A new versatile peroxidase from *Pleurotus*
F. J. Ruiz-Dueñas, S. Camarero, M. Pérez-Boada, M. J. Martinez and A. T. Martínez
Centro de Investigaciones Biológicas, CSIC, Velázquez 144, E-28006 Madrid, Spain

### Abstract
Lignin peroxidase (LiP) and manganese peroxidase (MnP) have been investigated in *Phanerochaete chrysosporium*. A third ligninolytic peroxidase has been described in *Pleurotus* and *Bjerkandera*. Two of these versatile peroxidases (VPs) have been cloned, sequenced and characterized. They have high affinity for Mn²⁺, hydroquinones and dyes, and also oxidize veratryl alcohol, dimethoxybenzene and lignin dimers. The deduced sequences show higher identity with LiP than MnP, but the molecular models obtained include a Mn⁴⁺-binding site. Concerning aromatic substrate oxidation, *Pleurotus eryngii* VP shows a putative long-range electron transfer pathway from an exposed tryptophan to haem. Mutagenesis and chemical modification of this tryptophan and the acidic residues forming the Mn⁴⁺-binding site confirmed their role in catalysis. The existence of several substrate oxidation sites is supported further by biochemical evidence. Residue conservation in other fungal peroxidases is discussed.

### Introduction
The key step in lignin biodegradation is oxidation by H₂O₂ catalysed by peroxidases [1]. Three peroxidases involved in lignin degradation are produced by white-rot fungi, the well-known lignin and manganese peroxidases (LiP and MnP), first described in *Phanerochaete chrysosporium*, and the versatile peroxidase (VP) recently described in *Pleurotus* and *Bjerkandera* species [2–4]. Biochemical and molecular characterization of *Phanerochaete chrysosporium* peroxidases has been reported because of their potential in paper pulp manufacturing and other biotechnological applications. LiP is characterized by its ability to oxidize high-redox-potential aromatic compounds including veratryl alcohol (VA) and MnP by Mn⁴⁺ oxidation, Mn⁴⁺ chelates acting as diffusing oxidizers. VP combines the catalytic properties of the two above peroxidases, being able to oxidize typical MnP and LiP substrates.

### A new versatile peroxidase
The ability of a peroxidase to oxidize both Mn⁴⁺ and aromatic compounds was reported first in *Pleurotus eryngii* [2]. Then, other VPs were iso-
lated from *Pl. pulmonarius* [5], *Pl. ostreatus* [6], *Bjerkandera adusta* [7] and *Bjerkandera* sp. [3]. These peroxidases are able to oxidize Mn²⁺ to Mn³⁺ (as MnP does), degrade the lignin model dimer veratrylglycerol-β-guaiacyl ether (used to demonstrate LiP ligninolytic activity) yielding veratraldehyde, and oxidize VA and p-dimethoxybenzene to veratraldehyde and p-benzoquinone respectively (as reported for LiP) [4,8]. Moreover, they oxidize hydroquinones and substituted phenols that are substrates for plant peroxidases [4]. Their pH optima for oxidation of Mn³⁺ (pH 5) and aromatic compounds and dyes (pH 3) differ, being similar to those of optimal MnP and LiP activity. The study on reducing substrates of *Pleurotus* and *Bjerkandera* VPs isolated from liquid cultures showed similar steady-state kinetic constants for both peroxidases [4]. Differences reported in VA affinity [3] were not confirmed in subsequent studies with the same fungal strains [9]. VP has high affinity for Mn³⁺ ($K_m$ 19–20 μM), ferulic acid, α-naphthol, and different hydroquinones ($K_m$ 6–22 μM), and dyes ($K_m$ 3–12 μM), but the affinities for VA ($K_m$ 300 μM) and substituted phenols ($K_m$ 90–300 μM) are lower. It shows also high affinity for H₂O₂ ($K_m$ 10 μM). VP has the highest activity on Mn³⁺ ($V_{max}$ 165–180 units/mg), moderate activities on different aromatic substrates (including VA) and 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonate) (ABTS) ($V_{max}$ 12–40 units/mg) and the lowest activity on guaiacol and other dyes ($V_{max}$ 5–10 units/mg). The VP affinities for H₂O₂ and Mn³⁺ are higher than obtained for *Ph. chrysosporium* peroxidases, but VA affinity is lower than found for LiP. The maximal activities on Mn³⁺ and VA are also lower than obtained for MnP and LiP respectively.

