Review

Microbial degradation of lignin: how a bulky recalcitrant polymer is efficiently recycled in nature and how we can take advantage of this

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Summary

Lignin is the second most abundant constituent of the cell wall of vascular plants, where it protects cellulose towards hydrolytic attack by saprophytic and pathogenic microbes. Its removal represents a key step for carbon recycling in land ecosystems, as well as a central issue for industrial utilization of plant biomass. The lignin polymer is highly recalcitrant towards chemical and biological degradation due to its molecular architecture, where different non-phenolic phenylpropanoid units form a complex three-dimensional network linked by a variety of ether and carbon–carbon bonds. Ligninolytic microbes have developed a unique strategy to handle lignin degradation based on unspecific one-electron oxidation of the benzenic rings in the different lignin substructures by extracellular haemperoxidases acting synergistically with peroxide-generating oxidases. These peroxidases posses two outstanding characteristics: (i) they have unusually high redox potential due to haem pocket architecture that enables oxidation of non-phenolic aromatic rings, and (ii) they are able to generate a protein oxidizer by electron transfer to the haem cofactor forming a catalytic tryptophanyl-free radical at the protein surface, where it can interact with the bulky lignin polymer. The structure–function information currently available is being used to build tailor-made peroxidases and other oxidoreductases as industrial biocatalysts.

Interest of microbial degradation of lignin

Lignin is a complex aromatic polymer, highly recalcitrant towards both chemical and biological degradation, characteristic of the cell wall of vascular plants (Fig. 1). Around 20% of the total carbon fixed by photosynthesis in land ecosystems is incorporated into lignin, being the second main constituent of plant biomass after cellulose. In addition of providing plant stems the rigidity required for growth on land, and waterproofing vascular tissues for sap circulation, a main role of lignin is to protect the cellulose polymer towards hydrolytic attack by most pathogen and saprophytic organisms. In spite of this, lignin-degrading microbes evolved simultaneously with the colonization of land by vascular plants in the Palaeozoic era, around 400 million year ago (Taylor and Osborne, 1996). Microbial degradation of lignin (Martínez *et al.*, 2005; Kersten and Cullen, 2007) represents a key step for closing the carbon cycle, since removal of the lignin barrier enabled the subsequent use of plant carbohydrates by other microorganisms.

Lignin removal is also a central aspect in industrial uses of cellulosic biomass, such as bioethanol production and manufacture of cellulose-based chemicals and materials, including paper. In the plant cell wall, lignin concentrates in the middle lamella, its most external layer acting as a cementing agent between fibres (Fig. 2, top). Cellulose pulp manufacture basically consists in breaking down (chemically or mechanically) the middle lamella in such a way that wood fibres are individualized (Sixta, 2006). Although in lower concentration than plant carbohydrates (cellulose and hemicelluloses) lignin is also present in secondary wall, the thicker cell-wall layer, where it is intimately associated to carbohydrates preventing their efficient hydrolysis in the production of bioethanol (Galbe and Zacchi, 2007). In the above industrial applications, biotechnology based on lignin-degrading microbes and their enzymes can contribute to more efficient and environmentally sound use of renewable lignocellulosic feedstocks for sustainable production of materials, chemicals, biofuels and energy.

Chemical bases of lignin recalcitrance

Although lignin precursors, i.e. the three classical *p*-hydroxycinnamyl alcohols and their recently reported acylated forms (Ralph *et al.*, 2004; Martínez *et al.*, 2008), are phenolic compounds (Fig. 1, top), the lignin polymer formed from these monolignols is basically non-phenolic (Fig. 1, bottom). In the last step of lignin biosynthesis, plant peroxidases (and maybe also laccases) oxidize monolignol to their phenoxy radicals (Higuchi, 1997). Chemical coupling between the resonant forms of these radicals results in a variety of phenolic dimeric structures (dilignols) that can be enzymatically oxidized again, the process finally leading to lignin polymer formation. However, due to predominance of the corresponding radical forms and higher stability of the coupling products, ether linkages between the phenolic position (C_4) and a side-chain (or aromatic ring) carbon of the *p*-hydroxyphenylpropenoid precursors (substructures A, B and D) are strongly predominant in the growing polymer. Due to the high frequency of these ether linkages, the aromatic lignin units are basically non-phenolic. Moreover, in contrast to cellulose formed by linear (anhydroglucose) chains and hemicelluloses that include short branches on a main polysaccharidic backbone, the lignin polymerization mechanism (based on resonant radical coupling) results in a complex three-dimensional network (Fig. 1, bottom) due to both chain branching and inter/intra-chain coupling during polymerization, as shown in updated lignin models (Gellerstedt and Henriksson, 2008).

Due to its non-phenolic aromatic nature, lignin units cannot be oxidized by low-redox-potential oxidoreductases, such as the plant peroxidases initiating the polymerization process. In fact, only a small group of highly specialized peroxidases secreted by ligninolytic fungi are able to degrade model compounds representing the main lignin substructures. The bulky nature of the heterogeneous lignin polymer forming a complex three-dimensional network represents an additional limitation for biodegradation since the enzyme accessibility is strongly reduced. To overcome this difficulty, two main strategies have been developed by ligninolytic organisms based on: (i) presence of catalytic residues widely exposed at the surface of ligninolytic peroxidases, and (ii) use of redox mediators participating in the enzymatic attack.

Ligninolytic organisms and enzymes

The first lignin structural models were available in the 1970s (Nimz, 1974; Adler, 1977) and new substructures are still being identified using modern analytical techniques (Karhunen *et al.*, 1995; Zhang *et al.*, 2006; del Río *et al.*, 2007). Before this date no contrasted information on lignin structure was available, preventing studies on its microbial or enzymatic degradation. Synthetic lignins and simple model compounds (incorporating radioactive labelling) were used to unravel the mechanisms of lignin attack by white-rot basidiomycetes, the only organisms that are able to extensively mineralize lignin (Eriksson *et al.*, 1990). The complexity shown by the first lignin models and the variety of compounds identified in early lignocellulose biodegradation studies (Chen and Chang, 1985) suggested a set of different degradative enzymes attacking the different lignin substructures and releasing different breakdown products. However, subsequent work using model compounds representative of the main substructures found in lignin revealed that lignin-degrading organisms just adopted the opposite strategy. These studies, instead of revealing a variety of enzymes catalysing the different reactions observed, showed that the latter were the result of an unspecific oxidative attack on the benzene rings of lignin units followed by different bond breakdown reactions due to the chemical instability of the cation radicals formed (Kirk and Farrell, 1987).

