Exploring the biochemical and biocatalytic properties of bacterial DyP-type peroxidases
Colpa, Dana Irene

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2018

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the “Taverne” license. More information can be found on the University of Groningen website: https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment.

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

Download date: 06-06-2024
Chapter 3:

Exploring the catalytic properties of DyP-type peroxidase *TfuDyP* by site-directed mutagenesis

Dana I. Colpa, Thomas Hilberath, Bastiaan de Wit and Marco W. Fraaije
Abstract
In this work we used site-directed mutagenesis to establish the role of various residues in catalysis by *TfuDyP*. Different point mutations in the heme pocket, next to heme propionate and in the predicted hydrogen peroxide tunnel were studied, together with mutations that were designed to shift the pH optimum of *TfuDyP*. Also mutations that might give information on whether the activity of *TfuDyP* is based on long-range electron transfer were analyzed. Mutagenesis of the fifth heme ligand (H338C), the catalytically active aspartate (D242N) and the residue next to this aspartate (N236K) underlined the importance of these residues in catalysis. These variants yielded soluble proteins that bound the heme cofactor but were not active towards Reactive Blue 19. Two other mutations highlighted the importance of the area around the heme propionate and catalytically active arginine on the pH range/optimum for activity of *TfuDyP*. A245R and N246L broadened the pH range for activity towards Reactive Blue 19 by one pH unit. The dependence of *TfuDyP* on long-range electron transfer for the oxidation of bulky substrates was studied by individually mutating the surface exposed tyrosines and tryptophans to phenylalanine. None of the tyrosine or tryptophan mutants lost its activity, indicating that *TfuDyP* is either flexible to use different tyrosines/tryptophans or that *TfuDyP* is not depended on long-range electron transfer for the oxidation of bulky substrates. This site-directed mutagenesis study has provided a better insight in how a DyP-type peroxidase performs catalysis and provides leads for future engineering of this family of biotechnologically interesting peroxidases.
Introduction

The family of DyP-type peroxidases was discovered relatively recently. The first member of this family was discovered two decades ago and named after its ability to degrade dyes: a dye decolorizing peroxidase (DyP). Members of the DyP-type peroxidase family have the advantage over plant/fungal peroxidases that they are mainly found in bacteria and are easily heterologously overexpressed in a bacterial host, e.g. *Escherichia coli*. The family of DyP-type peroxidases is divided in four subclasses by PeroxiBase based on sequence homology. Classes A-C are predominantly formed by bacterial enzymes while members of class D are mainly found in fungi. Class A is formed by Tat-dependently exported DyPs, while classes B and C are intracellular enzymes of which the smallest family members are found in class B. Classifications with three or five subclasses were also reported.

Several structurally and catalytically important residues were identified in DyP-type peroxidases. A histidine is found as the fifth ligand of the non-covalently bound heme cofactor, similar to peroxidases from the peroxidase-catalase superfamily. However, DyP-type peroxidases, which belong to the peroxidase-chlorite dismutase superfamily, differ from the peroxidase-catalase superfamily on the distal side of the heme. For the large family of peroxidase-catalases a histidine is found as acid-base catalyst together with an essential arginine, while for DyP-type peroxidases an aspartate and an arginine are found. The aspartate is part of the highly conserved GXDG-motif and functions (in most DyPs) as acid-base catalyst. The pH optimum for activity of DyPs is in the acidic range and is (partially) defined by this residue. For most DyP-type peroxidases both the aspartate and arginine are important for the activity of the enzyme. However, for DyPB from *Rhodococcus jostii* RHA1 (class B) only the arginine was reported to be essential. The role of these two residues on the activity of DyP-type peroxidases with respect to different substrates is still unclear. For EfeB/YcdB from *Escherichia coli* O157 (class A) it was shown that D235N had a strongly reduced activity towards catechol but was fully active towards guaiacol, while mutants of R347 showed a strongly reduced activity towards both substrates. Mutants of D220 in *TcDyP* from *Thermomonospora curvata* (class A) retained up to 85% of the wild-type activity towards guaiacol, while activity towards ABTS was lost. D142 mutants of YfeX from *Escherichia coli* O157 (class B) showed almost no activity, while mutant R232L lost its activity only towards Reactive Blue 19 and ABTS but was still for 50% or more active towards guaiacol and catechol. This suggests that DyP-type peroxidases oxidize different substrates via different pathways.

The substrate scope of DyP-type peroxidases is diverse and covers...
monophenolic compounds, bulky dyes and lignin model compounds.\textsuperscript{2,20–22} Substrate access to the active site is limited in DyP-type peroxidases. Small molecules, such as hydrogen peroxide, may enter the active site via a narrow channel on the distal side of the heme or potentially via the heme propionate pocket (in case the heme propionate is surface exposed).\textsuperscript{19,23,24} For two DyPs, TfuDyP and engineered AauDyP from Auricularia auricula-judae (class D), peroxygenase activity in the form of enantioselective sulfoxidation of small aromatic sulfides was reported.\textsuperscript{4,25} For enantioselective sulfoxidation substrates need to enter the active site and react with the oxoferryl heme species directly. However, bulky compounds such as dyes and lignin model compounds are too big to enter the active site of a DyP-type peroxidase. These compounds are proposed to react at an oxidation site on the protein surface. Substrate binding sites on the protein surface were observed for various peroxidases. Crystal structures of multiple plant and mammalian peroxidases showed substrate binding at the δ-edge of the heme.\textsuperscript{26} For other enzymes, e.g. manganese and ascorbate peroxidases, enzyme-substrate complexes with substrates bound at the surface exposed heme propionate (γ-edge of the heme) were revealed.\textsuperscript{26–28} And lastly, bulky substrates could be oxidized by a surface exposed radical oxidation site formed via long-range electron transfer (LRET). Tyrosines and tryptophans are redox-active amino acids that are able to propagate and stabilize a radical.\textsuperscript{29,30} LRET-pathways and surface exposed radical sites were observed in lignin and versatile peroxidases.\textsuperscript{31–33} In DyP-type peroxidases two potential surface exposed oxidation sites were noted. DyP from Bjerkandera adusta Dec 1 (class D) was crystalized with ascorbic acid and 2,6-dimethoxyphenol bound to the heme propionate pocket (the γ-edge of the heme) were reported.\textsuperscript{23,24,27,28} Yoshida et al and Roberts et al proposed that the heme propionate pocket might be the oxidation site of larger substrates, in line with ascorbate and manganese peroxidases.\textsuperscript{23,24,27,28} Furthermore, LRET-pathways were reported for three DyP-type peroxidases: AauDyP (class D), TcDyP (class A) and VcDyP from Vibrio cholerae (class B).\textsuperscript{6,29,34}

