated by the R257D mutation. Therefore, the current hypothesis is that the lack of LiP activity on RB5 would not be due to the steric hindrances mentioned above, and also suggested by other authors (Tsukihara *et al*., 2008), but to the existence of a partially-negative environment (including Asp264 homologous to VP Arg257, and Asp165 homologous to VP Ser158) compared with the positively-charged environment of VP Trp164 (including Lys264 in addition to Arg257) that would favour binding of anionic substrates.

On the other hand, it was demonstrated that three of the five exposed acidic residues around Trp171 in *P. chrysosporium* LiP affected the kinetics of VA oxidation (Smith and Doyle, 2006) and suggested that a partially acidic environment would stabilize the VA cation radical ($VA^{\bullet+}$) formed to act as an enzyme-bond mediator (Khindaria *et al*., 1997). Two of the acidic residues in LiP (Glu168 and Glu250) are conserved in VP, and the three others were introduced as single and/or multiple VP mutations. In contrast with the initial expectations that predicted a more efficient oxidation of VA, the N256D/R257D/A260F VP variant completely lost its activity on VA. Moreover, this and other variants bearing the R257D, N256D or S158E mutations also lost their activity on RB5. Interestingly, the RB5-oxidizing activity of the R257D variant was restored by adding VA to the reaction mixture. This suggested stabilization of $VA^{\bullet+}$ by a more acidic Trp164 environment in the mutated VP, similar to that found in LiP. Such behaviour is similar to the VA-mediated activity of LiP on RB5 and other substrates, and contrasted with RB5 direct oxidation by native VP (Heinfling *et al*., 1998*b*).

Finally, transient-state kinetic studies revealed that combination of the A260F and R257A mutations in a VP double variant strongly increased the apparent second-order rate constants for the reduction of Compounds I and II by VA (over 20-fold and 50-fold improvement, respectively). Moreover, the first-order rate and dissociation constants for Compound II reduction showed improvement of both the k_3 and K_{D3} constants. It is noteworthy that the k_{3app} for reduction of Compound II of the R257A/A260F variant by VA (68 mM^{-1} s^{-1}) was only slightly lower than reported for *P. chrysosporium* LiP (185 mM^{-1} s^{-1}); and its k_3 (46 s^{-1}) was even higher than that of LiP (34 s^{-1}) (Gelpke *et al*., 2002). The involvement of Phe267 in VA binding had been reported in LiP (Gelpke

et al., 2002). The VP A260F and R257L single variants also showed some decreases of VA K_{D3}, but the k_3 values were not comparable to that of the VP double variant, indicating that the simultaneous removal of Arg257 and incorporation of a phenylalanine residue (homologous to LiP Phe267) are required to obtain a VP variant with VA transient-state kinetic constants comparable to those of LiP. It has been suggested that the discrepancy between the strongly improved transient-state constants and the barely modified steady-state constants of the R257/A260F variant of VP could be due to slow dissociation of the enzyme–product complex (Ruiz-Dueñas *et al*., 2008*a*). However, if $VA^{\bullet+}$ is the product of VA oxidation by VP and LiP, and veratraldehyde is formed by subsequent reaction with O_2 (Schoemaker *et al*., 1994*b*), the latter reaction could also be the rate-limiting step in veratraldehyde formation by this variant.

Oxidation of low redox-potential substrates at the VP main haem channel

Sigmoidal curves are obtained for oxidation of different concentrations of low redox-potential 2,2′-azino-*bis*(3-ethylbenzothiazoline-6-sulphonate) (ABTS), *p*-hydroquinone, and 2,6-dimethoxyphenol by VP (Heinfling *et al*., 1998*b*; Ruiz-Dueñas *et al*., 2001) enabling the calculation of two sets of K_m and k_{cat} constants. This double kinetics suggested two independent oxidation sites, characterized by high and low specificity constants. This was confirmed by site-directed mutagenesis of VP Trp164 resulting in the removal of the high-specificity site for ABTS oxidation, whereas the low-specificity site remained (Ruiz-Dueñas *et al*., 2008*a*). Recently, a similar effect of the W164S mutation has been found on the VP oxidation of phenols.

Simultaneously, the location of the low specificity oxidation site has been identified by site-directed mutagenesis at the main haem access channel. This resulted in several-fold increased specificity constants for phenols after removing the side-chains of some amino acids increasing the channel size (Ruiz-Dueñas *et al*., 2008*b*). To confirm substrate oxidation at the haem channel in these improved variants, the mutations opening the haem channel were combined with the W164S mutation. Variants with specificity constants similar to those of the high specificity site in native VP were obtained in spite of the removal of Trp164.

These studies provided evidence on the oxidation of different substrates at the main haem channel of native VP, albeit with a lower specificity than observed at the catalytic Trp164 (that, in addition, is able to oxidize high redox-potential substrates that are not oxidized at the haem channel of native VP). This third substrate-oxidation site in VP is similar to the classical oxidation site reported in different peroxidases, including CIP (Smith *et al*., 1998; Tsukamoto *et al*., 1999). Interestingly, its operation is improved when the haem channel in VP, which is smaller than in CIP and plant peroxidases, is opened by site-directed mutagenesis. Studies currently being conducted include transient-state kinetics and crystallographic studies of these haem channel variants.