Moreover, using simple model compounds, it was possible to demonstrate that among the different oxidative enzymes produced by lignin-degrading organisms, only a group of basidiomycete haemperoxidases could directly attack the non-phenolic lignin network (Martínez, 2002; Hammel and Cullen, 2008). These enzymes include lignin peroxidase (LiP) initially described in *Phanerochaete chrysosporium*, the first basidiomycete whose genome was sequenced due to the interest on biological degradation of lignin (Martínez *et al.*, 2004), and a versatile peroxidase (VP) more recently reported in *Pleurotus* and *Bjerkandera* species, the former genus including species being able to degrade lignin selectively (Martínez *et al.*, 1994). VP is also able to oxidize Mn^{2+}, as reported for *P. chrysosporium* manganese peroxidase (MnP). The Mn^{3+} resulting from the action of these two peroxidases oxidizes phenolic compounds but, in the presence of unsaturated lipids, it can also oxidize non-phenolic lignin via peroxidation radicals (Bao *et al.*, 1994). The molecular evolution of ligninolytic peroxidases has been shown by a recent study (Morgenstern *et al.*, 2008).

Taking into account the unique characteristics of the microbial attack to lignin, compared with hydrolytic attack to other natural polymers including plant carbohydrates, the process was described as an 'enzymatic combustion' where enzymatically generated hydrogen peroxide oxidizes the lignin polymer in a reaction catalysed by the above-mentioned high-redox-potential peroxidases (Kirk and Farrell, 1987) (Fig. 2). Simultaneously to the discovery of ligninolytic peroxidases, several peroxide-generating oxidases were described in ligninolytic fungi, such as glyoxal oxidase (a copper radical enzyme), aryl-alcohol oxidase (AAO) and pyranose-2 oxidase (two flavoenzymes) (Martínez *et al.*, 2005). In *Pleurotus* species
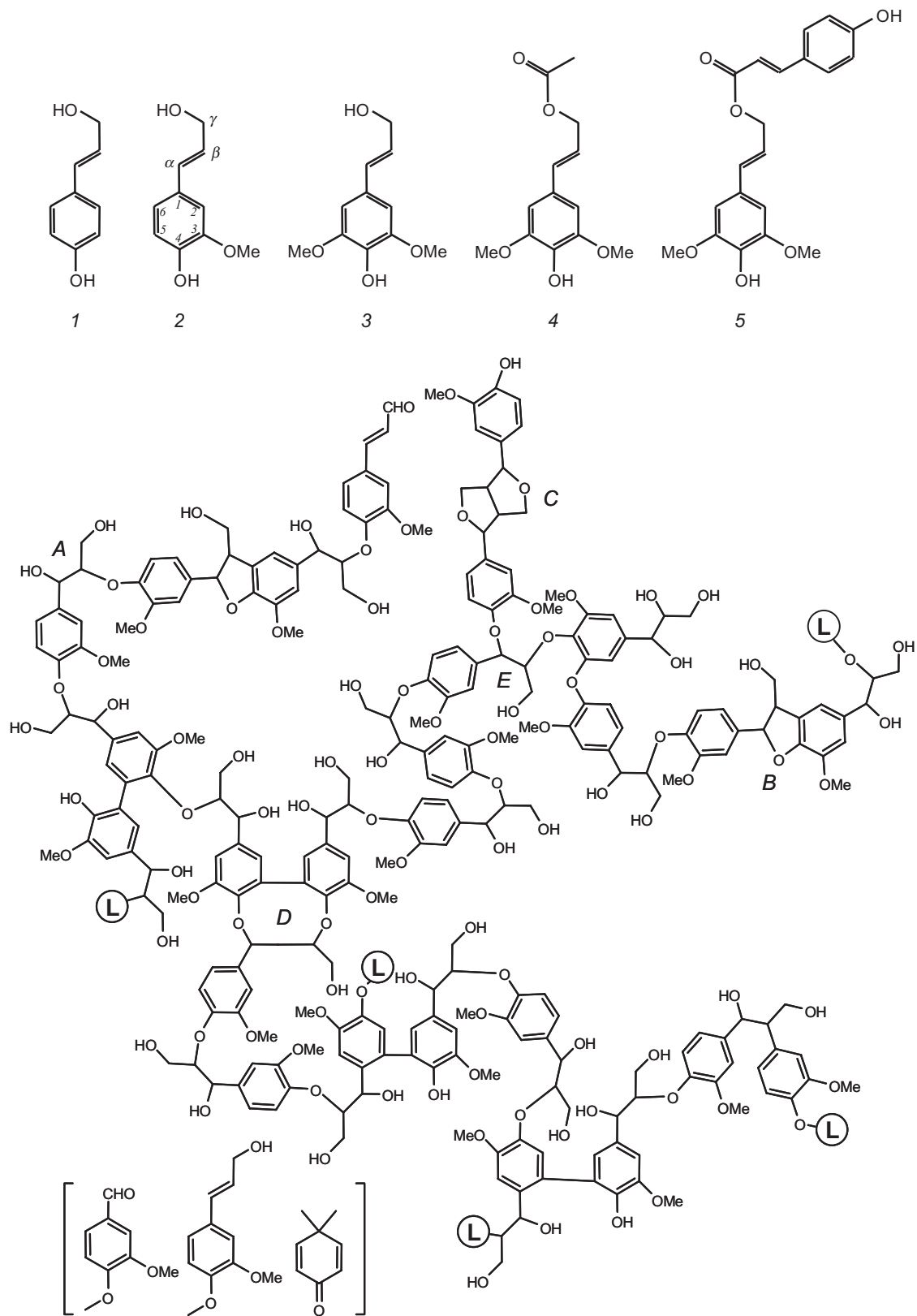
Fig. 1. Three classical and two acylated lignin precursors or monolignols (top), and structural model for gymnosperm lignin (bottom). Gymnosperms produce the simplest lignin type formed only by guaiacyl units derived from coniferyl alcohol (*2*). In contrast, angiosperm lignin also include *p*-hydroxyphenyl and sinapyl units derived from *p*-coumaryl (*1*) and sinapyl (*3*) alcohols, as well as a variable amount of acylated lignin often derived from sinapyl alcohol γ-esterified with acetic (*4*), *p*-coumaric acid (*5*) or other organic acids (Ralph *et al.*, 2004; Martínez *et al.*, 2008). A variety of ether and carbon–carbon inter-unit linkages are formed during monolignol radical polymerization resulting in β-O-4′ (*A*), phenylcoumaran (*B*), pinoresinol (*C*) and dibenzodioxocin (*D*) substructures, among others. Linkages to additional lignin chains are indicated (L-containing circles). Other minor structures (in brackets) include vanillin, coniferyl alcohol and dimethylcyclohexadienone-type units, the latter in new spirodienone substructures (Zhang *et al.*, 2006) (courtesy of G. Gellerstedt).
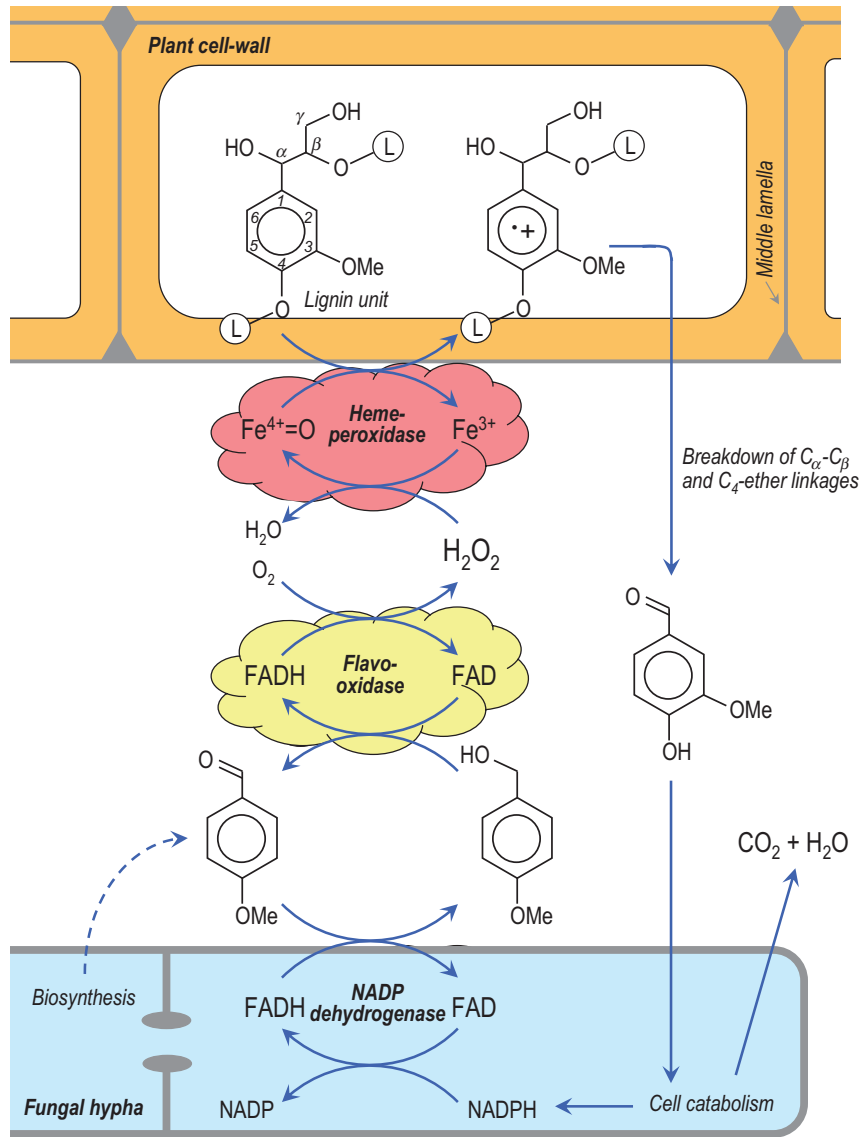

