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Review

Basidiomycete DyPs: Genomic diversity, structural–functional aspects, reaction mechanism and environmental significance

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ABSTRACT

The first enzyme with dye-decolorizing peroxidase (DyP) activity was described in 1999 from an arthroconidial culture of the fungus *Bjerkandera adusta*. However, the first DyP sequence had been deposited three years before, as a peroxidase gene from a culture of an unidentified fungus of the family Polyporaceae (probably *Irpex lacteus*). Since the first description, fewer than ten basidiomycete DyPs have been purified and characterized, but a large number of sequences are available from genomes. DyPs share a general fold and heme location with chlorite dismutases and other DyP-type related proteins (such as *Escherichia coli* EfeB), forming the CDE superfamily. Taking into account the lack of an evolutionary relationship with the catalase–peroxidase superfamily, the observed heme pocket similarities must be considered as a convergent type of evolution to provide similar reactivity to the enzyme cofactor. Studies on the *Auricularia auricula-judae* DyP showed that high-turnover oxidation of anthraquinone type and other DyP substrates occurs *via* long-range electron transfer from an exposed tryptophan (Trp377, conserved in most basidiomycete DyPs), whose catalytic radical was identified in the H_2O_2-activated enzyme. The existence of accessory oxidation sites in DyP is suggested by the residual activity observed after site-directed mutagenesis of the above tryptophan. DyP degradation of substituted anthraquinone dyes (such as Reactive Blue 5) most probably proceeds *via* typical one-electron peroxidase oxidations and product breakdown without a DyP-catalyzed hydrolase reaction. Although various DyPs are able to break down phenolic lignin model dimers, and basidiomycete DyPs also present marginal activity on non-phenolic dimers, a significant contribution to lignin degradation is unlikely because of the low activity on high redox-potential substrates.

From DyP discovery to screening of basidiomycete genomes

The first dye-decolorizing peroxidase (DyP[2]) was described as an extracellular enzyme from a basidiomycetous fungus only 15 years ago [1,2]. Since this date the number of known DyP-type enzymes has exponentially increased. EfeB, initially investigated as a substrate of the "twin-arginine" translocation system of *Escherichia coli* (as YcdB) was the first recognized bacterial DyP in 2006 [3]. Subsequently, the number of DyP-type genes from prokaryotes (up to several thousand bacterial and archaeal sequences) experienced an even more rapid growth than that of fungal DyPs (up to several hundred sequences from basidiomycetes and other fungi) due to the large number of sequenced genomes.

Bjerkandera adusta, the basidiomycete from which the first DyP (*Bad*DyP) was isolated, is a member of the order Polyporales that includes most wood-rotting fungi. Due to arthroconidia formation, the isolate was initially identified as *Geotrichum candidum* (an ascomycete-type fungus) [4], and subsequent ITS sequencing resulted in a new misidentification as the basidiomycete *Thanatephorus cucumeris* [5]. The situation was clarified by Ruiz-Dueñas et al. [6] who, after comparisons with authentic ITS

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[1] Current address: Department of Biotechnology, Delft University of Technology, Julianalaan 136, 2628BL Delft, The Netherlands.
[2] Abbreviations used: CDE, Cld, DyP and EfeB proteins (superfamily); Cld, chlorite dismutase; DyP, dye-decolorizing peroxidase; LiP, lignin peroxidase; LRET, long-range electron transfer; MnP, manganese peroxidase; VP, versatile peroxidase.

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sequences, concluded that the fungus belongs to *B. adusta*. Other basidiomycete DyPs have been cloned and sequenced, including those from an unidentified Polyporaceae species (Y.-H. Han, 1996, AAB58908 direct submission to GenBank), the termite mushroom *Termitomyces albuminosus* (Agaricales) [7], the oyster mushroom *Pleurotus ostreatus* (Agaricales) [8], the leaf-litter degrader *Mycetinis scorodonius* (syn. *Marasmius scorodonius*; Agaricales) [9], the jelly fungus *Auricularia auricula-judae* (Auriculariales) [10], and the wood rotter *Ganoderma lucidum* (Polyporales) (C.-P. Kung et al., 2010, ADN05763 direct submission to GenBank). Recently, a DyP was isolated from the selective lignin-degrader *Irpex lacteus* (family Polyporaceae) [11]. Although its complete sequence has not been published, the secretomic analysis of this fungus revealed that this DyP protein has up to 13 unique tryptic peptides (near 50% total coverage) exactly matching with those predicted by the AAB58908 sequence mentioned above [12]. This suggests that both peroxidases are the same protein and, therefore, that the unidentified Polyporaceae species from which the first peroxidase gene was isolated most probably corresponds to *I. lacteus* (also described as *Polyporus tulipifera*).

Many new basidiomycete DyP sequences have recently become available after analysis of numerous fungal genomes [13,14] as part of the DOE Joint Genome Institute (JGI; http://www.jgi.doe.gov) effort to find enzymes of interest for lignocellulose biorefineries. DyP sequences (from fungi, bacteria and archaea) have been classified into four (A–D) types [15], or subfamilies [16]. More than 138 relevant fungal sequences in Peroxibase (http://peroxibase.toulouse.inra.fr; September 2014 data) correspond to subfamily D, and only two to subfamily C. A specific comparison of basidiomycete DyPs from 16 sequenced genomes and some GenBank sequences is shown in the phylogram of Fig. 1. This figure also compares ten relevant residues of the *A. auricula-judae* DyP (*Aau*DyP used as reference in the present revision) with those in *Bad*DyP and other basidiomycete DyPs. It also shows the presence/absence of signal peptides and the number of tyrosine and tryptophan residues, discussed below, in each of the sequences. Seven DyP clusters are evident, and they display differences at the amino-acid sequence level that could affect catalytic properties. It is worth mentioning that more than 75% of the sequences are included in clusters I–III, the farthest from the phylogram root. Apparently, the initially increased mutational rate in basal branches (VI and VII) was decreased and stayed constant during further evolution leading to large homogeneous clusters (I–III). An example of this is provided by the residues equivalent to *Aau*DyP Val253 (Fig. 1), where a variety of aliphatic amino acids can be observed at clusters IV–VI, whereas a clear predominance of phenylalanine and valine residues is observed in clusters I and II, respectively (in a similar way, the highest variability in the number of tyrosine and tryptophan residues occurs in clusters IV–VII). However, the opposite tendency is also observed, for example in the residues equivalent to *Aau*DyP Asp395 and Tyr337 (Fig. 1). These two positions are highly conserved, except in clusters I and III, where changes of Asp395 into glutamic acid and of Tyr337 into phenylalanine, respectively, have occurred. These and other differences in relevant regions of basidiomycete DyP sequences are discussed in the following sections.