The incapacity of MnP to oxidize phenols, compared with VP activity on phenols and hydroquinones, is due to their inability to reduce MnP compound II, although both VP and MnP can oxidize phenols and some dyes via Mn³⁺ chelates. The ability to oxidize Mn³⁺ is a unique characteristic of MnP and VP. It had been reported that LiP isoenzyme H2 was able to oxidize Mn³⁺ [10] but recent results using recombinant enzyme show that this is not the case [11]. However, Mn³⁺ can be oxidized by superoxide generated directly [12] or indirectly [13] by other extracellular fungal enzymes. It is known that VA increases the ability of LiP to oxidize high-redox-potential substrates and phenols (its role as a redox mediator or enzyme stabilizer, including its contribution to closing the catalytic cycle, being a matter of controversy). However, VA does not increase oxidation of other substrates by VP, which is able to oxidize compounds that are not oxidized by LiP in the absence of VA. This is the case of several dyes [14] which are oxidized by VP, LiP, and MnP in the presence of 2 mM VA (VP/LiP/MnP+VA) with the following activities (in units/mg): Reactive Violet 5, 8/0/6; Reactive Black 5 (RBS), 5/0/5; Reactive Orange 96, 7/0/4; Reactive Red 198, 3/0/10; Reactive Blue 38, 10/1/5; and Reactive Blue 15, 11/1/10.

*Pl. eryngii* is being investigated for biological pulping due to its ability to degrade lignin selectively. When enzymes from lignocellulose cultures were investigated, two new peroxidases were isolated [8,15]. One of them corresponds to a second VP (isoenzyme PS1), whereas the other is a peroxidase closer to MnP than to VP. The activities of VP-PS1 on Mn³⁺, 2,6-dimethoxyphenol and methoxy-p-hydroquinone are similar to those of *Pl. eryngii* VP isolated from liquid cultures (isoenzyme PL). However, it shows lower activities on VA, p-dimethoxybenzene and lignin model dimers, suggesting a lower redox potential. During VP-PS1 oxidation of methoxy-p-hydroquinone, biphasic kinetics with apparent saturation in both micromolar and millimolar ranges were obtained (Fig. 1A), as found for ABTS oxidation by VP-PL [4]. Biphasic kinetics have been reported also for LiP (and S168W MnP variant) oxidation of some substrates [16]. Moreover, mutual inhibition between VP-PS1 oxidation of Mn³⁺ and RB5, an azo dye which is not oxidized by Mn³⁺, was found as reported for VP-PL [4]. A decrease of the velocity of Mn³⁺ oxidation was caused by RB5 but the $K_m$ was maintained, suggesting non-competitive inhibition (Fig. 1B).

**Gene cloning and peroxidase sequence**

DNA probes were obtained by PCR and reverse transcription-PCR, using primers corresponding to N-terminal and internal sequences (from tryptic peptides) of VP-PL, and used to select cDNA and genomic DNA clones containing the corresponding gene. The gene *pl* appeared as two alleles, sharing 99% of the sequence, which encode the two peroxidases isolated from liquid cultures [17]. The VP allelic variants PL1 and PL2 differ only in three amino acid residues but they could be identified because one of the substitutions is in the N-terminal region. A second gene was cloned encoding VP-PS1 produced in lignocellulose
cultures. [18]. Both *P. eryngii* genes (*pl* and *psl*) contain 15 introns and encode proteins with 361 amino acids (including a 30 amino acid signal peptide) in the case of VP-PL, and 370 amino acids (including a signal peptide of 31 amino acids) in the case of VP-PS1, showing 74% sequence identity. The presence of putative elements for oxidative stress response in the promoters of these genes could explain transcriptional regulation of *P. eryngii* VP by *H₂O₂* and other reduced oxygen species [19]. Their heterologous expression provided recombinant peroxidase with the same catalytic properties of wild VP [20].