It is possible to conclude that the versatile catalytic properties of *P. eryngii* VP are due to a hybrid molecular architecture that includes in the same protein different oxidation sites (for Mn^{2+}, high redox-potential aromatics, and low redox-potential phenols and dyes) related to those found in the other basidiomycete peroxidase families (MnP, LiP, and CIP, respectively).

Biotechnological interest of VP and other basidiomycete peroxidases

Xylanases were introduced in the bleaching plant of paper-pulp mills, as a substitute for chlorine-containing reagents (Viikari *et al*., 1994), at a time when barely any information on ligninolytic peroxidases was available. However, enzymes acting on lignin (ligninolytic peroxidases and laccases) are the biocatalysts of choice for environmentally sound bleaching (Bajpai, 2004; Sigoillot *et al*., 2005). Laccases have been known for decades and are already produced in high yields, however, ligninolytic peroxidases are not commercially available despite the considerable efforts devoted to improve their heterologous expression (Conesa *et al*., 2000, 2002; Gu *et al*., 2003; Lú-Chau *et al*., 2004; Eibes *et al*., 2008). Laccases have low redox-potential and can degrade lignin only in the presence of mediators, whose costs and potential environmental risks are the main obstacles for implementing the laccase-mediator systems at the mill scale. By contrast, some ligninolytic peroxidases do not require mediators to degrade high redox-potential compounds.

Among the different basidiomycete peroxidases, VP presents particular interest due to its catalytic versatility including the degradation of compounds that other peroxidases are not able to oxidize directly. VP versatility permits its application in Mn^{3+}-mediated or Mn-independent reactions on both low and high redox-potential aromatic substrates and dyes, among others. It has been shown recently that VP can be used to reoxidize Mn-containing polyoxometalates, which are efficient oxidizers in paper pulp delignification (Marques *et al*., 2008). Another aspect of VP catalysis, which has been reported recently and merits investigation in more detail for some applications, is its high activity in the presence of organic solvents (Rodakiewicz-Nowak *et al*., 2006).

The ability to degrade directly a variety of recalcitrant compounds represents a VP characteristic of the highest biotechnological interest. The use of aromatic mediators acting via their cation radicals (Schoemaker *et al*., 1994*b*) is required for LiP oxidation of simple phenols (Koduri and Tien, 1995), monomethoxylated aromatics

(Harvey *et al*., 1986), polymeric dyes including Poly R-478 (Harvey *et al*., 1995; Paszczynski and Crawford, 1991), PAH (Barr and Aust, 1994), high redox-potential dyes (Heinfling *et al*., 1998*b*), ferrocytochrome *c* (Sheng and Gold., 1998), and, most important, polymeric lignin (Hammel *et al*., 1993). By contrast, VP does not requires mediators to oxidize many of the above compounds, including phenolic and non-phenolic aromatic pollutants (Rodríguez *et al*., 2004), pesticides (Dávila-Vázquez *et al*., 2005), high redox-potential and polymeric dyes (Heinfling *et al*., 1998*a*; Kamitsuji *et al*., 2005; Tinoco *et al*., 2007), PAH (Wang *et al*., 2003), and lignin (Camarero *et al*., 2001; Moreira *et al*., 2007). This VP ability represents an important advantage with respect to LiP, since the cost of VA as a mediator can be saved. Moreover, for more efficient oxidation of some recalcitrant compounds VP can also be used in combination with redox mediators including natural phenolic compounds (Tinoco *et al*., 2007).

The abundant information on peroxidase structure–function relationships currently available has already been used to modulate the catalytic and operational properties of these enzymes (including peroxide inactivation resistance) as industrial biocatalysts using site-directed mutagenesis (Yeung *et al*., 1997; Wilcox *et al*., 1998; Timofeevski *et al*., 1999; Reading and Aust, 2000; Celik *et al*., 2001; Mester and Tien, 2001*b*; Miyazaki and Takahashi, 2001; Valderrama *et al*., 2002; Feng *et al*., 2003; Ryan *et al*., 2006; Pfister *et al*., 2007; Ruiz-Dueñas *et al*., 2008*b*). In those cases where the structural basis of the property to be improved is unknown, or too difficult to be predicted, directed evolution and/or combinatorial mutagenesis are the approach of choice (Cherry *et al*., 1999; Miyazaki-Imamura *et al*., 2003; Ryu *et al*., 2008). It is expected that genetic engineering will also contribute to overcoming the main drawback for the industrial utilization of VP and other ligninolytic peroxidases, i.e. their low yields in heterologous expression. An alternative approach is to engineer already commercial (i.e. high-yield expressed) peroxidases by introducing the ability to oxidize high-redox potential compounds by site-directed mutagenesis, based on the structure–function information already available. Using a combination of site-directed mutagenesis, saturation mutagenesis, directed evolution, and other molecular biology techniques, tailor-made biocatalysts will be available in the future enabling industrial exploitation of the unique catalytic abilities of the high redox-potential peroxidases (VP, LiP, and MnP) produced by the lignin-degrading basidiomycetes.

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