DyP general fold and active-site architecture

The crystal structures of basidiomycete *Bad*DyP [17–19] and *Aau*DyP [20,21] have been reported, together with those of several bacterial DyPs [22–26]. Their molecular structure is shared with other DyP-type peroxidases, as well as with chlorite dismutases (Clds), justifying definition of a CDE structural superfamily (for Cld, DyP, and EfeB proteins) with a common evolutionary origin [27,28]. This is illustrated in Fig. 2A–C, which shows the related crystal structures of *Aau*DyP, DyP-type *E. coli* EfeB [22] and *Dechloromonas aromatica* Cld [29], respectively (which share 13–15% sequence identities). These structures include two domains from an ancestral duplication, each containing a ferredoxin-like fold formed by a four-stranded antiparallel β-sheet (*Aau*DyP N-terminal and C-terminal domains including strands β1-β4 and β5-β7/β10, respectively) covered at one side by 2–3 helices (*Aau*DyP helices H2/H4 and H7/H9/H11, respectively) and one heme molecule at the C-terminal domain. A small extra β-sheet exists at the C-terminal domain formed by two β-strands (*Aau*DyP strands β8 and β9), although it is not always present as in the *E. coli* EfeB. The above overall structure is very different from that of the classical fungal and plant peroxidases, currently classified in the catalase–peroxidase superfamily [30]. The latter group includes largely helical proteins with the heme cofactor located at the interface between their two domains, 4–5 disulfide bonds and two structural Ca^{2+} ions (the two last features being absent from DyPs) [31,32]. Although the CDE core fold is the same, EfeB shows a higher number of loops than Cld, and this number is even increased in basidiomycete DyPs. The increasing complexity may hinder enzyme oligomerization by changing the dimerization interfaces, thus explaining the predominance of monomeric basidiomycete DyPs *vs*. oligomeric bacterial DyPs [24].

Details of the *Aau*DyP, *E. coli* EfeB and *D. aromatica* Cld heme pocket, all located in the C-terminal domain, are shown in Fig. 2D–F, respectively. The heme propionates in DyP-type peroxidases (Fig. 2D and E) adopt a characteristic disposition with the pyrrole D propionate below the plane of the heme (instead of above as found in other fungal peroxidases) and that of pyrrole A tilted into an unusual conformation due to several strong H-bonds [21] (note that pyrrole A appears as pyrrole C in this cited publication). In addition, the Cld heme is flipped with respect to the position found in DyP-type and other fungal peroxidases (Fig. 2F). The heme pocket architecture is similar to that found in other heme-containing peroxidases, resulting from an evolutionary convergence to provide similar reactivity properties to the cofactor. This is the case for the aspartic acid and arginine residues (*Aau*DyP Asp168 and Arg332) located over the heme plane (at the so-called distal side) that are expected to contribute to the heterolytic cleavage of H_2O_2 to form compound I, as distal histidine and arginine do in other fungal peroxidases [33]. This was demonstrated by the loss of *Aau*DyP activity after D168N and R332L single mutations, which resulted in an impaired reaction with H_2O_2 as shown by electronic absorption and electron paramagnetic resonance (EPR) spectra [20]. A central role of either the conserved aspartic acid or arginine residues in the reaction with H_2O_2 had been claimed in *B. adusta* [18] and bacterial DyPs [22,34], respectively, but both residues are required for H_2O_2 activation of *Aau*DyP, as mentioned above. As found for other DyPs [18], no compound II spectrum is observed during *Aau*DyP self-reduction at physiological pH 3 (see below), a fact that suggests faster reduction of compound II than of compound I, in contrast with other peroxidases [35]. It has been suggested that Asp168 in *A. auricula-judae* could also act as a gatekeeper controlling the access of reducing substrates to the heme [21]. Nevertheless, such mobility, which is also observed for distal arginine when comparing several crystal structures of other peroxidases, is most probably related to its contribution to heme reaction with H_2O_2 as suggested for *Bad*DyP [36] (note that most known basidiomycete DyP substrates are oxidized at the protein surface, as discussed below). Concerning other basidiomycete DyPs (Fig. 1), the only exception for the conserved arginine is found in a *Punctularia strigosozonata* putative DyP (JGI gene model 114678), where it is substituted by a similarly basic but smaller lysine residue. Regarding the conserved aspartic acid, the three sequences forming cluster VI show a glycine at this position. The effect of such large changes (in terms

