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DyP-type peroxidases: a promising and versatile class of enzymes

Dana I. Colpa · Marco W. Fraaije · Edwin van Bloois

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Abstract DyP peroxidases comprise a novel superfamily of heme-containing peroxidases, which is unrelated to the superfamilies of plant and animal peroxidases. These enzymes have so far been identified in the genomes of fungi, bacteria, as well as archaea, although their physiological function is still unclear. DyPs are bifunctional enzymes displaying not only oxidative activity but also hydrolytic activity. Moreover, these enzymes are able to oxidize a variety of organic compounds of which some are poorly converted by established peroxidases, including dyes, β -carotene, and aromatic sulfides. Interestingly, accumulating evidence shows that microbial DyP peroxidases play a key role in the degradation of lignin. Owing to their unique properties, these enzymes are potentially interesting for a variety of biocatalytic applications. In this review, we deal with the biochemical and structural features of DyP-type peroxidases as well as their promising biotechnological potential.

Keywords Peroxidase · Heme · Lignin degradation · Dye decolorization · Biocatalysis

Introduction

Peroxidases (EC1.11.1.x) represent a large family of oxidoreductases that typically use hydrogen peroxide as an electron acceptor to catalyze the oxidation of substrate molecules. The vast majority of these enzymes contain heme as

a cofactor [4] and are ubiquitously present in prokaryotes and eukaryotes. Peroxidases take center stage in a variety of biochemical processes, ranging from the biosynthesis of cell wall material to immunological host-defense responses [11, 25]. Heme-containing peroxidases were originally classified into two superfamilies: the plant peroxidases and the animal peroxidases [47]. Remarkably, some members of the peroxidase superfamily have been studied for more than a century like horseradish peroxidase (HRP), a prototype plant peroxidase [46]. In this respect, it was fascinating that the first member of a newly discovered peroxidase superfamily, the group of DyP-type peroxidases, was described in 1999 [16]. In this review, we discuss the biochemical and structural features of DyP-type peroxidases as well as their promising biotechnological potential.

Phylogenetic and structural comparison

Dye-decolorizing (DyP-type) peroxidases were first discovered in fungi and named after their ability to degrade a wide range of dyes [16]. Subsequently, additional members were found in the proteomes of other fungi as well as in several bacteria [40]. This indicates that these enzymes are widespread like other peroxidases. Interestingly, recent genome sequence analysis revealed that these enzymes are prominent in bacteria, whereas only a small number is found in fungi and higher eukaryotes. Their occurrence in archaea is even more limited. The most comprehensive overview of the DyP-type peroxidase superfamily is offered by the Interpro database. According to this database, the DyP superfamily currently comprises 5,019 enzymes of which 4,886 are found in bacteria, 122 in eukaryotes, and 11 in archaea. The growing number of putative DyP-type peroxidases identified in the proteomes of bacteria emphasizes our previous

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Fig. 1 Structural comparison of DyP from *Bjerkandera adusta* Dec1 (a), HRP from *Armoracia rusticana* (b), and human myeloperoxidase (hMPO) from *Homo sapiens* (c). α -helices are shown in green, β sheets are in blue, and the heme cofactor is in red. Close-up of key amino acids in the heme-surrounding region of DyP (d), HRP (e), and human myeloperoxidase (f). The proximal histidine of DyP (His308) and both the distal and proximal histidines of HRP (His42 and

His170) and human myeloperoxidase (His95 and His336) are indicated, as well as catalytically important residues of DyP (e.g., Asp171 and Arg329) and HRP (e.g., Arg38 and Phe41). Heme is covalently bound by Asp94, Glu242, and Met243 in human myeloperoxidase. PDB files used: DyP, 2D3Q, HRP, 1ATJ and hMPO, 1CXP (color figure online)

suggestion that this superfamily should be renamed into the superfamily of bacterial peroxidases [44]. Additionally, DyP-type peroxidases are, according to PeroxiBase, further sub-classified into the phylogenetically distinct classes A, B, C, and D. Many of the potential bacterial enzymes are putative cytoplasmic enzymes (class B and C), indicating that they are involved in intracellular metabolism. In contrast, enzymes belonging to class A contain a Tat-dependent signal sequence, which suggests that they function outside of the cytoplasm or extracellularly as previously confirmed by us and others [15, 39, 44]. Class D contains primarily fungal variants. For some of these peroxidases, it has been shown that they are involved in dye decolorization [40]. Nevertheless, the physiological function of the majority of DyP-type peroxidases is at present unclear, although evidence is accumulating that some bacterial variants are involved in the degradation of lignin [1, 2, 31]. This suggests that these enzymes can be regarded as the bacterial equivalents of the fungal lignin degrading peroxidases.