The aim of this study is to gain a better understanding of the catalytic machinery of DyP-type peroxidase TfuDyP. TfuDyP, a thermostable dimeric class A DyP-type peroxidase from Thermobifida fusca, is active on small phenolic compounds, bulky dyes and displays peroxygenase activity in the enantioselective sulfoxidation of aromatic sulfides.\textsuperscript{4,20} In earlier work we showed the importance of the fifth heme ligand H338 and catalytically active D242 of TfuDyP.\textsuperscript{4} H338 is required for proper heme binding. When this histidine was mutated to an alanine no Soret-band was observed in UV-vis absorbance spectra and the activity towards Reactive Blue 19 was reduced to 3%.\textsuperscript{4} D242 is part of the conserved GXXDG-motif of DyP-type peroxidases and is crucial for the activity of
Exploring the catalytic properties of \textit{TFuDyP} by site-directed mutagenesis

the enzyme. Mutant D242A was able to bind the heme cofactor but the activity towards Reactive Blue 19 was almost fully lost (0.7\% activity).\textsuperscript{4} Besides the catalytic aspartate also an arginine was shown to be essential for the activity of various DyP-type peroxidases.\textsuperscript{6–8,16–19} Rahmanpour et al solved the crystal structure of \textit{TFuDyP} which confirmed the close proximity of the Asp and Arg to the heme cofactor.\textsuperscript{16} In agreement with the work of van Bloois\textsuperscript{4}, Rahmanpour observed a strongly reduced enzyme activity for mutant D242A (D203A in truncated \textit{TFuDyP} used by Rahmanpour). D242A was not active towards phenolic compounds and showed reduced activity towards ABTS compared to the wild-type enzyme. Mutant R354Q (R315Q in truncated \textit{TFuDyP}) was not active towards ABTS nor towards phenolic substrates.

To gain a better understanding of \textit{TFuDyP} the effect of a variety of rationally designed point mutations were studied in this work. First, mutations were made around the heme propionate, in the $\text{H}_2\text{O}_2$-tunnel and in the heme pocket. In the heme pocket the fifth heme ligand, a histidine, was mutated to a cysteine as is found in heme-thiolate enzymes such as peroxigenases and cytochrome P450 monooxygenases.\textsuperscript{35,36} With this mutation we aimed to enhance the peroxygenase activity of \textit{TFuDyP}. Furthermore, mutations were made in the heme pocket with the aim to shift the pH optimum of \textit{TFuDyP} from an acidic to a more neutral pH range. Mutations that were proven beneficial in shifting the pH optimum of \textit{PpDyP} from \textit{Pseudomonas putida} MET94 (class B) were applied on \textit{TFuDyP}.\textsuperscript{8} Lastly we studied the dependence of \textit{TFuDyP} on long-range electron transfer (LRET) for the oxidation of bulky substrates. In order to study this, we mutated the surface exposed tyrosines and tryptophans individually to phenylalanine. Substitution to phenylalanine is a conservative mutation, but Phe is unlike Trp/Tyr not able to stabilize a radical.\textsuperscript{29} We also made a knock-out mutant in which all five tyrosines and seven tryptophans of \textit{TFuDyP} were mutated to phenylalanine.

\section*{Results and discussion}

\subsection*{Enzyme expression and purification}

Wild-type \textit{TFuDyP} and mutants thereof were heterologously overexpressed in \textit{Escherichia coli} TOP10. All enzymes were soluble and purified by His-tag affinity chromatography with yields of 20 to 35 mg enzyme per liter culture broth and a Reinheitszahl (Rz-value) of 1.0-1.5. Most enzymes were brown-red due to the heme cofactor and showed an UV-vis absorbance spectroscopy profile comparable to the wild-type protein, with a protein peak at 280 nm, a Soret band around 410 nm and one or two Q-bands between 450-600 nm depending on the oxidation state of the enzyme (Fig. 1).\textsuperscript{4} However, different features were
observed for mutants H338C and N236K. Purified TfuDyP N236K was green while mutant H338C was yellow. These differences were also visible in UV-vis absorbance spectroscopy (Fig. 1).

![UV-vis absorbance spectroscopy profile of purified enzyme TfuDyP wild-type (solid line) and mutants N236K (dashed line) and H338C (dotted line). The inset shows the color of the enzymes, from left to right: wild-type (brown-red), N236K (green) and H338C (yellow).](image)

**Figure 1.** UV-vis absorbance spectroscopy profile of purified enzyme TfuDyP wild-type (solid line) and mutants N236K (dashed line) and H338C (dotted line). The inset shows the color of the enzymes, from left to right: wild-type (brown-red), N236K (green) and H338C (yellow).