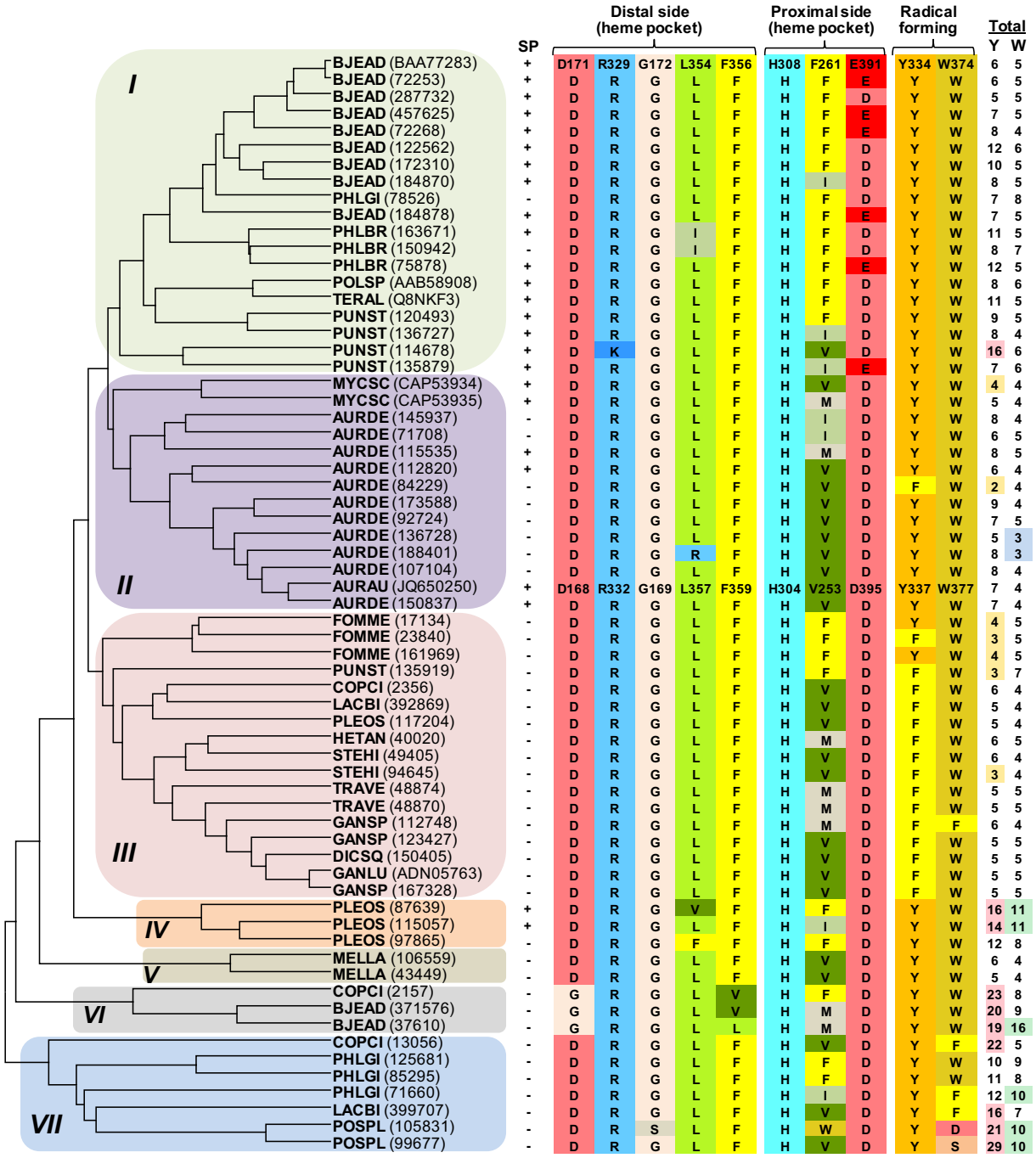

Fig. 1. Phylogram of basidiomycete DyPs. Up to a total 65 sequences of basidiomycete DyPs were obtained from the genomes of *Auricularia delicata* (AURDE), *B. adusta* (BJEAD), *C cinerea* (COPCI), *Dichomitus squalens* (DICSQ), *Fomitiporia mediterranea* (FOMME), *Ganoderma* sp. (GANSP), *Heterobasidion annosum* (HETAN), *Laccaria bicolor* (LACBI), *Melampsora laricis-populina* (MELLA), *Phlebia brevispora* (PHLBR), *Phlebiopsis gigantea* (PHLGI), *P. ostreatus* (PLEOS), *Postia placenta* (POSPL), *P. strigosozonata* (PUNST), *Stereum hirsutum* (STEHI) and *Trametes versicolor* (TRAVE) available at the JGI MycoCosm portal (http://genome.jgi-psf.org/programs/fungi) together with GenBank sequences of *A. auricula-judae* (AURAU), *Ganoderma lucidum* (GANLU), *M. scorodonius* (MYCSC), Polyporaceae species (POLSP) and *T. albuminosus* (TERAL) (PLEOS 87639 corresponds to GenBank CAK55151). According to Peroxibase (http://peroxibase.toulouse.inra.fr) only BJEAD (122562) belongs to DyP subfamily C, while all the other sequences are included in subfamily D. The phylogram obtained with MEGA5 [88] includes three main clusters (I–III) and four small clusters (IV–VII). The JGI and GenBank references are provided, together with indication of: (i) presence/absence of signal peptides (SP) [89]; (ii) residues equivalent to *Aau*DyP/*Bad*DyP heme distal residues (Asp168/Asp171, Arg332/Arg329, Gly169/Gly172, Leu357/Leu354 and Phe359/Phe356), heme proximal residues (His304/His308, Val253/Phe261 and Asp395/Glu391); (iii) radical-forming residues (equivalent to *Aau*DyP/*Bad*DyP Tyr337/Tyr334 and Trp377/Trp374); and (iv) total number of tyrosines (those significantly over or below the average on red and yellow background, respectively) and tryptophans (those significantly over or below the average on green and blue background, respectively). The above numbering corresponds to the predicted mature (i.e. signal peptide excluded) amino-acid sequences.