Fig. 2. Pictorial scheme of the enzymatic degradation of plant cell-wall lignin (L-containing circles represent the remaining lignin polymer) by *Pleurotus* VP, with contribution of extracellular flavooxidases (such as AAO) generating hydrogen peroxide during redox cycling of non-phenolic aromatic aldehydes (such as the fungal metabolite *p*-anisaldehyde) with participation of intracellular aryl-aldehyde dehydrogenase. Peroxidase one-electron oxidation of lignin units (the key step in the degradative process) results in an unstable cation radical that experiences different reactions including breakdown of C_α–C_β and C_4–ether linkages releasing the corresponding aromatic aldehydes (vanillin in the case of guaiacyl units) that can be intracellularly mineralized. In the case of *P. chrysosporium* LiP, lignin attack requires the presence of veratryl alcohol, probably as an enzyme-bound mediator, and hydrogen peroxide is mainly generated by glyoxal oxidase.

continuous production of hydrogen peroxide during redox cycling of anisaldehyde, an extracellular metabolite (Gutiérrez *et al.*, 1994) is produced involving mycelium-associated aryl-alcohol dehydrogenase (a NADPH dehydrogenase) working together with extracellular AAO (Guillén and Evans, 1994) (Fig. 2). In *P. chrysosporium*, glyoxal oxidase uses products from lignocellulose degradation as the enzyme-reducing substrates for generating the hydrogen peroxide required by the ligninolytic peroxidases (Kersten and Cullen, 2007). The unstable cation radicals formed during peroxidase attack to lignin model compounds experience different chemical reactions including breakdown of C_α–C_β and C_4–ether linkages resulting in the release of aromatic aldehydes, one of the main products found during enzymatic depolymerization of lignin (Fig. 2), together with other reactions such as demethoxylation (methanol release) and aromatic-ring cleavage (with formation of muconate-type structures) (Martínez *et al.*, 2005).

The involvement of ligninolytic peroxidases in wood lignin degradation has been recently supported by comparison of the genomes of *P. chrysosporium* (Martínez *et al.*, 2004), a model white-rot fungus characterized by its ability to remove wood lignin leaving a whitish cellulosic residue, and *Postia placenta* (Martinez *et al.*, 2009), a model brown-rot fungus characterized by its ability to remove wood polysaccharides leaving a brown lignin-enriched residue. This comparison showed a large set of ligninolytic peroxidase genes in the genome of *P. chrysosporium*, in agreement with previous studies (Cullen, 1997). In contrast, the *P. placenta* genome includes genes of oxidases and other enzymes involved in cellulose attack via Fenton chemistry, but contains an unique peroxidase gene related to the low-redox-potential peroxidase of the non-ligninolytic basidiomycete *Coprinopsis cinerea* (CIP), and completely lacks ligninolytic peroxidase genes (*lip*, *vp* or *mnp*).