**Figure 1**

Biochemical evidence for different substrate oxidation sites in *Pleurotus* VP

Biphasic kinetics for methoxy-p-hydroquinone oxidation by *P. eryngii* VP were obtained by Michaelis-Menten adjustment in the two steps (with substrate concentration on a logarithmic scale) resulting in two different apparent *Kₘ* values from the Lineweaver-Burk plot (inset) (A); and non-competitive inhibition of Mn²⁺ oxidation by the same peroxidase by 32 μM RB5, including Lineweaver-Burk plot (inset) (B).

The genomic or cDNA sequences of at least 29 fungal peroxidases are currently available from GenBank, including *Coprinus cinereus* peroxidase (CIP) and peroxidases from *Ph. chrysosporium* (LiP and MnP), *Poria subvermispora* (MnP), *Trametes versicolor* (LiP, MnP, two hypothetical peroxidases, and the so-called Mn-repressed peroxidase, MRP), *Pl. ostreatus* (MnP), *Phlebia radiata* (LiP) and *B. adusta* (LiP). The sequences of the two *P. eryngii* mature peroxidases are close to those of two *P. ostreatus* peroxidases recently deposited as MnP2 [21], showing 98% identity with VP-PS1, and MnP3 [22], sharing 77% identity with VP-PL. The sequence of the first MnP gene reported in *P. ostreatus* [23] is less closely related. It is interesting that the *P. eryngii* VP isoenzymes have higher sequence identity with *Ph. chrysosporium* LiP (up to 60% for VP-PL and 62% for VP-PS1) than MnP isoenzymes (up to 55% for VP-PL and 57% for VP-PS1).

**Peroxidase molecular structure**

*Ph. chrysosporium* LiP (isoenzyme H8) and MnP crystal models were reported in 1993-1994 [24-26]. Only the cytochrome *c* peroxidase crystal structure was known at that time. More recently, crystal structures of CIP (99% identical to the peroxidase from *Arthromyces ramosus*, an invalid species that could correspond to a *Coprinus* ana-morph) [27,28], *Ph. chrysosporium* LiP isoenzyme H2 [29,30], fungal chloroperoxidase [31] and several plant peroxidases [32-35] have been reported. Taking advantage of high sequence identity with *Ph. chrysosporium* LiP and MnP, and considering similar folding and helical topology of peroxidases in classes I and II, molecular models for *P. eryngii* VP isoenzymes were built by homology modelling [36]. Fig. 2 shows schematic representations of the predicted models [17,18]. Both include 12 predominantly α-helices; four disulphide bridges; two Ca²⁺-binding sites; several characteristic residues in the haem pocket, and putative substrate-oxidation sites (described below). Despite both VP isoenzymes having the same number of helices, the position of one of them is different: helix B' being characteristic of VP-PL and helix B' of VP-PS1 (VP-PL B' corresponding to VP-PS1 B'). Moreover, VP helix B'/B'' corresponds to helix B' of MnP and LiP. A small helix has been reported in CIP at the position of PS1 helix B' [28] and some helical conformation at this position exists in LiP. Therefore, VP, CIP and LiP could have 12 helices,
Figure 2
Molecular models for Pleurotus VP

Schematic molecular representation of peroxidases from liquid (left) and lignocellulose (right) cultures of P. eryngii after homology modelling with PROMOD using LiP, MnP and CIP crystal models as templates and refined by CHARMM [36]. The positions of α-helices, the haem group, Mn⁺⁺, and C- and N-termini are indicated. The atomic co-ordinates correspond to PDB entries 1A20 and 1QJR.

compared with 11 helices of MnP. The C-terminal tails of Pl. eryngii VP isoenzymes are a few residues shorter than that Ph. chrysosporium LiP, whereas Ph. chrysosporium MnP has a longer (18–26 residues) tail. When the predicted molecular models for Pl. eryngii VP were superimposed with the crystal models for Ph. chrysosporium peroxidases, lower distances between backbone Cα carbons were obtained with LiP than MnP. The root-mean-square distances for VP-PL were 1.24 Å (329 residues computed) after superimposition with LiP-H8 and 1.49 Å (324 residues) with MnP1, whereas those for VP-PS1 were 0.88 Å (335 residues) after superimposition with LiP-H8, and 1.33 Å (321 residues) with MnP1.