of residue size and charge) needs to be investigated to confirm the functionality and possible modification of catalytic properties in these three putative DyPs. The arginine and aspartic acid residues are conserved in *E. coli* EfeB (Fig. 2E), where they would contribute to the peroxidase activity of this protein. The arginine residue is also conserved in Cld (Fig. 2F) where it participates in chlorite conversion into chloride and O_2, by controlling the substrate binding and dismutation reaction [37]. Finally, one phenylalanine residue

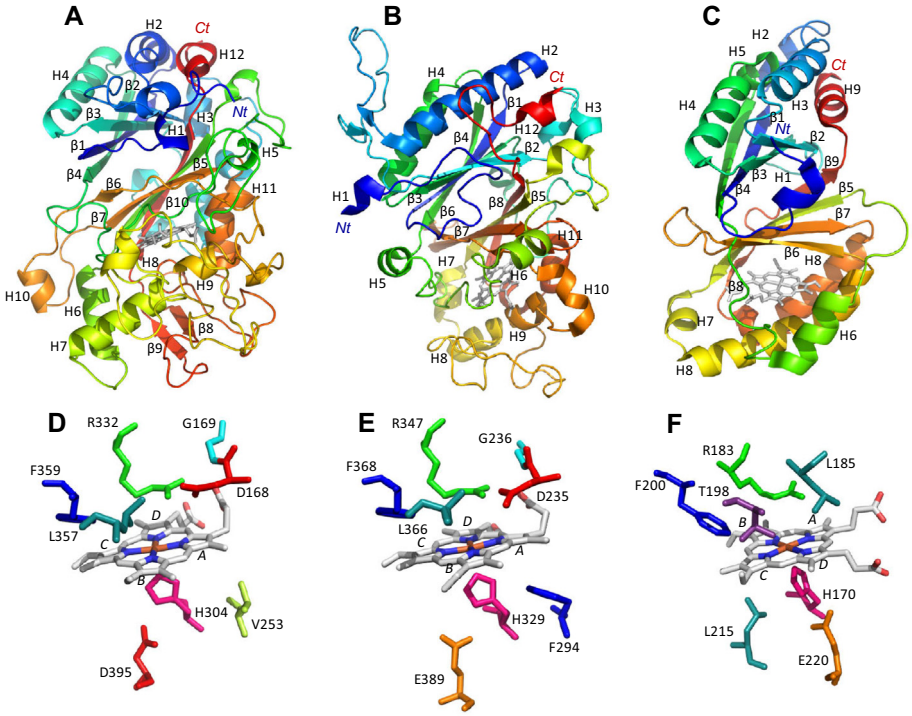

Fig. 2. Comparison of crystal structures. (A–C) Ribbon representation of recombinant *Aau*DyP (PDB 4W7J), DyP-type EfeB from *E. coli* (PDB 3O72) and Cld from *D. aromatica* (PDB 3Q09), respectively, colored from N-terminus (blue) to C-terminus (red) showing α-helices and β-strands. (D–F) Detail of the heme pocket of each protein showing several conserved residues with a possible role in catalysis, and heme position (A–D pyrrole ring names are in italics).

(*Aau*DyP Phe359) is also conserved at the distal side of many CDEs and fungal peroxidases.

At the other side of the heme, a proximal histidine (*Aau*DyP His304) acts as the fifth ligand of the heme iron (at 2.2 Å), being conserved in all basidiomycete DyPs (Fig. 1) and other CDEs (Fig. 2D–F), as found also for other fungal peroxidases [33], P450-type peroxygenases excluded [38,39]. A different histidine (*Bad*DyP His164) was initially proposed to play this role [5,17] but subsequent work highlighted the impossibility that this residue could act as fifth ligand of the heme in DyP [25], as confirmed when the complete crystal structure of *Bad*DyP was reported [18]. A neighbor acidic residue would contribute to modulate the heme electron-deficiency in DyP-type proteins by forming a hydrogen bond between the side-chain carboxylate and the histidine Nδ1, as the proximal aspartic acid does in other fungal peroxidases. The position occupied by this aspartic acid in *Aau*DyP (Asp395) and most basidiomycetes DyPs, is substituted by a glutamic acid in *Bad*DyP [18] and other five basidiomycete DyPs (Fig. 1), as well as in *E. coli* EfeB (Fig. 2E). This acidic residue is absent from Cld but a different glutamic acid occupies a neighbor position (Glu220 in Fig. 2F). Near the proximal histidine and aspartic acid residues of *Aau*DyP, Val253 occupies a position with respect to the heme cofactor equivalent to that occupied by a tryptophan residue in cytochrome *c* peroxidase and by a phenylalanine in fungal (and plant) peroxidases, one exception being *Coprinopsis cinerea* peroxidase, which has a leucine at this position [33]. This tryptophan is where a catalytic protein radical is located in H_2O_2-activated cytochrome *c* peroxidase [40,41] but radicals are not known at this position in other peroxidases. Either aromatic (phenylalanine) or aliphatic (valine, isoleucine or methionine) residues occupy this position in the different basidiomycete DyPs (Fig. 1) as well as in *E. coli* EfeB (Fig. 2E).

Basidiomycete DyP substrates

The natural substrates and degradative activities of DyPs remain to be clarified although their extended presence in forest soils has been detected at the transcript level [42], and their ability to improve wheat straw digestibility [11] and eucalypt pulp bleachability [43] has been shown, suggesting an environmental role in transformation of plant biomass and/or soil organic matter. The above agrees with the extracellular nature of all the basidiomycete DyPs isolated to date from *A. auricula-judae*, *B. adusta*, *Exidia glandulosa*, *I. lacteus*, *M. scorodonius*, *Mycena epipterygia* and *T. albuminosus* cultures [1,7,9–11,44,45], as well as with the presence of signal peptides in 40% of the DyP sequences included in Fig. 1. Interestingly, signal peptides are frequent among members of clusters I, II and IV (42–89% of sequences) and absent from the rest of the sequences analyzed, suggesting different physiological/environmental roles for DyPs in different clusters. However, the absence of a signal peptide does not necessarily mean that the active enzyme is not released to the extracellular medium using an alternative secretion mechanism or simply by hyphal autolysis (note that lignocellulose degradation is typically a secondary metabolic event), as discussed for other fungal oxidoreductases [46].