A third type of microbial oxidoreductases, laccases and related multicopper oxidases are produced by most ligninolytic basidiomycetes (Baldrian, 2006) as well as by eubacteria and actinomycetes growing on lignocellulosic materials (Sharma *et al.*, 2007). The low redox potential of laccases prevents their direct action on lignin. However, they can degrade this and other recalcitrant compounds in the presence of redox mediators, as discussed below. Laccases also play a variety of other physiological roles including mushroom morphogenesis, detoxification and humification, among others (Claus, 2004). Moreover, they seem involved in generation of lignocellulose-degrading hydroxyl radical (via Fenton chemistry) in both white-rot (Guillén *et al.*, 2000) and at least some brown-rot basidiomycetes, in agreement with the presence of laccase genes in the genome of *P. placenta* (http://genome.jgi-psf.org/Pospl1/Pospl1.home.html).
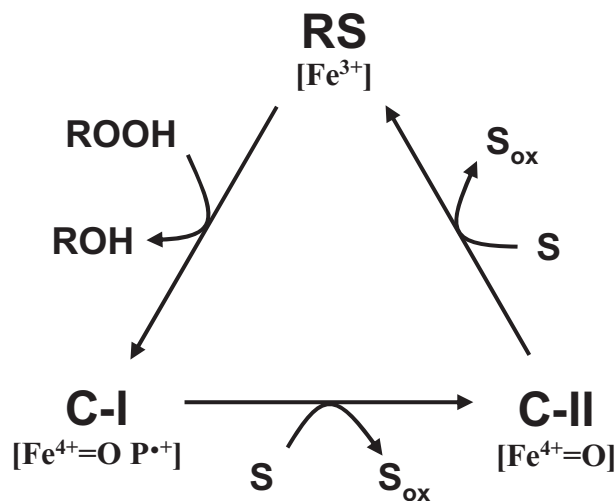

Fig. 3. General catalytic cycle of peroxidases (Dunford, 1999). The cycle includes two-electron oxidation of the enzyme resting state (RS, containing Fe^{3+}) by hydroperoxide to yield compound-I (C-I; containing Fe^{4+}-oxo and porphyrin cation radical), whose reduction in two one-electron steps results in the intermediate compound-II (C-II; containing Fe^{4+}=O after porphyrin reduction) and then the resting form of the enzyme, with concomitant oxidation of two substrate molecules (S; which could be low-redox-potential phenols and dyes, or Mn^{2+} in the cases of MnP and VP).

General determinants of ligninolytic peroxidase structure and activity

All prokaryotic, fungal and plant haemperoxidases share a common folding and helical topology described in cytochrome *c* peroxidase (CCP), the first peroxidase whose crystal structure was reported (Poulos *et al.*, 1980) and only more recently (Gajhede *et al.*, 1997) in the best-known peroxidase, horseradish peroxidase (HRP). It is noteworthy that LiP, an enzyme that was completely unknown until 1983 (Glenn *et al.*, 1983; Tien and Kirk, 1983), was the second peroxidase whose crystal structure was reported (Piontek *et al.*, 1993; Poulos *et al.*, 1993). The rapid progresses of LiP structure–function studies, as well as the fact that two groups reported simultaneously its discovery and crystal structure, demonstrate the interest on high-redox-potential peroxidases.

As mentioned above, all peroxidases require hydrogen peroxide, or other hydroperoxides, to activate the haem cofactor yielding the so-called compound-I in a common catalytic cycle (Dunford, 1999) (Fig. 3). Compound-I contains a reactive Fe^{4+} oxo complex with a cation radical at the porphyrin ring, formed by two-electron oxidation of the Fe^{3+}-containing haem of the resting enzyme. One-electron oxidation of one substrate molecule yields compound-II, where the porphyrin cation radical has been reduced. The remaining Fe^{4+}=O in compound-II oxidizes a second substrate molecule, and the enzyme returns to its ferric resting state to initiate a new catalytic cycle.
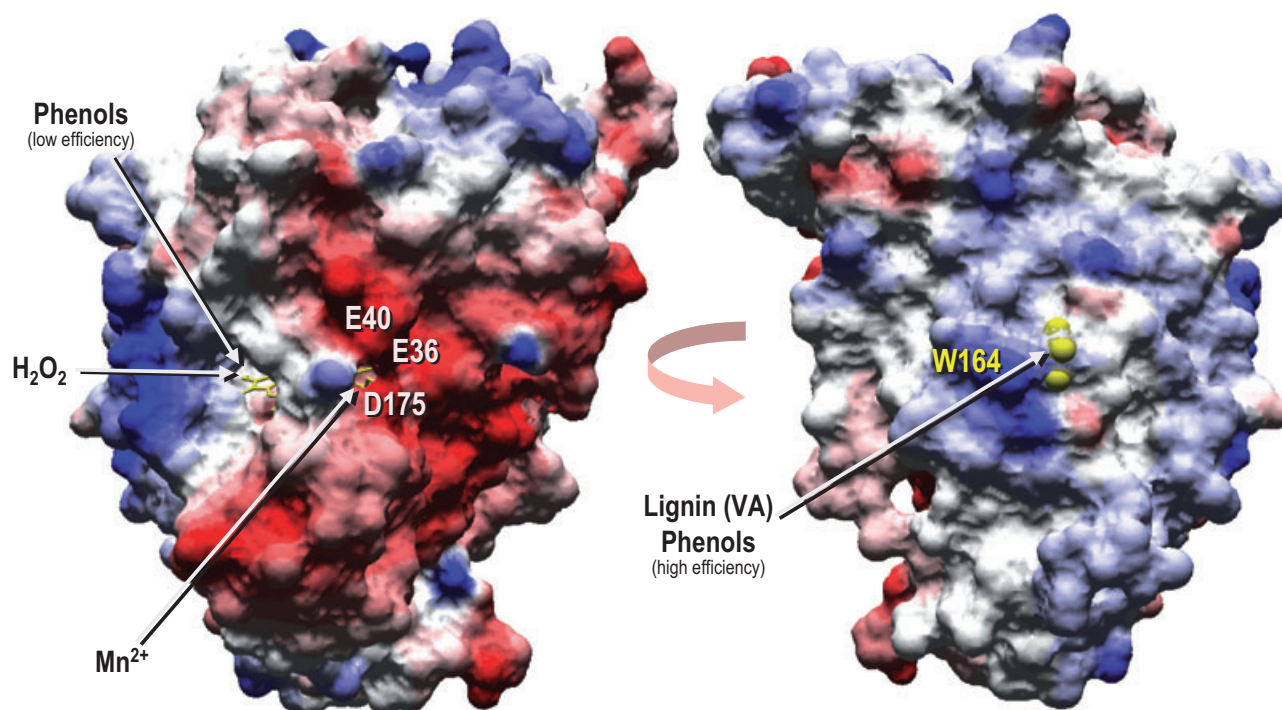
Fig. 4. Two different views of the solvent access surface in a ligninolytic peroxidase (*P. eryngii* VP; PDB entry 2BOQ) revealing (left) the main haem access channel enabling hydrogen peroxide entrance for activation of the haem cofactor (in yellow) located in a central pocket (low-redox-potential phenols and dyes can also be oxidized at this channel albeit with low efficiency), and the Mn^{2+}-oxidation channel formed by three acidic residues (Glu-36, Glu-40 and Asp-175); as well as an approximately 180° rotated view (right) of the same peroxidase showing the partially exposed side-chain (yellow van der Waals spheres including hydrogen atoms) of the catalytic tryptophan (Trp-164) involved in oxidation of high-redox-potential compounds, such as veratryl alcohol (VA) and lignin models, as well as in high-efficiency oxidation of some phenols and dyes, by long-range electron transfer (LRET) to the haem cofactor (surface colours correspond to electrostatic charge).