### Mutation of heme ligand H338 to cysteine

TfuDyP is the only native DyP reported to date to perform enantioselective sulfoxidations. Even though the overall conversion was low, TfuDyP was shown to enantioselectively sulfoxidize four aromatic sulfides of which thioanisole (methyl phenyl sulfide) was the best substrate with an enantiomeric excess (ee-value) of 61% for the $R$-sulfoxide. This peroxygenase activity clearly showed that some substrates access the active site of TfuDyP and react with the oxoferryl heme directly, an activity which is typical for peroxygenases and cytochrome P450 monooxygenases. Recently, AauDyP from Auricularia auricula-judae was successfully engineered to perform enantioselective sulfoxidations. The F359G variant of AauDyP gave a 95-99% conversion of thioanisole and methyl-$p$-tolyl sulfide with up to 99% excess of the $S$-products. Peroxygenases and cytochrome P450 monooxygenases are heme-thiolate enzymes, with a proximal cysteine functions as fifth ligand to the heme iron instead of a histidine as is found in DyP-type peroxidases. To enhance the sulfoxidation activity of TfuDyP we
mutated the proximal heme ligand H338 to a cysteine. TfuDyP variant H338C was purified as a soluble enzyme that bound, in contrast to an earlier studied variant H338A, a heme cofactor. The enzyme was however yellow instead of red and showed an UV-vis absorbance spectrum which significantly differed from the wild-type enzyme (Fig. 1). For TfuDyP H338C a broader Soret band, a lower Rz-value (0.4) and no Q-bands were observed. The observed UV-vis spectrum is comparable to the results observed for TcDyP variant H312C, which also showed a broader Soret band and a lower Rz-value when compared to the wild-type enzyme. TcDyP variant H312C not only bound the heme cofactor, it showed some residual activity towards ABTS (0.5%), hydroquinone (HQ, 1.1%) and guaiacol (5.4%). TfuDyP H338C did not show any significant residual activity towards Reactive Blue 19 or in the (enantioselective) sulfoxidation of thioanisole.

**Effect of mutations in the predicted hydrogen peroxide tunnel**

TfuDyP and other DyP-type peroxidases have a narrow tunnel from the surface of the enzyme to the heme iron. We made two mutations in this tunnel to make the tunnel narrower and more hydrophobic. G243 from the highly conserved GXXDG-motif and G356 were mutated to alanine. The modification of G243 to alanine reduced the initial activity towards Reactive Blue 19 at pH 3.5 to 15% of the wild-type activity (Fig. 2). Mutation G356A had a smaller effect on the enzyme: the activity decreased to 53% of the wild-type activity. Remarkably, the sulfoxidation of thioanisole was unaffected by both mutations: both the conversion in two hours and the enantioselectivity were comparable to the wild-type enzyme (Table 1).

**Table 1**: Conversion of thioanisole by different mutants of TfuDyP.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>ee R-product (%)a</th>
<th>Conversion (%)b</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>77</td>
<td>23</td>
</tr>
<tr>
<td>G243A</td>
<td>79</td>
<td>23</td>
</tr>
<tr>
<td>A245R</td>
<td>79</td>
<td>37</td>
</tr>
<tr>
<td>N246L</td>
<td>77</td>
<td>20</td>
</tr>
<tr>
<td>H338C</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>G356A</td>
<td>81</td>
<td>20</td>
</tr>
</tbody>
</table>

[a] The enantiomeric excess (ee) of the R-sulfoxide was calculated by \( ee = (A_R - A_S)/(A_R + A_S) \times 100\%, \) in which the peak areas (A) of the R- and S-sulfoxide products were determined by chiral GC. [b] Conversion of thioanisole (S) after incubation at 30 °C, 100 rpm for two hours. Conversion = \( (1 - ([S]/[S_i])) \times 100\% \).
D242N and N246L, designed to shift the pH optimum of TfuDyP, are also located in the predicted hydrogen peroxide tunnel. Mutant N246L, which forms a hydrogen bond with the backbone of R354 and makes the entrance of the hydrogen peroxide tunnel more hydrophobic, shifted the pH range for activity slightly (Fig. 2). Mutant D242N was inactive.

Effect of mutations in the heme propionate pocket
One possible site for substrate binding and oxidation in TfuDyP is the surface exposed heme propionate pocket (Fig. 3). Substrate binding in such a pocket is well known for various heme peroxidases. Manganese and ascorbate peroxidases were for instance crystallized in complex with their substrates bound to the heme propionate pocket.26–28 Even though DyP-type peroxidases are structurally unrelated to these peroxidases, a surface exposed heme propionate is found in many DyP-type peroxidases.16,23,24,29 To study the importance of this pocket to TfuDyP we mutated an alanine directly next to the surface exposed heme propionate to positively charged arginine, negatively charged glutamate and hydrophobic valine. The initial activity of mutants A245E and A245V towards Reactive Blue 19 at pH 2.5-3.5 decreased slightly to 50-80% of the wild-type activity. Interestingly, variant A245R was active at a broader pH range. The wild-type enzyme is active towards Reactive Blue 19 at pH 2.5-4.0, while mutant A245R is active up to pH 5.0 (Fig. 2). The activity at pH 4.0 increased fourfold and

Figure 2. Initial activity of TfuDyP wild-type and mutants G243A, G356A, A245R and N246L towards Reactive Blue 19 at pH 2.5-5.0.
initial activities of respectively 0.31 and 0.11 s\(^{-1}\) were measured at pH 4.5 and 5.0. The conversion of thioanisole by A245R was increased significantly while the mutant enzyme retained its enantioselectivity (Table 1).