DyP-type peroxidases are unrelated at the primary sequence level to peroxidases of the plant and animal superfamilies. They also lack the typical heme-binding motif of plant peroxidases, comprising one proximal histidine, one distal histidine, and one crucial arginine (Fig. 1) [25, 40, 46]. Moreover, DyP-type peroxidases and plant peroxidases both bind the heme cofactor non-covalently, unlike animal peroxidases, which bind the heme cofactor covalently [49] (Fig. 1). All DyP-type peroxidases contain the so-called GXXDG motif in their primary sequence, which is part of the heme-binding region. This motif is important for peroxidase activity because replacement of the conserved aspartate by an alanine or asparagine inactivates the enzyme, while heme-binding is not affected [42, 44]. Based on these results, it was proposed that the conserved aspartate of the GXXDG motif is functionally similar to the distal histidine of plant peroxidases [4, 25]. However, the catalytic role of this conserved aspartate was put into question by a recent study. It was shown that substitution of the

aspartate of the GXXDG motif of *E. coli* EfeB/YcdB by an asparagine only marginally affected the peroxidase activity of this enzyme [23].

A limited number of fungal and bacterial DyP-type peroxidases have been characterized in some detail, including elucidation of their crystal structures [5, 23, 30, 37, 42, 50, 51]. While DyP peroxidases from the different subclasses often exhibit a remarkable low sequence similarity, their overall structural topology is highly conserved. Structurally, DyP-type peroxidases comprise two domains that contain α -helices and anti-parallel β -sheets, unlike plant and mammalian peroxidases that are primarily α -helical proteins (Fig. 1) [46, 49]. Both domains in DyP-type peroxidases adopt a unique ferredoxin-like fold and form an active site crevice with the heme cofactor sandwiched in between. The heme-binding motif contains a highly conserved histidine in the C-terminal domain of the enzyme (Fig. 1), which seems to be an important heme ligand and is therefore functionally similar to the proximal histidine of plant peroxidases [23, 42, 50, 51]. To test the role of the proximal histidine of DyP-type peroxidases as a heme ligand, we replaced this residue by an alanine in *TfuDyP* from *Thermobifida fusca*. This resulted in a loss of heme, which demonstrates that this residue is indeed an important heme ligand of DyP-type peroxidases [44]. In addition, fungal DyP peroxidases also contain a conserved histidine in the N-terminal domain of the enzyme, which was previously assigned as heme ligand [41]. However, this residue does not contribute to heme binding according to the available structures [42]. Clearly, more structural studies are required to unveil the molecular details by which DyP peroxidases catalyze oxidations.

Biochemical properties

The biochemical properties of only a few DyP-type peroxidases of fungal and bacterial origin have been analyzed thus far. An overview of the DyP peroxidases characterized thus far is shown in Table 1. These enzymes are typically 50–60 kDa, while several bacterial variants are somewhat smaller (about 40 kDa). All characterized DyP peroxidases contain non-covalently bound heme (proto heme IX) as cofactor [19, 39, 42, 44, 50, 51]. In addition, several oligomeric states have been reported, ranging from monomers to hexamers [16, 23, 24, 39, 44, 50, 51]. It has been well established that the catalytic mechanism of plant and animal peroxidases proceeds via formation of compound I. This is the first (high-oxidation) intermediate in the reaction cycle of peroxidases and is formed by a reaction between H_2O_2 and the Fe(III) resting state of the enzyme. It is therefore generally assumed that this is also the case for DyP-type peroxidases. Although the exact details about

their catalytic cycle are still unclear, several recent studies point towards major differences between the catalytic mechanism of DyP peroxidases and other peroxidases. Based on four novel structures of a fungal DyP, it was proposed that the aspartate of the GXXDG motif swings into a proper position that is optimal for interaction with H_2O_2 , thereby enabling compound I formation [48]. This crucial role of the conserved aspartate as a catalytic residue agrees well with the results of the mutagenesis studies on a fungal and a bacterial DyP as discussed above. However, it is in contrast to, for example, plant peroxidases where the distal histidine functions as an acid–base catalyst and compound I formation is assisted by an essential arginine (Fig. 1) [46]. Furthermore, analysis of the peroxidative cycle of DypB from *Rhodococcus jostii* RHA1 established that its conserved aspartate is not required for peroxidase activity because replacement of this residue by alanine had a marginal effect on the reactivity towards H_2O_2 and the formation of compound I. Rather, a conserved arginine of DypB was found to be essential for peroxidase activity [35]. It therefore appears that DyP-type peroxidases employ different residues as acid–base catalyst(s) during their catalytic cycle.