The molecular structure of haemperoxidases includes two domains, probably originating from ancestral gene duplication, delimiting a central cavity where the haem cofactor is located (Li and Poulos, 1994; Banci, 1997). In most cases, the access of both the enzyme-oxidizing (peroxide) and -reducing substrates to the haem cofactor is produced through a main access channel (Fig. 4, left). Taking into account the high reactivity of both compound-I and compound-II, this internal location of the cofactor most probably plays a dual role. First, it prevents unspecific reduction of the activated enzyme by a variety of reductants different from its specific target substrate. Second, it prevents intermolecular reaction resulting in oxidation of surface susceptible amino acids (e.g. tyrosine residues) leading to enzyme inactivation (e.g. by dimerization reactions). Oxidation of Mn^{2+} by basidiomycete MnP and VP is also produced through a specific access channel enabling entering of the cation to reach one of the haem propionates (Fig. 4, left). The edge of this channel includes three acidic residues that bind Mn^{2+} specifically enabling its high-efficiency oxidation.

A detail of the haem environment and other neighbour amino acid residues in a ligninolytic peroxidase (*Pleurotus eryngii* VP) is shown in Fig. 5. These include two axial histidine residues, the so-called proximal (His-169) and distal (His-47) histidines, and other conserved residues (Martínez, 2002). Proximal histidine acts as the fifth ligand of the haem iron together with the four nitrogens of the tetrapyrrolic macrocycle, while distal histidine together with a conserved arginine (VP Arg-43) contributes to iron reaction with hydrogen peroxide in compound-I formation (Hiner *et al.*, 2002). Two aromatic residues are also highly conserved at the proximal (Phe-186) and distal (Phe-46) sides of the haem of many peroxidases, in CCP being two tryptophan residues one of them playing a direct role in catalysis as mentioned below. Two more residues at the proximal (Asp-231) and distal (Asn-78) sides of the haem establish hydrogen bonds with the two histidines. Two structural Ca^{2+} ions are located in conserved binding sites at the two peroxidase domains contributing to folding. The above-mentioned Mn^{2+}-oxidation site of VP (and MnP) is shown at the right side of haem, being formed by three acidic residues positioning the cation near one of the haem propionates for direct electron transfer (Gold *et al.*, 2000; Ruiz-Dueñas *et al.*, 2007). Finally, a tryptophan residue (Trp-164) is shown at the left side of the haem, being involved in oxidation of high-redox-potential aromatic compounds (together with contiguous Leu-165) as
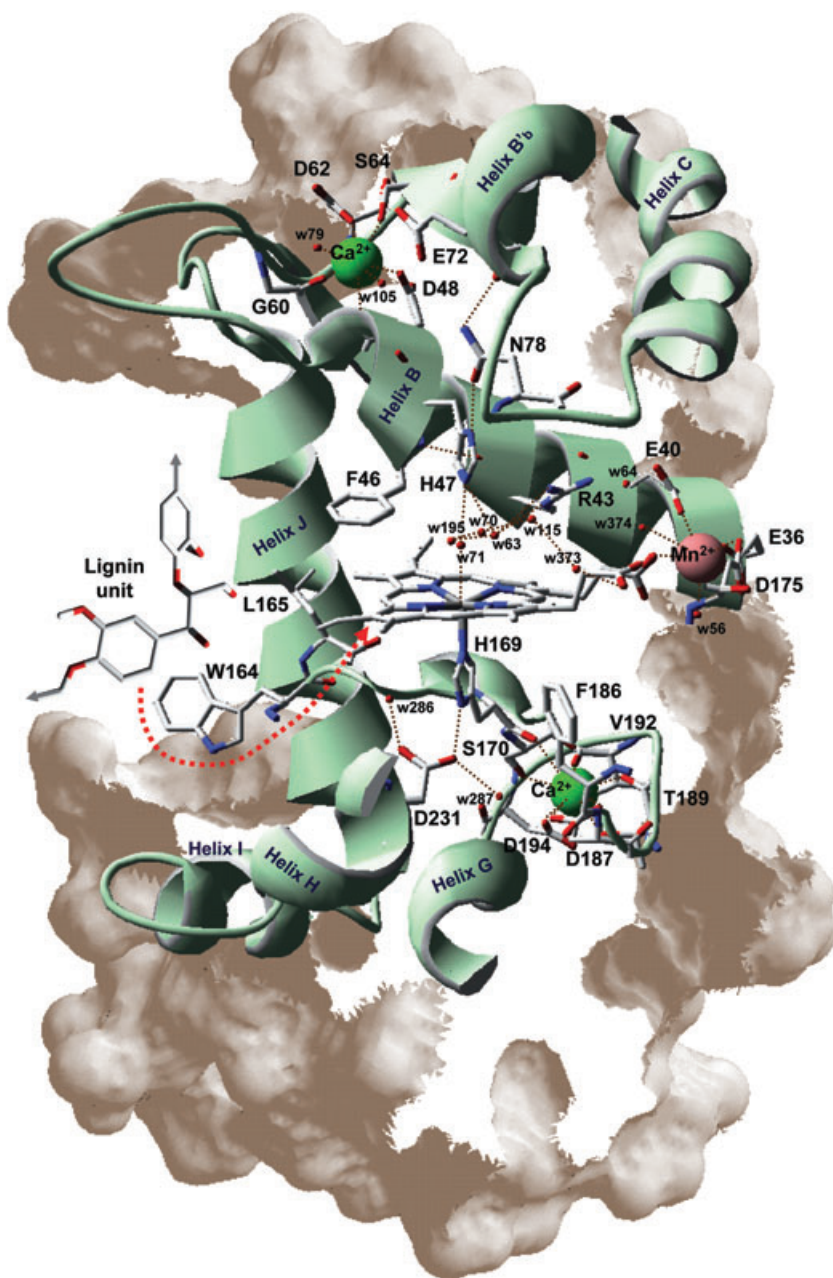
Fig. 5. Details of haem environment and other structurally and catalytically relevant residues in *P. eryngii* VP. His-169 (the fifth ligand of haem iron), Phe-186 and Asp-231 (corresponding to His-176, Phe-193 and Asp-238 in LiP-H8) are shown at the proximal side of the haem, while His-47, Phe-46, Arg-43 and Asn-78 (corresponding to His-47, Phe-46 and Arg-43 in LiP-H8) are shown at the distal side. Glu-36, Glu-40 and Asp-175 (corresponding to Ala-36, Glu-40 and Asn-182 in LiP-H8) constitute the site of oxidation of Mn^{2+} (red van der Waals sphere) near the internal propionate of haem, while Trp-164 (corresponding to Trp-171 in LiP-H8) is responsible for oxidation of lignin units and other aromatic compounds by LRET (red arrow) to the activated haem cofactor via Leu-165 (corresponding to Leu-172 in LiP-H8). Finally, the ligands of the two structural Ca^{2+} ions (green spheres) are indicated at the proximal (Ser-170, Asp-187, Thr-189, Val-192 and Asp-194) and distal (Asp-48, Gly-60, Asp-62 and Ser-64) sides (corresponding, respectively, to Ser-177, Asp-194, Thr-196, Ile-199 and Asp-201; and Asp-48, Gly-66, Asp-68 and Ser-70 in LiP-H8). Several water molecules are also shown including those completing Mn^{2+}, Ca^{2+} and haem Fe^{3+} coordination.