**Figure 3.** Surface exposed heme propionate pocket of *TfuDyP*. Heme and residues D242, A245, H338 and R354 are shown in sticks. The heme cofactor is shown in orange. Heme ligand H338, residue A245 and catalytically active D242 and R354 are shown in green. The active site and the position of A245 are shown on the left while the protein surface is shown on the right. The picture is based on a model created by Yasara, the crystal structure (PDB: 5fw4) is missing a loop in this area.

**Broadening the pH range for activity**

DyP-type peroxidases show a pH optimum for activity in the acidic range.\(^2\text{–}^4,6\text{–}^8\) For the applicability of these enzymes in for instance cascade reactions with other enzymes it would be desirable to shift the pH optimum to a more neutral pH range.\(^3^7\) Shifting the pH optimum through site-directed or random mutagenesis was shown before to be successful for various enzymes.\(^8,3^8\text{–}^4^0\) Mendes et al shifted the pH optimum of *PpDyP* (class B) by four pH units by mutagenesis of the catalytically active aspartate to an asparagine (D132N, GXXDG).\(^8\) The pH optimum of *PpDyP* D132N was shifted from 4.3 to 7.4 for ABTS as substrate. Mutagenesis of N136L, a residue that forms a hydrogen bond with the backbone of the catalytically active arginine, had a smaller effect and shifted the pH optimum to 5.6. Mutagenesis of the arginine (R214L) itself had an opposite effect; it shifted the pH optimum to 3.6. These results are in line with the \(pK_a\) of the side chains of these residues: aspartate and arginine have a \(pK_a\) of respectively 3.9 and 12.5 in water.\(^6,8\) Mutagenesis of the same residues in *BsDyP* (class A) had a smaller effect: D240N shifted the pH optimum for activity on ABTS
Chapter 3

from 3.8 to 4.4.\textsuperscript{41} Brissos et al shifted the pH optimum for activity of \textit{PpDyP} even further by random mutagenesis to a pH of 8.5.\textsuperscript{40}

We aimed to shift the pH optimum for activity of \textit{TfuDyP} to a higher pH range by applying the mutations that were proven beneficial for \textit{PpDyP} and \textit{BsDyP} on \textit{TfuDyP}. The catalytically active aspartate was mutated to an asparagine (D242N) and N246 was mutated to a leucine. N246 forms a hydrogen bond with the catalytically active arginine (R354). The mutations discovered by Brissos et al could not be applied on \textit{TfuDyP}: the sequence identity between \textit{TfuDyP} and \textit{PpDyP} is only 29%. Besides these two mutations we mutated the residue next to the catalytically active aspartate to a lysine to potentially shift the $pK_a$ of this aspartate to a higher range (N236K, $pK_a$ of lysine is 10.5 in water). In contrast to the results obtained for \textit{PpDyP} and \textit{BsDyP}, mutagenesis of the catalytically active aspartate of \textit{TfuDyP} (D242N) resulted in an inactive enzyme.\textsuperscript{8,41} \textit{TfuDyP N246L} broadened the pH range for activity towards Reactive Blue 19 slightly (Fig 2). It showed 51\% of the wild-type activity towards Reactive Blue 19 at pH 3.5. The initial activity at pH 4.0 increased threefold and at pH 4.5 and 5.0 initial activities of 0.93 and 0.11 s\textsuperscript{-1} were measured, respectively. \textit{TfuDyP N236K} showed different features, the enzyme was green, showed an Rz-value of 0.6 and a plateau between 450-700 nm in the UV-vis absorbance spectrum (Fig. 1). The color of mutant N236K might be shifted to green due to a change in the heme environment by the positively charged lysine directly above the heme, or due to a heme modification.\textsuperscript{42–44} \textit{TfuDyP N236K} was not activity towards Reactive Blue 19.

Surface exposed tyrosines and tryptophans

Small (phenolic) substrates can enter the active site of DyP-type peroxidases and react with the oxoferryl heme directly. Direct evidence for such a mechanism is the enantioselective sulfoxidation of thioanisole and related small aromatic sulfides by \textit{TfuDyP} and engineered \textit{AauDyP}.\textsuperscript{4,25} Large substrates, e.g. dyes and lignin model compounds, are however too large to enter the active site. For \textit{AauDyP} (class D), \textit{TcDyP} (class A) and \textit{VcDyP} (class B) long-range electron transfer pathways between the heme and surface exposed tyrosines and tryptophans were demonstrated.\textsuperscript{6,29,34,45} Tyrosines and tryptophans are the only amino acids that can stabilize and propagate a radical.\textsuperscript{29,30} In \textit{AauDyP} and \textit{TcDyP} mainly a tryptophan is used while in \textit{VcDyP} mainly a tyrosine is used. After mutagenesis of these residues, radicals were formed by another surface exposed Tyr/Trp.

We studied the effect of mutagenesis of all surface exposed tyrosines and tryptophans of \textit{TfuDyP}. \textit{TfuDyP} contains in total five tyrosines and seven tryptophans of which four tyrosines and four tryptophans are partially surface
exposed and within 20 Å of the heme cofactor (Fig. 4 and Supporting Information Table S1). These eight residues were individually mutated to phenylalanine. Phenylalanine was chosen because of its resemblance to tyrosine and tryptophan by being an aromatic residue, it is however unable to form a stable radical.²⁹ Individually mutating the eight surface exposed tyrosines and tryptophans that are within 20 Å from the heme had no or only a small effect on the expression level, the heme content and the activity of TfuDyP towards Reactive Blue 19 at pH 3.0 (Fig. 5). From these results it was concluded that individual tyrosines or tryptophans are not solely responsible for the oxidation of large substrates. In case TfuDyP uses long-range electron transfer for the oxidation of bulky substrates, multiple tyrosines/tryptophans are involved and TfuDyP is capable of using a different pathway if one of the Tyr/Trp is knocked-out. These results are in line with the results recorded for AauDyP, VcDyP and TcDyP: knocking out one Tyr/Trp did not completely abolish the activity of these enzymes.⁶,²⁹,³⁴,⁴⁵ A knock-out mutant of TfuDyP in which all twelve tyrosines and tryptophans were substituted with a phenylalanine could not be expressed. Further research is required to study whether the oxidation of bulky substrates by TfuDyP is based on LRET.