Substrate-oxidation sites in a versatile peroxidase

It is known that H₂O₂ attains the distal side of haem through the access channel present in all peroxidases. However, identification of sites involved in substrate oxidation remain elusive. The Mn-interaction site of MnP constitutes one exception. Although Mn⁺⁺ oxidation at the haem edge had been suggested [37], an Mn-interaction site near the internal haem propionate was first postulated in theoretical [38] and crystal models [26] and then confirmed by site-directed mutagenesis (SDM) and X-ray diffraction of MnP/Mn⁺⁺ complexes [39–41]. As shown in Fig. 3, a putative Mn-interaction site similar to that of MnP (E35, E39 and D179) was identified in Pl. eryngii VP-PL (E36, E40 and D175) and VP-PS1 (E36, E40 and D181). The three acidic residues delimit a small access channel in front of haem propionates. Mn⁺⁺ enters this second haem-access channel being co-ordinated by carboxylate oxygens of the internal propionate and the three acidic residues, and one electron is transferred to haem via the propionate arm. No such site exists in LiP (Fig. 3) and, therefore, it lacks the ability to oxidize Mn⁺⁺ efficiently, as confirmed by recent studies [11]. Substitution of one of these residues (D175) in Pl. eryngii VP-PL by SDM results in the loss of Mn⁺⁺ oxidation ability [42]. Multiple sequence alignment of fungal peroxidases showed that the above three residues are conserved in Ph. chrysosporium, Po. subvermispora and T. versicolor MnPs, as well as in all Pleurotus peroxidases and T. versicolor MRP [43], being absent from LiP sequences, with one exception [44], and CIP.

No crystal structure of LiP has been reported containing VA. It was generally considered, and recently confirmed [34], that substrate oxidation by horseradish peroxidase takes place in the vicinity of the haem. Therefore, when LiP-H8 was
crystallized, the VA molecule was modelled at the haem-access channel, H-bonded to H82 and Q222 [25]. These residues are conserved in *P. eryngii* VP-PS1 (H82 and Q222), which has a main haem-access channel remarkably similar to that of LiP [15], but not in VP-PL.

Despite the possibility of direct electron transfer to haem by some VP and LiP substrates, long-range electron transfer (LRET) should also be considered due to: (i) the high molecular size of some of the substrates; (ii) the small size of the haem-access channel, and (iii) the distance between the channel opening and the haem edge. The first LRET pathway in LiP was suggested by Schoemaker et al. [45]. It would initiate in LiP-H82 and proceed via P83 and N84, H-bonded to distal histidine (Fig. 3). Such a pathway does not exist in *P. chrysosporium* MnP and *P. eryngii* VP-PL, but it can be identified in VP-PS1 (H82, A83 and N84). Unfortunately, there is no ad-