described in the next section. The VP structure also includes several molecules of water, including those completing the characteristic coordinations of Mn^{2+} and Ca^{2+} ions. Another water molecule (w71) could act as the sixth ligand of the haem Fe^{3+} at a position close to that occupied by the $Fe^{4+}=O$ oxygen of compound-I and compound-II, as shown for HRP (Berglund *et al.*, 2002).

It has been shown that the redox potential of peroxidase compound-I and compound-II depends of the haem environment characteristics, as shown by ^{1}H-NMR spectra of the cyanide adduct of the enzyme (Banci *et al.*, 1991; 2003). These included, as one of the most important factors, the position and more or less marked imidazolate

character of the proximal histidine side-chain, whose N_{1} acts as the fifth ligand of the haem iron. The strength of this bond affects the electron deficiency of iron and, consequently, the reactivity of the activated enzyme. The characteristics of the proximal histidine side-chain that are affected by H-bonding to other residues (such as Asp-231 in Fig. 5) (Banci *et al.*, 1995) and the position of helix F where this residue is located (Piontek *et al.*, 1993) affect the chemical shift of its Hε1 signal (which was found at about −8 p.p.m. in LiP, −16 p.p.m. in VP, −22 p.p.m. in CCP and −32 p.p.m. in HRP spectra) in agreement with the reported higher redox potential of ligninolytic peroxidases compared with plant peroxidases (Millis *et al.*, 1989).

Direct peroxidase oxidation of lignin: long-range electron transfer (LRET) mechanism

When the crystal structure of *P. chrysosporium* LiP was reported, it was assumed that this peroxidase would oxidize high-redox-potential aromatic compounds at the main haem access channel. In fact, veratryl alcohol was modelled at the crystal structure channel to determine the eventual contacts (Poulos *et al.*, 1993). This is the classical peroxidase substrate oxidation site, as shown in plant HRP and in the low-redox-potential fungal CIP (Smith and Veitch, 1998). However, the main haem access channel of LiP is significantly narrower than those of CIP or HRP, and the hypothesis of long-range electron transfer (LRET) oxidation of lignin at the protein surface, initially proposed by Du and colleagues (1992) and Schoemaker and colleagues (1994), was adopted by other authors.

Protein LRET requires the existence of an amino acid residue susceptible to form a stable radical (preferably a tyrosine, tryptophan or histidine residue) located at the protein surface and adequately connected to the haem cofactor for electron transfer. The above authors suggested two residues (Trp-171 and His-82) as the origin of two LRET pathways for oxidation of high-redox-potential aromatic compounds by LiP. Several years later a third pathway was proposed (initiating at His-239) (Johjima *et al.*, 1999). No pathway starting at a tyrosine residue has been proposed since all ligninolytic peroxidases cloned up to date (a total of near 50 sequences) are free of tyrosine residues (to prevent oxidative inactivation) with the only exception of a *Trametes cervina* LiP discussed below.

The same three putative LRET pathways were identified in *P. eryngii* VP, and their eventual operation was investigated by site-directed mutagenesis by Pérez-Boada and colleagues (2005). This study definitively showed that only the pathway starting at the exposed tryptophan (Trp-164 of isoenzyme VPL) was operative for oxidation of high-redox-potential aromatic compounds including veratryl alcohol. The position of this residue at the vicinity of the haem cofactor is illustrated in Fig. 5, which also shows a leucine residue involved in the electron transfer; and the solvent exposed aromatic side-chain of this tryptophan is shown in Fig. 4 (right). The catalytic role of a homologous tryptophan has also been shown in VP from other fungi (Tinoco *et al.*, 2007; Tsukihara *et al.*, 2008). Formation of the tryptophanyl-free radical postulated by Du and colleagues (1992) was confirmed by low-temperature electron paramagnetic resonance (EPR) of peroxide-activated VP (Pogni *et al.*, 2006).

