Exploring the biochemical and biocatalytic properties of bacterial DyP-type peroxidases
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Chapter 1:

Introduction and outline of the thesis

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

Dana I. Colpa, Marco W. Fraaije and Edwin van Bloois

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Introduction
Peroxidases (EC 1.11.1.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 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. Heme-containing peroxidases were originally classified into two superfamilies: the plant peroxidases and the animal peroxidases. Remarkably, some members of the peroxidase superfamily have been studied for more than a century like horseradish peroxidase (HRP), a prototype plant peroxidase. 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. In this chapter, 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. Subsequently, additional members were found in the proteomes of other fungi as well as in several bacteria. 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 (February 2018) comprises 12,670 enzymes of which 11,877 are found in bacteria, 741 in eukaryotes, and 52 in archaea. Additionally, DyP-type peroxidases are, according to PeroxiBase, further sub-classified into the phylogenetically distinct classes A, B, C, and D. Alternative classifications

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More recently heme-containing peroxidases were reclassified in four independently evolved superfamilies: the peroxidase-catalase superfamily, the peroxidase-cyclooxygenase superfamily, the peroxidase-chlorite dismutase superfamily and the peroxidase-peroxygenase superfamily. The peroxidase-catalase superfamily is formed by members of the previously called ‘superfamily of bacterial, fungal and plant heme peroxidases’. The ‘superfamily of the animal heme-dependent peroxidases’ contains enzymes from all kingdoms of life and was renamed to the peroxidase-cyclooxygenase superfamily. The peroxidase-chlorite dismutase superfamily consists of three protein families that share a common fold: DyP-type peroxidases, chlorite dismutases and EfeB, previously called the CDE-superfamily. In this chapter the original classification will be used.
with three and five classes have also been proposed.\textsuperscript{10,11} Subfamilies C and D are grouped together in the classification with three classes. An overview of the DyP-type peroxidases characterized thus far is shown in Table 1 and a phylogenetic tree is shown in Figure 1. 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.\textsuperscript{12–14} Class D contains primarily fungal variants. For some of these peroxidases, it has been shown that they are involved in dye decolorization.\textsuperscript{7} 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.\textsuperscript{15–19} This suggests that these enzymes can be regarded as the bacterial equivalents of the fungal lignin degrading peroxidases.

![Phylogenetic tree of the DyP-type peroxidases characterized thus far (February 2018). The sequence alignment and phylogenetic tree were made by Geneious version 8.1.9 using ClustalW alignment with GONNET as cost matrix for the sequence alignment and neighbor-joining as tree build method.](image-url)
Table 1. DyP-type peroxidases characterized thus far (February 2018).

<table>
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<th>Class</th>
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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. 2). Moreover, DyP-type peroxidases and plant peroxidases both bind the heme cofactor non-covalently, unlike animal peroxidases, which bind the heme cofactor covalently (Fig. 2). 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. 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. 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 *Escherichia coli* EfeB/YcdB by an asparagine only marginally affected the peroxidase activity of this enzyme.

A limited number of fungal and bacterial DyP-type peroxidases have been characterized in some detail, including elucidation of their crystal structures, see Table 1. While DyP-type 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. 2). 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. 2), which seems to be an important heme ligand and is therefore functionally similar to the proximal histidine of plant peroxidases. 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. In addition, fungal DyP-type peroxidases also contain a conserved histidine in the N-terminal domain of the enzyme, which was previously assigned as heme ligand. However, this residue does not contribute to heme binding according to the available structures. Clearly, more structural studies are required to unveil the molecular details by which DyP-type peroxidases catalyze oxidations.
**Biochemical properties**