The ability of the above DyPs (fungal and recombinant proteins) to oxidize 2,6-dimethoxyphenol, guaiacol, other substituted phenols, azo dyes (such as Reactive Black 5), ascorbic acid, β-carotene, veratryl alcohol and even lignin model dimers has been reported, although on the two latter substrates with variable results due to low activity [1,9–11,19,44,47,48]. However, anthraquinone dyes such as Reactive Blue 19, Reactive Blue 5 and others [4] are the best substrates of these enzymes, together with the general oxidoreductase substrate ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), as revealed by high turnover numbers (often over 200 s^{-1}) [7,11,47]. In addition to the above oxidation reactions, oxidation of aromatic sulfides has been described for bacterial DyPs [49]. Moreover, oxidation of Mn(II) to Mn(III), a reaction characteristic of basidiomycete manganese peroxidases (MnPs) and versatile peroxidases (VPs) [50], has also been reported for bacterial DyPs, with two putative Mn-binding sites being tentatively identified in crystal structures [26,51]. Optimal pH values reported

for the oxidative reactions of DyP [1,11,44] span those of the class II peroxidases (pH 2.5–4.5) although a drastically acidic condition (pH 1.8) is often used for oxidation of the highest redox-potential substrates investigated, namely veratryl alcohol (3,4-dimethoxybenzyl alcohol) and lignin model dimers [10,47]. Three related aspects of DyP catalysis and environmental roles are discussed in the following three sections concerning: (i) the need for long-range electron transfer (LRET) from the enzyme surface for oxidation of dyes and other bulky substrates; (ii) the mechanism of substituted anthraquinone dye cleavage; and (iii) the possible contribution of DyPs to lignin biodegradation.

Electron transfer in basidiomycete DyPs

Dyes known as the typical substrates of basidiomycete DyPs cannot directly interact with the buried heme cofactor, and the same applies for other bulky additional substrates including lignin-related compounds (see below), pointing to LRET from radical-forming exposed residues. *Aau*DyP possesses several aromatic residues potentially able to form such reactive radicals, including seven tyrosines and four tryptophans (Fig. 3) [21,52]. The average number in basidiomycete DyPs is 8.8 tyrosines and 5.7 tryptophans per protein molecule (Fig. 1). This high number of tyrosine residues contrasts with the absence, or low number, of tyrosines in ligninolytic peroxidases from the same group of fungi. The rationale for the absence of tyrosine residues in LiPs and VPs is enzyme protection against oxidative inactivation (from coupling and other reactions between tyrosine phenoxy radicals). Therefore, it is possible to presume that DyPs naturally act in a less oxidative environment than the above ligninolytic peroxidases [53]. The aromatic surface residues in *Aau*DyP were recently investigated for their tendency to harbor a reactive radical by a combination of spectroscopic, site-directed mutagenesis and quantum mechanics/molecular mechanics (QM/MM) methods, after heterologous expression and crystallization of the recombinant enzyme [20].

In the above study, formation of a mixed Trp377 and Tyr337 radical was detected by EPR of peroxide-activated recombinant DyP at physiological pH 3 (Fig. 4A), the corresponding signal being different from those of "pure" tryptophanyl and tyrosyl radicals previously found in *Pleurotus eryngii* VP [54] and *Trametes cervina* lignin peroxidase (LiP) [55], respectively. Formation of a Tyr337 radical in *Aau*DyP isolated from a fungal culture had been indirectly shown in spin-trapping experiments [52]. Very recently, a tryptophanyl radical has been reported in this enzyme after H_2O_2 activation at pH 6.8 [56]. The absence of a tyrosyl component in the EPR signal suggests that the proton-coupled electron transfer for the oxidation of Tyr337 is altered when moving from acidic pH (mixed Trp377/Tyr337 signal) to non-physiological neutral pH (Trp377 signal). The fact that *Aau*DyP has no activity at neutral pH [10,44] is most probably due to the decreased redox potential of aromatic radicals when pH increases [57,58]. Formation of a protein radical potentially involved in catalysis had also been reported in a bacterial DyP [23], but the type of residue involved could not be identified from the EPR spectrum. Finally, radicals of conserved tryptophan residues have been reported in Cld, although a direct role in catalysis is not expected [59]. The EPR results from H_2O_2-activated *Aau*DyP are in agreement with spin presence in both Trp377 and Tyr337 (and the heme cofactor) as shown in QM/MM simulations. Formation of exposed protein radicals requires the

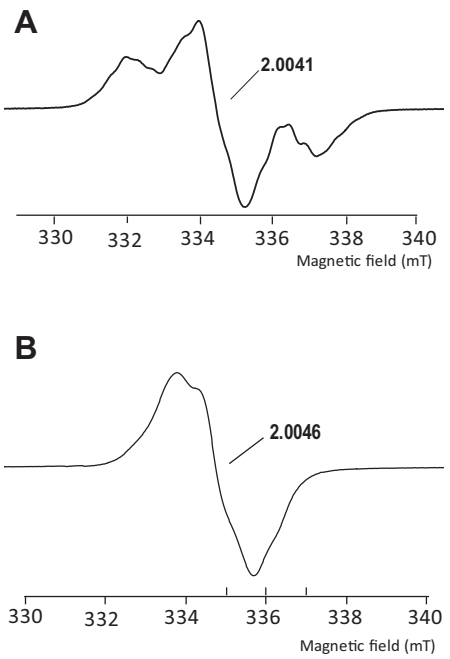

Fig. 4. EPR detection of mixed tryptophanyl/tyrosyl and tyrosyl radicals in H_2O_2-activated *Aau*DyP (A) and its W377S variant (B), respectively, at physiological pH. The spectra were acquired at pH 3, less than 10 s after the addition of H_2O_2 and rapid freezing, under the following conditions: ν = 9.39 GHz, 0.2 mT modulation amplitude, and 1 mW microwave power. Adapted from Linde et al. [20]. The g values are indicated on both spectra.