The involvement of the homologous tryptophan residue of LiP (Trp-171) in oxidation of veratryl alcohol and, more interestingly, of a tetrameric lignin model compound
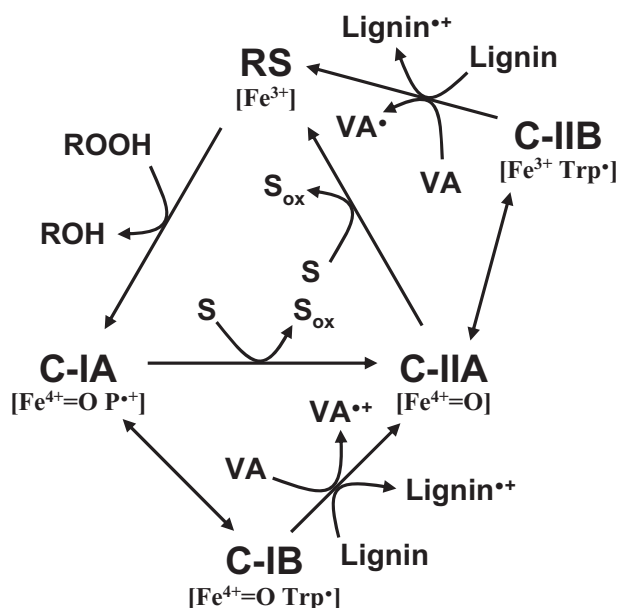

Fig. 6. Extended catalytic cycle proposed for ligninolytic peroxidases (LiP and VP). In addition to normal compound-I and compound-II of Fig. 3 (now C-I$_A$ and C-II$_A$), C-I$_B$ (containing Fe^{4+}=O and tryptophan radical) and C-II$_B$ (containing Fe^{3+} and tryptophan radical) are included, being involved in oxidation of high-redox-potential compounds such as veratryl alcohol (VA) and lignin units to their corresponding cation radicals. C-I$_B$ and C-II$_B$ are formed by LRET to the activated haem cofactor. Adapted from Pérez-Boada and colleagues (2005).

was also confirmed by mutagenesis (Doyle *et al.*, 1998; Mester *et al.*, 2001). Although the LiP tryptophanyl radical was not directly detected, as reported by VP EPR, indirect evidence for its presence in the activated enzyme was obtained by adduct formation (Blodig *et al.*, 1999).

A difference between the two ligninolytic peroxidases concerns the surface environment of the catalytic tryptophan, which in LiP has a partial negative charge, whereas in VP some acidic residues are substituted by basic residues (Fig. 4, right). A noteworthy characteristic of VP is its ability to oxidize directly a series of high-redox-potential substrates that LiP is able to oxidize only in the presence of veratryl alcohol (see below). This fact is related to the catalytic tryptophan environment, and a charge inversion VP variant (R257D) was obtained that exhibited a LiP-type behaviour during oxidation of high-redox-potential dyes (Ruiz-Dueñas *et al.*, 2008).

After demonstrating the involvement of a protein radical in substrate oxidation by ligninolytic peroxidases, an extended catalytic cycle has been proposed for VP (Fig. 6) that in general terms can be also applied to *P. chrysosporium* LiP. In this cycle, compound-I$_B$ containing Fe^{4+}=O and tryptophan radical, and compound-II$_B$ containing Fe^{3+} and tryptophan radical, are included together with normal compound-I and compound-II (containing Fe^{4+}=O and porphyrin radical, and Fe^{4+}=O respectively) that are

now called compound-I$_A$ and compound-II$_A$. Compound-I$_B$ and compound-II$_B$ (formed by LRET to the activated haem cofactor) represent a small percentage of the total activated enzyme (Pogni *et al.*, 2006) being in equilibrium with the corresponding compound-I$_A$ and compound-II$_A$.

A different LiP form lacking a catalytic tryptophan residue has been reported in *T. cervina* (Miki *et al.*, 2006). This unique peroxidase presents an exposed tyrosine at a different position of the molecule that seems to play the same role of the above tryptophan. Interestingly, a plant peroxidase using a tyrosyl radical for oxidation of bulky phenols such as sinapyl alcohol (structure *3* in Fig. 1) has been recently reported (Sasaki *et al.*, 2008).

Redox mediators in lignin degradation

The use of small chemical oxidizers acting as redox mediators represents a second alternative to overcome the difficulties associated to the limited access of the bulky lignin polymer to the activated cofactor of peroxidases and other oxidoreductases. Enzyme-mediator systems have been extensively investigated in the case of laccases after the work of Bourbonnais and Paice (1990) reporting that synthetic mediators expanded the application potential of these low-redox-potential oxidoreductases enabling oxidation of non-phenolic lignin model compounds. These mediators are low-molecular-mass compounds that: (i) form stable free radicals oxidizing compounds that the enzyme alone is not able to oxidize, and (ii) diffuse away from the enzyme and can easily penetrate the lignocellulose matrix. It has been recently shown that some phenolic lignin precursors or degradation products can be used as 'natural' laccase mediators in industrial processes, and suggested that some of them could play a similar role in nature (Camarero *et al.*, 2005). However, the real significance of the laccase-mediator systems in natural biodegradation of lignin is still to be demonstrated.

The existence of an exposed protein radical transferring electrons to the haem via a LRET pathway enables LiP direct oxidation of veratryl alcohol and lignin model compounds, including non-phenolic tetramers (Mester *et al.*, 2001). However, LiP requires the presence of veratryl alcohol or other compounds forming aromatic cation radicals, to oxidize polymeric lignin and high-redox-potential dyes (Harvey *et al.*, 1986). This contrasts with the 'veratryl alcohol-independent' activity of VP oxidizing different compounds including lignin (Heinfling *et al.*, 1998a; Camarero *et al.*, 2001). The role of veratryl alcohol cation radical as a real mediator in LiP reactions has been matter of controversy, taking into account the short half-life of this species in aqueous media (Candeias and Harvey, 1995). However, the acidic environment surrounding LiP Trp-171 could stabilize the cation radical that would act as an enzyme-bound mediator (Khindaria *et al.*, 1996).

Biotechnological interest and future trends

Microbial oxidoreductases, including both peroxidases and laccases, have been investigated for biotechnological application including paper pulp bleaching in chlorine-free sequences (Paice *et al.*, 1995; Bajpai, 2004; Sigoillot *et al.*, 2005). Ligninolytic peroxidases, in contrast to laccases and low-redox-potential peroxidases that are used in several industrial sectors (e.g. textiles, detergents, food and beverages, etc.), are not still commercially available due to different reasons including the low levels of enzyme obtained from natural and recombinant hosts. Moreover, these oxidoreductases in most cases are not suitable biocatalysts as they are produced in nature. Industrial processes often require enzymes recognizing specific substrates or proceeding under extreme conditions (e.g. high hydrogen peroxide concentration or extreme pH and temperature).