Figure 4. Protein structure of TfuDyP visualizing the heme cofactor in red and the two monomeric units in gray and green. The five tyrosines and seven tryptophans of TfuDyP are shown in orange and blue sticks respectively. The picture is based on the crystal structure with pdb code: 5fw4.¹⁶ The numbering in this figure is based on the full protein sequence.
Linde et al compared the tyrosine and tryptophan content of basidiomycete DyPs to other ligninolytic peroxidases (versatile peroxidases (VP) and lignin peroxidases (LiP)) from the same organisms. VPs and LiPs show a low number or even the absence of tyrosines, in contrast to DyPs. Low abundance of tyrosines protects the enzyme from oxidation damage due to the formation and the subsequent (coupling) reactions of tyrosyl radicals. From these results, Linde et al presumed that DyPs function in a less oxidizing environment than VPs or LiPs. LRET-pathways and surface exposed radical sites have however been recorded for lignin and versatile peroxidases. The lower number of Tyr/Trp in VPs and LiPs presumably promotes one specific LRET-pathway on the one hand and protects the enzyme from oxidation damage on the other hand. We compared the tyrosine and tryptophan content of thirty-eight well-studied DyP-type peroxidases from all four subclasses to the average amino acid composition of proteins. The thirty-eight DyPs contained on average 1.2 ± 0.5% tryptophans and 1.9 ± 0.9% tyrosines (Supporting Information Table S2). When compared to the average values recorded for prokaryotic/eukaryotic intra/extracellular proteins (1.01-1.44% tryptophans and 2.60-4.03% tyrosines), DyP-type peroxidases show an average tryptophan content and a slightly lower percentage of tyrosines (Supporting Information Table S3). These results show no indication for a relatively high or low tyrosine or tryptophan content in DyPs which would be expected when LRET-pathways are a main contributor to the catalysis of DyP-type peroxidases.

Conclusions
In this work we studied the effect of seventeen rationally designed point mutations and one 12-fold mutant in TfuDyP (Fig. 6). Mutations were made...
in the heme pocket, in the \( \mathrm{H}_2\mathrm{O}_2 \)-tunnel, next to the heme propionitate, and with the aim to shift the pH optimum of \( \text{TfuDyP} \). Furthermore, mutations were made to study the dependence of \( \text{TfuDyP} \) on LRET for the oxidation of bulky substrates. All designed \( \text{TfuDyP} \) variants were overexpressed as soluble enzymes and bound the heme cofactor. Mutagenesis of the fifth heme ligand (H338C), of the catalytically active aspartate (D242N) and of the residue next to this aspartate (N236K) had a great impact on enzyme activity. Although these mutant enzymes still bound the heme cofactor, the heme environment was significantly changed and these mutants were inactive towards Reactive Blue 19. The usually brown-red color of \( \text{TfuDyP} \) was shifted to yellow and green for H338C and N236K, respectively. Variant H338C was designed to increase the peroxygenase activity of \( \text{TfuDyP} \) as it was inspired on peroxygenases and cytochrome P450 monooxygenases in which a cysteine is found as the fifth heme ligand.\textsuperscript{35,36} \( \text{TfuDyP} \) H338C did not show any significant activity towards Reactive Blue 19 or thioanisole. To increase the peroxygenase activity of \( \text{TfuDyP} \) in future work, mutations that increased the peroxygenase activity of \( \text{AauDyP} \) could be introduced in \( \text{TfuDyP} \), or the predicted hydrogen peroxide tunnel could be broadened to give the aromatic substrates a better access to the heme iron.

**Figure 6.** Protein structure of \( \text{TfuDyP} \) in which the heme cofactor is shown in sticks and the \( \mathrm{C} \alpha \) atoms of the mutated residues are shown in spheres. Heme ligand H338A is shown as a red sphere, mutations N236K, D242N, G243A, A245(E/V/R), N246L, G356A are shown in cyan. All five tyrosines (yellow spheres) and seven tryptophans (blue spheres) were mutated to phenylalanine in the 12-fold mutant; eight of these mutations were also studied individually. The figure is based on the crystal structure with pdb code: 5fw4, chain A.\textsuperscript{16}
In order to shift the pH-optimum of _TfuDyP_ to a more neutral pH range, mutations that were proven beneficial in shifting the pH-optimum of _PpDyP_ and _BsDyP_ were introduced in _TfuDyP_. However, the mutation that shifted the pH-optimum of _PpDyP_ by four pH units (D132N, in _TfuDyP_ D242N) was not beneficial for _TfuDyP_. _TfuDyP_ D242N was not active towards Reactive Blue 19. Variant N246L, which forms a hydrogen bond with the backbone of the catalytically active arginine, had a small and similar effect on the pH range for activity as observed for _BsDyP_ and _PpDyP_. In _PpDyP_ and _BsDyP_ this mutation shifted the pH optimum slightly, in _TfuDyP_ it broadened the pH range for activity towards Reactive Blue 19 from pH 2.5-4.0 to 2.5-5.0. A similar broadening of the pH range for activity was observed for a mutant of the residue next to N246, A245R which is located in the heme propionate pocket. This area around the heme propionate and close to the catalytically active arginine is important for the pH optimum for activity of _TfuDyP_. In future work, the pH optimum for activity of _TfuDyP_ might be shifted to a more neutral pH range by exploring a library of _TfuDyP_ mutants based on random mutagenesis around the heme cofactor, including the area around the surface exposed heme propionate.