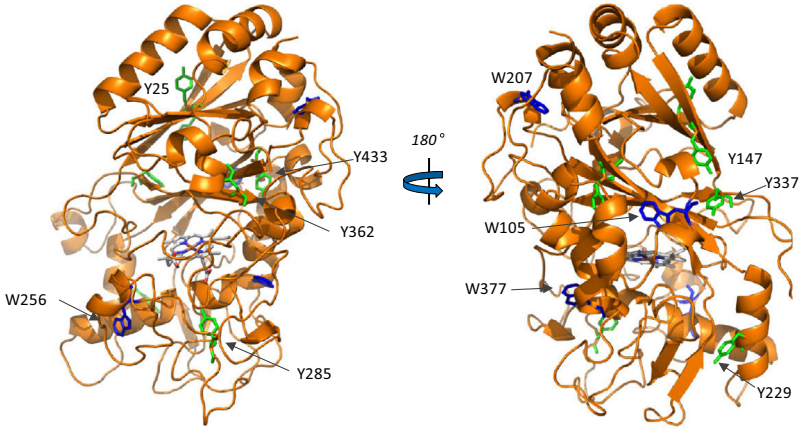

Fig. 3. Location of four tryptophan (blue sticks) and seven tyrosine (green sticks) residues on ribbon representation of the recombinant *Aau*DyP crystal structure (PDB 4W7J) from two different orientations (180° rotation). The heme cofactor is also shown as CPK-colored sticks. See Fig. 2A for helix and β-strand identification.

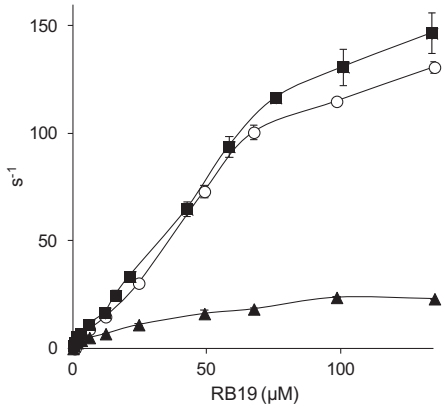

Fig. 5. Kinetics of Reactive Blue 19 oxidation by native *Aau*DyP (circles) and its Y337S (squares) and W377S (triangles) variants. Reactions were performed using 10 nm enzyme, 0.1 mM H_2O_2 and different RB19 concentrations. Means and 95% confidence limits are shown. Adapted from Linde et al. [20].

existence of LRET pathways from the corresponding residue to the peroxide-activated heme cofactor. Several pathways have been suggested from aromatic residues in *Aau*DyP [21,52], and more advanced QM/MM calculations using the e-pathway approach [60] show that the most probable pathway from reactive Trp377 includes the Pro310 side chain, the Arg309 and Arg306 carbonyls, and the Ile305 backbone to access the heme cofactor *via* His304 [20]. Interestingly, not only is Trp377 conserved in basidiomycete DyPs (91% of the sequences in Fig. 1) but also the above Pro310 and Arg306 putatively involved in LRET are conserved (in 95% and 100% of the 65 sequences analyzed, respectively).

The above conclusions were confirmed by obtaining the *Aau*DyP W377S variant in which the tryptophanyl radical disappears, leaving an EPR tyrosyl radical signal (Fig. 4B). As shown in Fig. 5, the W377S variant (triangles) showed strongly decreased enzymatic activity compared with native DyP (circles), revealing the central role in catalysis of this tryptophan residue. In contrast, the Y337S variant activity (squares) remains unaffected, which suggests that the corresponding tyrosyl radical, although detected by EPR, has low relevance in catalysis. A main role of Tyr337 in catalysis, instead of Trp377, had been suggested after chemical modification of both residue types in the enzyme isolated from an *A. auricula-judae* culture [21,52]. This discrepancy could be due to the low concentration of *N*-bromosuccinimide used for tryptophan modification in the above study, in agreement with the site-directed mutagenesis results mentioned above. The residual activity observed in the W377S variant (Fig. 5) suggests an accessory substrate oxidation site. Crystallographic structures of the *Bad*DyP with 2,6-dimethoxyphenol and ascorbic acid show both substrates at the entrance of a heme channel, connected to the exposed heme propionate through an H-bond network [19] reminiscent of ascorbate oxidation site in ascorbate peroxidase [61]. This channel does not exist in *Aau*DyP, but this peroxidase has a funnel-shaped channel [21] that could act as an accessory oxidation site, as recently suggested [56].

*Aau*DyP Trp377 is largely conserved in basidiomycete DyPs, being substituted by a phenylalanine residue in three of the sequences and by non-aromatic residues in two other sequences (Fig. 1). Tyr337, although conserved in many basidiomycete DyPs, is substituted by a phenylalanine residue in most of the cluster III sequences (Fig. 1), suggesting that its role is not to form a catalytic radical, in agreement with experimental results. Difficulties in *in vitro* activation of *Aau*DyP variants, where Tyr337 has been substituted by a nonaromatic residue, suggest a contribution to protein folding. In conclusion, Trp377 in *Aau*DyP, and probably also in other basidiomycete DyPs where this residue is conserved (Fig. 1), has been identified as responsible for high-turnover activity on dyes and other DyP substrates *via* a LRET pathway initiated at a tryptophanyl radical, as described for substrate oxidation by ligninolytic LiP and VP [35,62,63]. Interestingly, *T. cervina* LiP has a surface tyrosine involved in lignin oxidation [55], whose tyrosyl radical does not act as such, but rather after forming a reactive adduct [64]. This is because, although affected by pH and environment conditions [65], the redox potential of phenoxy radicals is generally not high enough to oxidize recalcitrant molecules, as shown for example by the W164Y mutation of the catalytic tryptophan of *P. eryngii* VP [66].