Remarkably, DyP-type peroxidases are able to oxidize substrates that are too large to fit in the active site. DypB, for instance, shows saturation kinetics towards the large molecules of kraft lignin [30]. Long-range electron transfer (LRET) between the heme cofactor and the surface of DypB was suggested as a potential mechanism. More recently, an LRET pathway to the surface of *AauDyPI* of *Auricularia auricula-judae* was identified [37, 38]. Residues Tyr337 and Leu357 facilitate electron transfer from the heme cofactor of *AauDyPI* to the surface of this fungal DyP, forming a surface-exposed oxidation site that might react with bulky substrates. Tyr337 is conserved in fungal DyPs. A comparable, but not identical, long-range electron pathway is present in lignin peroxidases (LiP) from the plant superfamily of peroxidases (Fig. 2) [9, 37]. For instance, in LiP from *Phanerochaete chrysosporium*, a surface-exposed tryptophan was shown to be the interaction site of veratryl alcohol [12].

The most distinguishing feature of DyP peroxidases is their unparalleled catalytic properties. Firstly, these enzymes are active at low pH, which is most likely dictated by the aspartate of the GXXDG motif that functions as an acid–base catalyst at low pH for at least a subset of DyP peroxidases [42, 44]. Secondly, DyP peroxidases exhibit a unique substrate acceptance profile. These enzymes are able to degrade various dyes efficiently and in particular anthraquinone dyes, which are poorly accepted by plant and animal peroxidases. Furthermore, DyP-type peroxidases display poor activity towards azo dyes and small non-phenolic compounds unlike plant and animal peroxidases

Table 1 DyP-type peroxidases characterized thus far

Subfamily	Protein name	Organism	Crystal structure	References
A	EfeB/YcdB	<i>Escherichia coli</i> O157	(2Y4E with PPIX) (2WX6 apo form)	[23]
	DyPA	<i>Rhodococcus jostii</i> RHA1		[2]
	<i>TfuDyP</i>	<i>Thermobifida fusca</i>		[44]
	<i>BsDyP</i> (YwbN)	<i>Bacillus subtilis</i>		[15, 32]
B	DyPB	<i>Rhodococcus jostii</i> RHA1	3QNR + 3QNS	[2, 30]
	TyrA	<i>Shewanella oneidensis</i>	2IIZ + 2HAG	[50, 51]
	<i>BtDyP</i>	<i>Bacteriodes thetaiotaomicron</i>	2GVK	[51]
	<i>PpDyP</i>	<i>Pseudomonas putida</i>		[34]
	<i>DyPPa</i>	<i>Pseudomonas aeruginosa</i> PKE117		[19]
	DyP2	<i>Amycolatopsis</i> sp. 75iv2	4G2C	[5]
C	AnaPX (<i>AnaDyP</i>)	<i>Anabaena</i> sp. PCC 7120		[24]
	DyP (<i>BadDyP</i> / <i>TcDyP</i>)	<i>Bjerkandera adusta</i> Dec 1 formerly called: <i>Thanatephorus cucumeris</i> Dec 1 and <i>Geotrichum candidum</i> Dec 1	2D3Q	[16, 42]
D	<i>AauDyP</i> I (AjP I)	<i>Auricularia auricula-judae</i>	4AU9	[20, 37]
	MsP1 (<i>MscDyP</i> 1)	<i>Mycetinis scorodonius</i> formerly called: <i>Marasmius scorodonius</i>		[33]
	MsP2 (<i>MscDyP</i> 2)	<i>Mycetinis scorodonius</i> formerly called: <i>Marasmius scorodonius</i>		[33]
	TAP (<i>TalDyP</i>)	<i>Termitomyces albuminosus</i>		[14]
	<i>PoDyP</i>	<i>Pleurotus ostreatus</i>		[13]
Class unknown	<i>EglDyP</i> ^a	<i>Exidia glandulosa</i>		[21]
	<i>MepDyP</i> ^a	<i>Mycena epipterygia</i>		[21]
	<i>AauDyP</i> II (AjP II) ^a	<i>Auricularia auricula-judae</i>		[20]
	<i>I.lacteus DyP</i> ^a	<i>Irpex lacteus</i>		[31]
	<i>BlDyP</i>	<i>Brevibacterium linens</i> M18		[43]
	EfeX ^b	<i>Escherichia coli</i> O157		[10]

^a Potentially class D^b Potentially class B **Fig. 2** Structural comparison of the residues involved in long-range electron transfer of *AauDyP* from *Auricularia auricula-judae* (a) and *LiP* from *Phanerochaete chrysosporium* (b). Residues Tyr337 and Leu357 of *AauDyP* and Trp171 of *LiP* are involved in long-range electron transfer. PDB files used: *AauDyP*, 4AU9 and *LiP*, 1LLP (color figure online)

[2, 7, 8, 16, 23, 24, 28, 29, 36, 39, 44, 50, 51]. Moreover, we have recently established that *TfuDyP* is able to oxidize aromatic sulfides enantioselectively, similar to plant peroxidases, thereby expanding their biocatalytic scope [17, 44–46]. Intriguingly, DyP peroxidases appear to be multifunctional enzymes displaying not only oxidative activity but also hydrolytic activity [33, 40].