**Figure 3**

**Residues involved in electron transfer to haem in different ligninolytic peroxidases**

Representation of Mn-binding sites in *P. chrysosporium* isoenzyme MnP1 and *P. eryngii* VP-PL and VP-PS1, and three hypothetical long-range electron-transfer pathways from superficial tryptophan and histidine residues in *P. chrysosporium* LiP isoenzyme H8 (from W171, H239 and H82) and *P. eryngii* VP-PL (from W164 and H239) and VP-PS1 (from W170 and H82), that from superficial tryptophan being confirmed by SDM. The atomic co-ordinates correspond to PDB entries 1LLP, 1MNP, 1A20 and 1QJR.
dional information to show if this hypothetical pathway is operative. Two additional LRET pathways have been proposed in LiP. One of them would initiate in another superficial histidine (LiP-H8 H239) and proceed via D238 to the proximal histidine [46] (Fig. 3). This is lacking from MnP and *Pleurotus eryngii* VP-PS1 being present in VP-PL (H232 and D231). Despite results from dehydrogenation polymer (synthetic lignin) oxidation suggesting that the H239 LRET pathway could be involved in lignin degradation by LiP, no SDM confirmation has been reported. Finally, a third LRET pathway from a superficial tryptophan was proposed after observation of Cα-hydroxylated W171 in wild-type LiP as well as in the recombinant peroxidase exposed to a few H2O2 equivalents (whereas W171 was present before H2O2 exposition) [30,47]. This is initiated in LiP W171 and proceeds via LiP-H8 M172 (or LiP-H2 L172) H-bonded to the porphyrin ring (Fig. 3). Its involvement in VA oxidation has been confirmed by tryptophan bromination and SDM [30,48] and a transient radical has been detected [49] revealing that W171 is a redox-active residue in LiP. Similar pathways can be identified in both *Pleurotus* isoenzymes (VP-PL W164 and L165, and VP-PS1 W170 and L171) and its operation has also been confirmed by both chemical and SDM modification of VP-PL W164 [42]. Multiple sequence alignment showed that the above tryptophan is conserved in all LiP sequences, as well as in *Pleurotus* peroxidases (except MnP3) and *T. versicolor* MRP, being absent from *Ph. chrysosporium*, *Po. subvermispora* and *T. versicolor* MnP, and CIP.

It is interesting that W171 at the LiP surface is located in an acidic environment that could stabilize the VA cation radical formed after one electron transfer. In this way, it could act as a mediator for oxidation of other compounds [48,50]. A comparison with VP shows that LiP W171 is surrounded by four acidic residues (D165, E168, E250 and D264) whereas only two are located near VP-PL W164 (E161 and E243) and VP-PS1 W170 (E167 and E249). This could be related to the fact that VA does not exert a stimulating (mediating) effect in VP-catalysed oxidations.

The existence of different sites involved in oxidation of Mn⁴⁺ and other substrates by VP is supported also by non-competitive inhibition between oxidation of Mn⁴⁺ and high-redox-potential dyes by *Pleurotus* VP-PS1 (Fig. 1B) and VP-PL1 [4]. This suggests two sites with different affinities, which are not affected by the presence of the alternative substrate acting as inhibitor. Moreover, the biphasic kinetics found for some substrates (Fig. 1A) [4] could suggest the existence of more than one site for oxidation of the same molecule. If these substrates are oxidized at the enzyme surface, it is possible to imagine several electron-transfer pathways involved in oxidation of each of them. Substrate oxidation at these sites would depend on both their affinity for the specific compound and their redox potential, being an inverse function of the length of the electron-transfer pathway to haem. Despite LiP having a much narrower substrate specificity than VP, SDM suggests that, in addition to the VA-oxidation site involving W171, oxidation of some dyes is produced in the vicinity of the haem channel [48].

The results presented have been obtained with the financial support of the European contracts AIR2-CT93-1219 and QLK3-99-590, and the Spanish Biotechnology Programme.

References

An old activity in the cytochrome P450 superfamily (CYP51) and a new story of drugs and resistance

S. L. Kelly1, D. C. Lamb, M. Cannieux, D. Greetham, C. J. Jackson, T. Marczyno, C. Ugochukwu and D. E. Kelly
AberBiocentre, Institute of Biological Sciences, University of Wales Aberystwyth, Aberystwyth SY23 3DA, Wales, U.K.

Abstract

Cytochrome P450 51 (CYP51) is sterol 14α-demethylase, known also as Erg1lp in yeast. First studied in yeast, where it is one of three CYPs in the genome, it has subsequently gained attention as the only CYP found so far in different kingdoms of life. As such it is central to considerations of CYP evolution. Recent use of CYP51-inhibiting antifungal drugs, such as fluconazole, has also been associated with dramatic CYP51 evolution to numerous resistant forms in fungal pathogens. CYP51 has also been discovered in mycobacteria where antifungal azoles have effect and might be of value against tuberculosis. Evolutionary and therapeutic aspects of CYP51 studies are discussed.

Introduction

Cytochromes P450 (CYPs) are studied extensively and the main engine for the field has been the applied significance of these mono-oxygenase genes/enzymes in drug metabolism, carcino-