Two mutations were made in the predicted hydrogen peroxide tunnel to analyze the effect of making the tunnel narrower: G243, which is part of the highly conserved GXXDG-motif of DyP-type peroxidases, and G356 were mutated to alanine. The activity of variants G243A and G356A towards Reactive Blue 19 was reduced to respectively 15 and 53% of the wild-type activity at pH 3.5. This result underlines the importance of the conserved motif and/or the presence of a glycine at position 243 in the narrow hydrogen peroxide tunnel of _TfuDyP_. Mutation N246L, which broadened the pH range for activity towards Reactive Blue 19 by one unit, is located in the hydrogen peroxide tunnel and makes this tunnel more hydrophobic. Besides broadening the pH range for activity it reduced the activity at the optimal pH, pH 3.5, to 51%. The enantioselectivity and the total conversion of thioanisole in two hours were not influenced by the mutations in the hydrogen peroxide tunnel. This is remarkable since thioanisole is expected to access the heme pocket via the same tunnel to react with the oxoferryl heme. Another possibility might be that this substrate enters the heme pocket via the heme propionate pocket. Mutations that influence/reduce the activity towards one type of substrates, but not towards another type, were observed before for various DyP-type peroxidases. These results support the hypothesis that different types of substrates get oxidized at different locations in or on the surface of DyP-type peroxidases.

Long-range electron transfer from the protein surface to the heme has been reported for _AauDyP_, _TcDyP_ and _VcDyP_. These enzymes were observed
to be flexible in the used pathway and were able to form radicals on more than one tyrosine/tryptophan. Mutating the surface exposed tyrosines and tryptophans of \textit{TfuDyP} to phenylalanine individually yielded proteins with a similar activity as the wild-type enzyme towards Reactive Blue 19 at pH 3.0. From this it was concluded that, if the activity of \textit{TfuDyP} towards bulky substrates is based on LRET and surface exposed Tyr/Trp radicals, more than one Tyr/Trp could be used by \textit{TfuDyP} as was observed for \textit{AauDyP}, \textit{TcDyP} and \textit{VcDyP}.\textsuperscript{6,29,34} Unfortunately, a “knock-out” \textit{TfuDyP} in which all five tyrosines and seven tryptophans were exchanged for a phenylalanine did not express. Another possibility for the activity towards bulky substrates might be that the activity of \textit{TfuDyP} is not based on surface exposed radicals formed by LRET, but that bulky substrates get oxidized in the heme propionate pocket. Binding of ascorbic acid and 2,6-dimethoxyphenol to this pocket was also observed in the crystal structure of DyP from \textit{Bjerkandera adusta} Dec 1.\textsuperscript{23} To study the oxidation site(s) for bulky substrates further, more Tyr/Trp knock-outs could be studied. Furthermore it would be very interesting to co-crystalize DyP-type peroxidases from all four subclasses with a variety of substrates.

\textbf{Materials and methods}

\textbf{Chemicals, reagents and enzymes}

Oligonucleotide primers were obtained from Sigma-Aldrich. Restriction enzyme \textit{DpnI} was obtained from New England Biolabs. Media components, reagents and salts were obtained from Merck, Sigma, Aldrich, BD and Fisher Scientific. Reaction Blue 19 was obtained from Acros Organics.

\textbf{Site directed mutagenesis}

Plasmid pBAD/Myc-His A-\textit{TfuDyP} was used for the overexpression of \textit{TfuDyP}.\textsuperscript{4} Point mutations (A245E, A245R, A245V, D242N, G243A, G356A, H338C, N236K, N246L, Y276F, Y357F, Y359F, Y403F, W179F, W215F, W289F, W363F) were made by site directed mutagenesis using \textit{PfuUltra} (I/II) hotstart PCR master mix (Agilent technologies). The twelve fold mutant of \textit{TfuDyP}, in which all five tyrosines and seven tryptophans were mutated to a phenylalanine, was ordered as gene fragment from GenScript. The native \textit{TfuDyP} gene in plasmid pBAD/Myc-His A-\textit{TfuDyP} was exchanged with the twelve fold mutant by In-Fusion cloning (ClonTech). The obtained plasmids were used to transform \textit{Escherichia coli} strain TOP10.

\textbf{Protein expression and purification}

\textit{TfuDyP} (mutants) were expressed and purified as described before, with minor
changes. In short, 100 mL cultures were grown in Terrific Broth (TB) medium and induced with 0.02% L-arabinose at an OD$_{600}$ ~ 0.6. All cultures were supplemented with 50 μg/mL ampicillin. Cells were harvested by centrifugation 3000 x g, for 30 min at 4 °C (Eppendorf centrifuge 5810R). Pellets were resuspended in buffer A (50 mM potassium phosphate, 500 mM NaCl, 5% glycerol, pH 8.0) supplemented with cOmplete EDTA-free protease inhibitor cocktail (Roche). After breaking the cells by sonication, the enzymes were purified from the cell-free extract using Ni-NTA agarose (Qiagen) or a 1-mL His-Trap HP column (GE Healthcare). Columns were washed with Buffer A and Buffer A supplemented with 20 mM imidazole. Enzymes were eluted with Buffer A containing 300 mM imidazole. The buffer was exchanged to Buffer B (50 mM potassium phosphate, 150 mM NaCl, 10% glycerol, pH 7.5) using an Econo-Pac 10DG desalting column (BioRad). Purified enzymes were concentrated, flash frozen in liquid nitrogen and stored at -20 °C.