It is interesting to mention that most of the predicted amino-acid sequences from basidiomycete genomes shown in Fig. 1 include the heme-pocket and radical-forming residues described in the two previous sections, suggesting that they encode functional peroxidases with DyP-type activity.

Substituted-anthraquinone dye cleavage mechanism by DyP

The ability of DyPs to oxidize and partially decolorize some industrially important dyes is the feature that gave this enzyme its name. Two dyes that have received much attention are the anthraquinonoid pigments Reactive Blue 5 (Fig. 6, structure **1**) and Reactive Blue 19. Sugano et al. [67] showed that *Bad*DyP cleaved Reactive Blue 5 to give, among other products, phthalic acid (structure **2**) and an amine (structure **3**). These authors proposed a unique hydrolase (or oxygenase) mechanism for the reaction, which has been mentioned in subsequent reviews [56,68,69]. However, the possibility should be considered that the reaction actually proceeds by a conventional peroxidase-catalyzed route. This alternative hypothesis is consistent with the observation that, in contrast with the frequent assumption that DyPs are the only hemeperoxidases to oxidize anthraquinone dyes efficiently [21,70], horseradish peroxidase also decolorizes Reactive Blue 5 [2] and the same has been observed for *P. eryngii* VP (unpublished).

The site of initial peroxidase attack on Reactive Blue 5 has not been determined, but from inspection of its structure two sequential one-electron oxidations of the *p*-phenylenediamine moiety on one of the anthraquinone end rings appear likely. The rationale for this assumption is that *p*-phenylenediamines are good peroxidase substrates [71]. The initial products are imines and, in the case of Reactive Blue 5, product **4** would likely be formed initially. However, product **4** would not persist, because imines hydrolyze rapidly in water [72]. The anticipated anthraquinonoid product of Reactive Blue 5 oxidation by a peroxidase is thus the anthracenetetrone **5**, formed with release of the amine (structure **3**) that was found by Sugano et al. [67]. Product **5** is then a reasonable precursor of phthalic acid (structure **2**) because anthracenetetrones are unstable. For example, unsubstituted anthracenetetrone was reported to undergo spontaneous cleavage to yield phthalic acid among other products [73].

DyP "ligninolysis"

Some features of DyPs suggest that they could participate in lignin degradation. As described above, *Aau*DyP forms surface-exposed reactive radicals similar to catalytic tryptophanyl radicals in ligninolytic LiPs and VPs, whose role is to mediate oxidation of bulky substrates. A variety of DyPs from fungi and bacteria have been shown to oxidize large dye molecules [10,49,67], and the exposed tryptophan residue potentially involved in catalysis is conserved in most basidiomycete DyPs, as discussed above. Moreover, DyP coding genes are more abundant in the genomes of efficiently ligninolytic wood decay basidiomycetes (white-rot

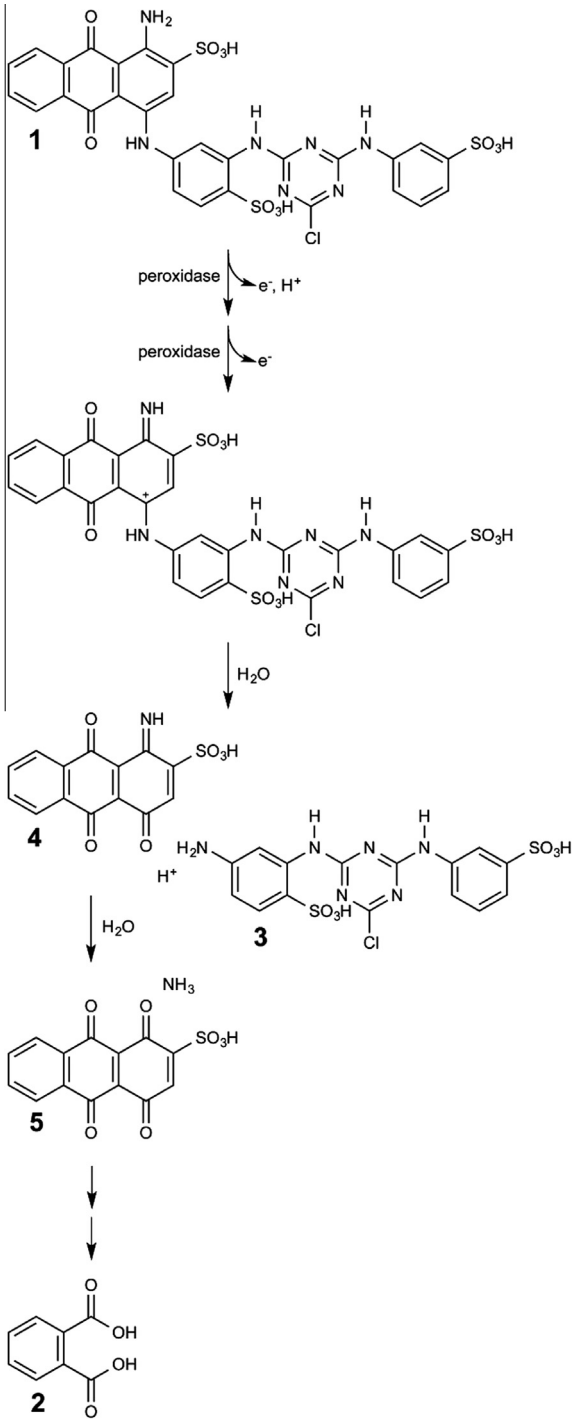

Fig. 6. Abbreviated pathway for the oxidation of Reactive Blue 5 (structure **1**) by a conventional peroxidase mechanism, followed by spontaneous cleavage of the anthracenetetrone (structure **5**) released. See the corresponding text for identity of the different intermediate compounds.

fungi) than in those of wood decay basidiomycetes that degrade lignin inefficiently (brown-rot fungi) [13].