The biochemical properties of about forty DyP-type peroxidases of fungal and bacterial origin have been analyzed thus far. These enzymes are typically 50-60 kDa, while several bacterial variants are somewhat smaller (about 40 kDa). All characterized DyP-type peroxidases contain non-covalently bound heme (protoheme IX) as cofactor\textsuperscript{13,14,28,31,35,43} in addition, several oligomeric states have been reported, ranging from monomers to hexamers.\textsuperscript{6,13,14,23,28,35,38} 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 $\text{H}_2\text{O}_2$ 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-type
peroxidases and other peroxidases. Based on four novel structures of a fungal
DyP, it was proposed that the aspartate of the GXXDG-motif functions as acid-
base catalyst and swings into a proper position that is optimal for interaction
with $\text{H}_2\text{O}_2$.\textsuperscript{54} The aspartate catalyzes compound I formation through the
transfer of a proton from the proximal to the distal oxygen atom of $\text{H}_2\text{O}_2$, hence
facilitating heterolytic cleavage of the O–O bond of $\text{H}_2\text{O}_2$ (Fig. 3). Compound I,
an oxoferryl iron with a porphyrin-based cation radical, reacts consecutively
with two equivalents of substrate to return via compound II (Fe(IV)) back to the
resting state of the heme (compound 0, Fe(III)). 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. 2).\textsuperscript{5} Furthermore, analysis of the peroxidative cycle of DypB from
\textit{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 $\text{H}_2\text{O}_2$ and the formation of compound
I. Rather, a conserved arginine of DypB was found to be essential for peroxidase
activity.\textsuperscript{55} 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.\textsuperscript{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 \textit{AauDyPI}
of \textit{Auricularia auricula-judae} was identified.\textsuperscript{42,56} Residues Tyr337 and Leu357
facilitate electron transfer from the heme cofactor of \textit{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 transfer pathway is present in lignin peroxidases
(LiP) from the plant superfamily of peroxidases (Fig. 4).\textsuperscript{42,57} For instance, in LiP
from \textit{Phanerochaete chrysosporium}, a surface-exposed tryptophan was shown
to be the interaction site of veratryl alcohol.\textsuperscript{58}

The most distinguishing feature of DyP-type 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
**Figure 3.** Schematic representation of the proposed mechanism of DyP-type peroxidases in case substrate AH reacts with the heme directly. Aspartate is shown as acid-base catalyst for the formation of compound I, oxoferryl iron with a porphyrin-based cation radical. Compound I will be reduced in two one-electron reductions back to the resting state, thereby forming two substrate radicals.

**Figure 4.** 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.
functions as an acid-base catalyst at low pH for at least a subset of DyP-type peroxidases. Secondly, DyP-type 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. Moreover, we have relatively recently established that TfuDyP is able to oxidize aromatic sulfides enantioselectively, similar to plant peroxidases, thereby expanding their biocatalytic scope. Intriguingly, DyP-type peroxidases appear to be multifunctional enzymes displaying not only oxidative activity but also hydrolytic activity.

**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. 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-type peroxidases appear remarkable robust, as shown by us and others. Furthermore, our characterization of TfuDyP showed that this enzyme is expressed well heterologously in *E. coli*. 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-type 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-type peroxidases are involved in the biodegradation of lignocellulosic material, which is highly resistant to (bio)chemical degradation. For example, DypB from *Rhodococcus jostii* RHA 1 showed activity towards polymeric lignin as well as lignin model compounds. Additionally, the hydrolytic degradation of wheat straw was increased by external addition of DyP from *Irpex lacteus*. Together, these studies show that DyP-type peroxidases act as ligninolytic enzymes, thereby pointing towards a major role of these enzymes in the microbial degradation of lignin. Moreover, it was reported that two fungal DyP-type peroxidases are able to degrade β-carotene. 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 and the respective fungal DyP-
type 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. With regards to this issue, it is important to emphasize that, as noted earlier, DyP-type peroxidases are remarkably abundant in the proteomes of bacteria, including many pathogenic bacteria, while these enzymes are absent in mammals. This indicates that DyP-type peroxidases could be promising, novel anti-microbial (pro)drug targets. This notion is supported by a recent study that showed that a DyP-type peroxidase from *Pseudomonas fluorescens* GcM5-1A is toxic to cells of the Japanese black pine.

**Conclusions**

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-type peroxidases exhibit unique reaction features by displaying novel substrate specificities and reactivities. Additionally, DyP-type 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. Despite the promising biocatalytic potential of DyP-type peroxidases, much more work is needed to fully characterize the catalytic mechanism of DyP-type 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-type peroxidases from all the various subclasses are therefore required, preferably in combination with different ligands. The limited number of DyP-type 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-type 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.
Aim and outline of the thesis
The research in this thesis was financed by the Netherlands Organization for Scientific Research (NWO) under the graduate program: synthetic biology for advanced metabolic engineering, project number 022.004.006.