**UV-vis absorption spectroscopy**
The purified enzymes were analyzed by UV-vis absorption spectroscopy (V-660 spectrophotometer, Jasco) as described before. Absorbance spectra were recorded between 250-800 nm at ambient temperature. The predicted molecular extinction coefficients at 280 nm were used to calculate the protein concentrations (ProtParam tool, Expasy): $TfuDyP$, ε$_{280 \text{ nm}}$ = 45,950 M$^{-1}$ cm$^{-1}$; $TfuDyP$ with mutation Tyr to Phe, ε$_{280 \text{ nm}}$ = 44,460 M$^{-1}$ cm$^{-1}$ and $TfuDyP$ with mutation Trp to Phe, ε$_{280 \text{ nm}}$ = 40,450 M$^{-1}$ cm$^{-1}$. Absorbance spectra of the oxidized and reduced enzymes were taken after the addition of 100 μM H$_2$O$_2$ or a few grains of sodium dithionite, respectively.

**Activity towards Reactive Blue 19**
Activity towards Reactive Blue 19 was measured as described before. In short, oxidation of Reactive Blue 19 was followed at 595 nm at ambient temperature (V-660 spectrophotometer, Jasco). Reaction mixtures contained 25 μM Reactive Blue 19, 100 μM H$_2$O$_2$ and 20 nM enzyme in a citric acid/Na$_2$HPO$_4$ buffer of pH 2.5-5.5.

**Peroxygenase activity: sulfoxidation of thioanisole**
Sulfoxidation of thioanisole was studied as described before with minor changes. A 250 mM stock of thioanisole was prepared in ethanol. Reaction mixtures of 1 mL contained 2.5 mM thioanisole, 1.0 mM H$_2$O$_2$ and 2.0 μM enzyme in a 25 mM citrate buffer of pH 3.5. Reactions were incubated in 4 mL glass vials at 30 °C, 100 rpm for two hours (Innova 44 incubator shaker, New Brunswick). Samples
of 0.5 mL were taken after two hours. Substrate and products were extracted and analyzed by chiral GC as described before. In short, the substrates and products were extracted by 0.5 mL tert-butyl methyl ether containing 0.1% mesitylene as internal standard. Samples were vortexed for one minute with the extracting solvent and centrifuged at 16,000 x g for two minutes to improve phase separation. The organic fractions were collected and the reaction mixtures were extracted for a second time by the same procedure. The organic fractions were combined and dried over anhydrous magnesium sulfate, followed by centrifugation at 16,000 x g for two minutes. The supernatant was analyzed by chiral GC (6890 GC system, Hewlett Packard) and a Chiraldex G-TA column (30 m x 0.25 mm x 0.125 mm, Grace Alltech). Program: (1) 35 ºC for 1 min, (2) gradient of 35 to 170 ºC with increment of 10 ºC/min, (3) 170 ºC for 8 minutes and (4) 170 to 35 ºC at 10 ºC/min. With an inlet temperature of 180 ºC, inlet volume of 2 μL and a split ratio of 1:20.

Acknowledgement
This work was supported by the NWO graduate program: synthetic biology for advanced metabolic engineering, project number 022.004.006, The Netherlands.
References


Exploring the catalytic properties of TfuDyP by site-directed mutagenesis


Supporting information

Table S1. Location and orientation of tyrosine and tryptophan residues in TfuDyP.

<table>
<thead>
<tr>
<th>Residue</th>
<th>Distance to heme (Å)</th>
<th>Location and orientation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y276</td>
<td>14.4</td>
<td>Surface exposed -OH</td>
</tr>
<tr>
<td>Y357</td>
<td>13.0</td>
<td>Backbone surface exposed, in a tunnel in dimer interface</td>
</tr>
<tr>
<td>Y359</td>
<td>9.7</td>
<td>Backbone surface exposed, in a tunnel in dimer interface</td>
</tr>
<tr>
<td>Y403</td>
<td>9.2</td>
<td>Surface exposed -OH</td>
</tr>
<tr>
<td>W179</td>
<td>16.3</td>
<td>Surface exposed -NH</td>
</tr>
<tr>
<td>W215</td>
<td>15.1</td>
<td>Backbone and part of the indole are surface exposed, NH-group faces inside</td>
</tr>
<tr>
<td>W289</td>
<td>8.3</td>
<td>Surface exposed -NH</td>
</tr>
<tr>
<td>W363</td>
<td>17.2</td>
<td>Surface exposed -NH</td>
</tr>
<tr>
<td>Y422</td>
<td>30.6</td>
<td>Surface exposed -OH</td>
</tr>
<tr>
<td>W111</td>
<td>26.2</td>
<td>Internal</td>
</tr>
<tr>
<td>W270</td>
<td>22.0</td>
<td>Backbone and part of the indole are surface exposed, NH-group faces inside</td>
</tr>
<tr>
<td>W378</td>
<td>12.0</td>
<td>Internal</td>
</tr>
</tbody>
</table>

Tyr/Trp that were not mutated in this study

<table>
<thead>
<tr>
<th>Residue</th>
<th>Distance to heme (Å)</th>
<th>Location and orientation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Lower the number by 39 to obtain the amino acid number of the same residues in the truncated version of TfuDyP used by Rahmanpour et al, PDB entry 5FW4.16.