However, it is important to consider another aspect of the problem as well: a peroxidase that cleaves lignin directly must be able to oxidize the non-phenolic alkoxyaromatic structures that predominate in this bulky and recalcitrant polymer. The only exception currently recognized is that some ligninolytic peroxidases may oxidize certain fungal metabolites such as veratryl alcohol to produce aryl cation radical intermediates, which then serve instead of the peroxidase as proximal oxidants of lignin [74,75]. However, a highly oxidizing peroxidase is still required for this proposed mechanism, because veratryl alcohol is also a non-phenolic alkoxyaromatic compound similar in structure to the monomeric subunits of lignin.

Thus far, DyPs do not appear well suited for the above requirement. Although some fungal DyPs oxidized a non-phenolic arylglycerol-β-aryl ether dimeric model compound, which represents the principal structure in lignin, the specific activities found were at most 4% of that exhibited by *Phanerochaete chrysosporium* LiP [44]. These fungal DyPs were also reported to oxidize veratryl alcohol, but with very low activities [10,44]. For example, the k_{cat}/K_m of the most active *Aau*DyP toward veratryl alcohol was reported to be around $1–5 \times 10^2\ M^{-1}\ sec^{-1}$ [10,47], whereas the corresponding values for LiPs on this proposed non-phenolic mediator are on the order of $10^4\ M^{-1}\ sec^{-1}$ [76,77]. Although the estimated redox potential of basidiomycete DyPs is higher than that of generic fungal peroxidases, as defined by Floudas et al. [13], it is still lower than that of basidiomycete LiP [48], resulting in the lower activities found for DyPs on high redox potential substrates. The bacterial DyPs investigated to date appear to be even less oxidizing than the fungal enzymes, and limited to the oxidation of less recalcitrant phenolic lignin models *via* reactions that are stimulated by Mn(II) [26,78,79].

Some attempts have been made to assess the ligninolytic ability of bacterial DyPs on lignocellulose-related substrates. An enzyme from *Rhodococcus jostii*, for example, released colored products from a nitrated milled wheat lignin, and also caused changes in the UV-absorbing material extractable from wheat straw lignocellulose [78]. However, the changes observed were small and it is not yet clear that they resulted from cleavage of lignin structures in these relatively uncharacterized substrates. On the other hand, it was recently reported that a small quantity of a C_α-oxidized G (guaiacyl)-H (*p*-hydroxyphenyl) dimeric product was released from lignocellulose by treatment with *Pseudomonas fluorescens* DyP in the presence of Mn(II) [79]. It was proposed that this product originates from lignin breakdown, although wheat straw lignin has only around 6% H units [80]. The above difficulties in demonstrating extensive ligninolysis by DyP could be addressed by attempting to depolymerize high molecular weight ^{14}C-labeled synthetic lignins with bacterial or fungal DyPs and then analyzing the products for depolymerization by gel permeation chromatography. Alternatively, evidence for depolymerization can be obtained by ^{13}C NMR analysis of ^{13}C-labeled synthetic lignins after peroxidase treatment [81]. A key advantage of synthetic lignins for this application is that they contain no low molecular weight impurities such as non-lignin aromatics that can confound the analysis.

An aspect that has not yet been investigated for DyPs is the possibility that they could oxidize naturally occurring phenols to yield phenoxy radicals, which then might act as diffusible ligninolytic redox mediators, as suggested earlier for basidiomycete laccases [82]. Although this hypothesis requires further study, it is pertinent to note that mediator systems of this type do not, thus far, appear able to cleave nonphenolic lignin structures [83]. On the other hand, DyPs from at least four bacterial species are able to oxidize Mn(II) [26,51,79,84], although with lower catalytic efficiency than MnPs and VPs [50,85]. Nevertheless, the Mn(III) formed by these peroxidases can only oxidize the phenolic units in lignin, which are minor due to the polymerization mechanism. In general, the cleavage of non-phenolic lignin by Mn(II)-oxidizing peroxidases is thought to occur only indirectly *via* peroxidation of unsaturated fungal lipids [86,87]. Therefore, the most likely contribution of DyPs and other low redox potential peroxidases to lignin metabolism is probably in the oxidative degradation of phenolic residues and in the subsequent modification of lignin-derived soil organic matter.

Conclusions

Structurally, basidiomycete (and other) DyPs are part of the CDE superfamily, being phylogenetically unrelated to the catalase–peroxidase superfamily. However, they present functional similarities concerning both the oxidative and reductive half-reactions, as a result of evolved structural convergences. On the oxidative side, these convergences include similar heme pocket architecture with key residues helping heme oxidation by H_2O_2, and also modulating the electron deficiency of the resulting activated cofactor. On the reductive side, the similarities include a strategy like that of basidiomycete ligninolytic peroxidases, which enables DyPs to abstract electrons from bulky substrates at exposed tryptophanyl radicals, and then to transfer them to the heme cofactor *via* LRET pathways. Due to lower redox-potentials (as compared with ligninolytic peroxidases) DyPs seem to be unable to degrade lignin, although they can oxidize low redox potential natural phenolic compounds. Moreover, basidiomycete DyPs can exploit LRET pathways to oxidize dyes of anthropogenic origin *via* typical peroxidase reactions.

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