The aim of the research presented in this thesis was to broaden the knowledge on class A DyP-type peroxidases. Two DyPs were selected and used as model enzymes: TfuDyP from Thermobifida fusca YX and SviDyP from Saccharomonospora viridis DSM 43017. TfuDyP was isolated and characterized by van Bloois et al as a thermostable, Tat-dependently secreted peroxidase which was easily overexpressed in Escherichia coli. The substrate scope of this enzyme covers dyes and monophenolic compounds while it also shows peroxygenase activity in the enantioselective sulfoxidation of aromatic sulfides. SviDyP is homologous to TfuDyP (42% sequence identity) and has the advantage over TfuDyP that it is active at a slightly higher pH range.

DyP-type peroxidases are named after their ability to degraded recalcitrant dyes. Previous studies focused predominantly on anthraquinone and azo dyes. To study the biotechnological potential of DyP-type peroxidases further, an extensive substrate profiling study was performed with TfuDyP as model enzyme. Chapter 2 presents the activity of TfuDyP on thirty dyes from seven distinct classes, three natural carotenoids and various lignin model compounds.

For their activity DyP-type peroxidases rely on a tightly bound heme cofactor. On the proximal side of the heme a histidine functions as the fifth ligand of the heme iron, while on the distal side two catalytically important residues are found: an aspartate and an arginine. The oxidation site(s) of small and large substrates are presumably different and not fully understood. Some small compounds are known to enter the heme pocket and react with the heme cofactor directly. Large compounds, e.g. bulky dyes and lignin model compounds, are however too large to enter the active site. Chapter 3 describes a mutagenesis study on TfuDyP with the aim to identify more catalytically important residues or residues that determine the pH optimum of the enzyme. This chapter presents the effect of mutations in the heme pocket, in the predicted hydrogen peroxide tunnel and close to the surface exposed heme propionate. Some (DyP-type) peroxidases depend on long-range electron transfer for the activity of substrates that are too large to enter the active site. To explore whether TfuDyP is dependent on such a mechanism, mutagenesis was performed on the surface exposed tyrosines and tryptophans. For the biotechnological applicability of DyP-type peroxidases, it would be beneficial to shift the pH optimum for activity
to a more neutral pH range. Mutations that were proven beneficial in shifting the pH optimum of \textit{PpDyP} and \textit{BsDyP} were studied in \textit{TfuDyP}.\textsuperscript{72,73}

For the industrial applicability of DyP-type peroxidases a high protein overexpression level is desired. Many DyP-type peroxidases are from bacterial origin and are therefore relatively easily heterologously overexpressed in a bacterial host. This is in stark contrast to peroxidases from eukaryotes. Even though \textit{TfuDyP} originates from \textit{T. fusca} and is easily overexpressed in \textit{E. coli}, increasing the overexpression level to 200 mg per liter culture broth led to an almost inactive enzyme. \textbf{Chapter 4} discusses the correlation between the activity of \textit{TfuDyP} and the expression level in \textit{E. coli}. Analysis of the protein by UV-vis absorbance spectroscopy and high-resolution mass spectroscopy on the extracted heme cofactor revealed the reason for the inactivity of the enzyme.

Most enzymes in nature are involved in metabolic pathways in which the product of one enzyme is the substrate of another enzyme. In some cases this even led to the fusion of enzymes to bi/multifunctional protein complexes. Peroxidases and oxidases form catalytically logical combinations and are often coexpressed in nature: peroxidases require hydrogen peroxide for their activity, a by-product of the oxidases. \textbf{Chapter 5} describes the first successful recombinant expression of artificial oxidase-peroxidase fusion enzymes. We fused \textit{SviDyP} to four distinct oxidases: alditol oxidase (HotAldO), chitooligosaccharide oxidase (ChitO), eugenol oxidase (EugO) and 5-hydroxymethylfurfural oxidase (HMFO).\textsuperscript{74–77} Special attention was paid to exploring the potential applicability of the designed oxidase-peroxidase fusions. Two fusion enzymes were used in one-pot two-step cascade reactions while the other two fusion enzymes could be applied as biosensor for the detection of various sugars.
References


DyP-type peroxidases


Chapter 1