Biotechnological potential

Plant peroxidases are attractive biocatalysts because of their broad substrate range, neutral pH optimum, and ability to catalyze reactions such as halogenations, epoxidations, hydroxylations, and enantioselective oxidations, often accompanied with good yields [27]. However, the exploitation of these enzymes is hampered by their notoriously difficult heterologous expression and limited stability. With regards to the latter, it is interesting to note that

DyP peroxidases appear remarkably robust, as shown by us and others [22, 26, 44]. Furthermore, our characterization of *TfuDyP* showed that this enzyme is expressed well heterologously in *E. coli* [44]. Combined, this shows that the bacterial enzymes are a promising alternative for known peroxidases of fungal origin because of the difficulties in fungal genetics and protein expression. The potential of DyP peroxidases as useful biocatalysts for industrial applications is further emphasized by their ability to degrade a variety of synthetic dyes, indicating that these enzymes can be used for the bioremediation of dye-contaminated waste water. Moreover, several recent studies showed that DyP peroxidases are involved in the biodegradation of lignocellulosic material, which is highly resistant to (bio)chemical degradation. For example, DypB from *Rhodococcus jostii* RHA1 showed activity towards polymeric lignin as well as lignin model compounds [2]. Additionally, the hydrolytic degradation of wheat straw was increased by external addition of DyP from *Irpex lacteus* [31]. Together, these studies show that DyP peroxidases act as ligninolytic enzymes, thereby pointing towards a major role of these enzymes in the microbial degradation of lignin [6]. Moreover, it was reported that two fungal DyP-type peroxidases are able to degrade β -carotene [33]. The degradation of β -carotene is of interest for the food industry, enabling the enzymatic whitening of whey-containing foods and beverages. This specific application was patented recently and the respective fungal DyP peroxidase is marketed under the name MaxiBright by DSM. The discovery of novel antimicrobial targets has become a pressing matter due to the vast increase of antibiotic-resistant, pathogenic bacteria [3]. With regards to this issue, it is important to emphasize that, as noted earlier, DyP peroxidases are remarkably abundant in the proteomes of bacteria, including many pathogenic bacteria, while these enzymes are absent in mammals. This indicates that DyP peroxidases could be promising, novel anti-microbial (pro)drug targets. This notion is supported by a recent study that showed that a DyP peroxidase from *Pseudomonas fluorescens* GcM5-1A is toxic to cells of the Japanese black pine [18].

Conclusions and perspectives

The group of DyP-type peroxidases comprises a newly identified superfamily of peroxidases, which are unrelated in sequence and structure to well-known peroxidases belonging to the plant or animal superfamilies. DyP peroxidases exhibit unique reaction features by displaying novel substrate specificities and reactivities. Additionally, DyP peroxidases can be remarkably robust and combined this unveils their potential use as biocatalysts in a variety of biotechnological applications. However, these enzymes are

only active under acidic conditions, which severely restrict their number of applications. It is therefore desirable to alter their pH optimum by enzyme redesign to broaden their applicability. Conceivably, this could be achieved by constructing DyP variants of which the active site more closely resembles that of plant peroxidases because these plant enzymes are highly active at a neutral pH. This can be achieved by, for example, replacing the conserved aspartate of the GXXDG motif with a histidine because the pK_a of the aspartic acid side chain is lower than the pK_a of the histidine side chain. However, a recent study with such variants of DypB from *R. jostii* RHA1 showed that DyP peroxidases are unable to utilize the histidine efficiently as a proton acceptor unlike plant peroxidases [35]. This clearly demonstrates that more rigorous engineering is required to obtain a pH-optimized DyP variant. Despite the promising biocatalytic potential of DyP peroxidases, much more work is needed to fully characterize the catalytic mechanism of DyP peroxidases, their heme biochemistry, as well as the exact role of the catalytic residues and in particular the function of the conserved aspartate. Additional high-resolution structures of DyP peroxidases from all the various subclasses are therefore required, preferably in combination with different ligands. The limited number of DyP peroxidases characterized so far has established that these enzymes exhibit a vastly different substrate scope than plant and animal peroxidases, using, however, a restricted set of diagnostic substrates. It is therefore desirable that more and diverse substrates should be tested in order to fully understand their biocatalytic scope. Lastly, future studies should be aimed at investigating the potential of DyP peroxidases in the biodegradation of lignocellulosic material and as novel microbial (pro)drug targets. In conclusion, it can be expected that the growing number of DyP-type peroxidases biochemically and structurally characterized will fully delineate their biotechnological potential. This will also provide new leads for the construction of improved variants suitable for biotechnological applications.

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