Laccases have low redox potential and can degrade recalcitrant compounds only in the presence of redox mediators. However, the laccase-mediator system has became extremely popular in biodegradation studies, as well as for processing and functionalization of lignocellulosic fibres (Riva, 2006; Widsten and Kandelbauer, 2008). In spite of 'natural' laccase mediators have been reported as potential substitutes of synthetic -N(OH)- compounds in different applications (Camarero *et al.*, 2007; Cañas *et al.*, 2007; Gutiérrez *et al.*, 2007), economic issues related to the cost of the mediator, and environmental concerns related to the eventual release of toxic compounds during the enzymatic treatment are main drawbacks for the industrial implementation of this enzymatic system in the pulp and paper and other industrial sectors.

In contrast to laccases, some ligninolytic peroxidases do not require mediators to degrade high-redox-potential compounds. Indeed, they should be the enzymes of choice for removing lignin or transforming high-redox-potential aromatic compounds in different applications. The rapid acquisition of knowledge on the structure and function of these enzymes over the last years has been used to modulate their catalytic and operational properties using site-directed mutagenesis in a variety of studies (Timofeevski *et al.*, 1999; Reading and Aust, 2000; Celik *et al.*, 2001; Mester and Tien, 2001; Feng *et al.*, 2003). In those cases where the structural basis of the property to be improved is unknown, or too difficult to be predicted, directed evolution or saturation mutagenesis is the approach of choice (Cherry *et al.*, 1999; Miyazaki-Imamura *et al.*, 2003; Ryu *et al.*, 2008). Some future trends in the industrial use of basidiomycete peroxidases are discussed below.

Considerable efforts have been devoted during the last years to improve the expression of ligninolytic and other peroxidases in different fungal hosts using a variety of strategies. Among others, strong promoters, protease deficient strains, molecular chaperones and external sources of haem have been used with only partially successful results (Stewart *et al.*, 1996; Conesa *et al.*, 2000; 2002; Gu *et al.*, 2003; Lú-Chau *et al.*, 2004; Wang *et al.*, 2004; Eibes *et al.*, 2009). New strategies based on converting low-redox-potential peroxidases, easily to express at levels of several grams per litre, into high-redox-potential peroxidases are being developed taking advantage of the structure–function knowledge accumulated. In this sense, CIP has been successfully modified and transformed into a LiP-like enzyme by introducing a catalytic tryptophan, at the same time that the acidity of the local environment of this residue was increased (Smith and Doyle, 2006). Although the mutated enzyme was not so efficient as a true LiP, it is possible to predict that additional modifications will yield an enzyme with the expected catalytic activity.

Hydrogen peroxide inactivate all peroxidases after several cycles of catalysis as a consequence of the competition between productive and unproductive electron sources (including enzyme components) in a process described as a suicide inactivation (Valderrama *et al.*, 2002). Different attempts aimed to improve hydrogen peroxide stability of ligninolytic peroxidases have been performed, mainly removing easily oxidizable and conformationally unstable amino acid residues located at the peroxide-binding side of haem with very promising results (Miyazaki and Takahashi, 2001; Miyazaki-Imamura *et al.*, 2003). Recently, a novel peroxidase from *Raphanus sativus* has been identified and characterized as the only known case of a haemperoxidase intrinsically resistant to hydrogen peroxide (Gil-Rodríguez *et al.*, 2008). In-depth analysis of its structure will give the first structural evidences of peroxide stability to be used to increase the peroxide stability of ligninolytic and other peroxidases.

pH and temperature inactivation of ligninolytic peroxidases is associated to the release of the two structural Ca^{2+} involved in stabilization of the molecular architecture (Sutherland and Aust, 1996; George *et al.*, 1999; Lú-Chau *et al.*, 2004). It has been described that losing these ions causes hexacoordination of the haem iron preventing LiP and MnP activation by hydrogen peroxide, although more recently a decrease in redox potential has been suggested as the main inactivation cause in VP (Verdín *et al.*, 2006). Some authors have succeeded stabilizing peroxidases by avoiding the lose of Ca^{2+} by adding extra disulfide bridges (Reading and Aust, 2000). Generation of the appropriate disulfide bridges could not only make these enzymes resistant to high pH and temperature, but it

could also be used to increase their redox potential. Thermal inactivation has been also related to destabilizing interactions between adjacent acidic side-chains. Mutations removing one of these interactions in CIP improved its thermal stability by 134-fold (Cherry *et al.*, 1999). Curiously these two residues are conserved in ligninolytic peroxidases (LiP, MnP and VP), and additional interactions between acidic residues can be observed in other regions of their molecular structures. It is expected that substitution of one amino acid residue of these acidic couples will promote enzyme stabilization towards high temperature, as described for CIP.

Among ligninolytic peroxidases, VP presents especial biotechnological interest due to different reasons including: (i) catalytic versatility by combination of different substrate oxidation sites, and (ii) ability to degrade some compounds of interest that LiP and MnP (as well as CIP) are not able to oxidize directly. Its catalytic versatility permits the application of VP in Mn^{3+}-mediated or Mn-independent reactions on both low- and high-redox-potential substrates. The possibility to degrade directly a variety of recalcitrant compounds represents a considerable advantage compared with LiP since the cost of veratryl alcohol required as mediator can be saved. Among the different compounds of industrial and/or environmental interest that VP can transform, polycyclic aromatic hydrocarbons (Wang *et al.*, 2003), phenolic and non-phenolic aromatic pollutants (Rodríguez *et al.*, 2004), pesticides (Dávila-Vázquez *et al.*, 2005) and a variety of industrial dyes (Heinfling *et al.*, 1998b) can be cited (including, among others, Reactive Blue 38 and other azo dyes, Reactive Black 5 and other phthalocyanine dyes, anthracene and derivatives, benzo[a]pyrene, pyrene, 2,4-dichlorophenol and pentachlorophenol). For some applications the use of VP in combination with redox mediators can also be considered (Tinoco *et al.*, 2007). Among them, the use of VP to reoxidize Mn^{2+}-containing polyoxometalates is an interesting possibility (Marques *et al.*, 2008), since these highly promising catalysts for environmentally friendly delignification are very difficult to be chemically reoxidized.

The promiscuity of ligninolytic fungi and their enzymes oxidizing aromatic xenobiotics and other recalcitrant compounds is due to the involvement of the lignin-degrading enzymatic machinery in many of these reactions. To overcome the bulky nature and structural heterogeneity of the lignin polymer, these microorganisms have developed a highly unspecific extracellular system being able to subtract one electron directly from the benzenic rings of the different lignin units. High-redox-potential extracellular peroxidases, often forming catalytic radicals at the protein surface, are the key enzymes in this initial attack yielding partially oxidized products whose catabolism is finally completed intracellularly.

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