Table S2. Tyrosine and tryptophan content of DyP-type peroxidases. Sequences of members from class A and D are in the mature form, without the predicted (TAT) signal sequences predicted by the TatP 1.0 and the SignalP 4.1 servers.54,55
<table>
<thead>
<tr>
<th>Class</th>
<th>DyP</th>
<th>PDB</th>
<th>Organism</th>
<th>Residue (#)</th>
<th>W (#)</th>
<th>Y (#)</th>
<th>W (%)</th>
<th>Y (%)</th>
<th>W + Y (%)</th>
<th>UniProtKB accession code</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>BsDp</td>
<td>5MJH</td>
<td>Bacillus subtilis</td>
<td>372 4 10</td>
<td>1.1</td>
<td>2.7</td>
<td>3.8</td>
<td>P39597</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>DtpA</td>
<td>2GVK</td>
<td>Streptomyces lividans TK24</td>
<td>386 6 4</td>
<td>1.6</td>
<td>1.0</td>
<td>2.6</td>
<td>A0A076MAJ9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>DyP</td>
<td>3QNR</td>
<td>Pseudomonas fluorescens Pf-5</td>
<td>396 4 6</td>
<td>1.0</td>
<td>1.5</td>
<td>2.5</td>
<td>Q4KB1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>DyP</td>
<td>5FW4</td>
<td>Rhodococcus jostii RHA1</td>
<td>379 6 6</td>
<td>1.6</td>
<td>1.6</td>
<td>3.2</td>
<td>Q0S4I5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>EfeB</td>
<td>2Y4F</td>
<td>Escherichia coli O157</td>
<td>388 4 10</td>
<td>1.0</td>
<td>2.6</td>
<td>3.6</td>
<td>P31545</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>SvDp</td>
<td>5JXU</td>
<td>Thermonospora curvata</td>
<td>369 7 3</td>
<td>1.9</td>
<td>0.8</td>
<td>2.7</td>
<td>D1A807</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TfuDp</td>
<td>5FW4</td>
<td>Thermobifida fusca</td>
<td>396 7 5</td>
<td>1.8</td>
<td>1.3</td>
<td>3.0</td>
<td>Q4KB1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(ScoDp)</td>
<td>4GT2</td>
<td>Streptomyces coelicolor ATCC BAA-471</td>
<td>386 6 4</td>
<td>1.6</td>
<td>1.0</td>
<td>2.6</td>
<td>Q9ZBW9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(TceDp)</td>
<td>4GS1</td>
<td>Thermobifida cellulosityica</td>
<td>393 7 5</td>
<td>1.8</td>
<td>1.3</td>
<td>3.1</td>
<td>U3KRF5</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Average class A 1.54 ± 0.40 1.46 ± 0.67 3.00 ± 0.43

<table>
<thead>
<tr>
<th>Class</th>
<th>DyP</th>
<th>PDB</th>
<th>Organism</th>
<th>Residue (#)</th>
<th>W (#)</th>
<th>Y (#)</th>
<th>W (%)</th>
<th>Y (%)</th>
<th>W + Y (%)</th>
<th>UniProtKB accession code</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>BDp</td>
<td>2GVK</td>
<td>Bacteroides thetaiotaomicron</td>
<td>316 2 10</td>
<td>0.6</td>
<td>3.2</td>
<td>3.8</td>
<td>Q8A8E8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>BdDp</td>
<td>3QNR</td>
<td>Dictyostelium discoideum</td>
<td>306 2 10</td>
<td>0.7</td>
<td>3.3</td>
<td>3.9</td>
<td>Q556V8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>DyB</td>
<td>5FW4</td>
<td>Rhodococcus jostii RHA1</td>
<td>350 2 8</td>
<td>0.6</td>
<td>2.3</td>
<td>2.9</td>
<td>Q0SE24</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>DyPb</td>
<td>295 6 4</td>
<td>Pseudomonas fluorescens Pf-5</td>
<td>324 3 6</td>
<td>0.9</td>
<td>1.9</td>
<td>2.8</td>
<td>Q4KB97</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>DyPa</td>
<td>299 6 7</td>
<td>Pseudomonas aeruginosa PKE117</td>
<td>335 2 5</td>
<td>0.6</td>
<td>1.5</td>
<td>2.1</td>
<td>I6Y4U9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Eldyp</td>
<td>5VJ0</td>
<td>Enterobacter lignolyticus</td>
<td>299 4 9</td>
<td>1.3</td>
<td>3.0</td>
<td>4.3</td>
<td>E3G9I4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mdyp</td>
<td>335 2 5</td>
<td>Mycobacterium tuberculosis H37Rv</td>
<td>287 5 5</td>
<td>1.7</td>
<td>1.7</td>
<td>3.5</td>
<td>Q8BH5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pypdyp</td>
<td>311 2 12</td>
<td>Pseudomonas putida</td>
<td>302 2 9</td>
<td>0.7</td>
<td>3.0</td>
<td>3.6</td>
<td>Q9KQ59</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>YfeX</td>
<td>5GT2</td>
<td>Escherichia coli O157:H7 str. Sakai</td>
<td>299 4 7</td>
<td>1.3</td>
<td>2.3</td>
<td>3.7</td>
<td>P76536</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Average class B 1.03 ± 0.53 2.46 ± 0.73 3.49 ± 0.79
<table>
<thead>
<tr>
<th>Class</th>
<th>DyP</th>
<th>PDB</th>
<th>Organism</th>
<th>Residue (#)</th>
<th>W (%)</th>
<th>Y (%)</th>
<th>W + Y (%)</th>
<th>UniProtKB accession code</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>AnaPX</td>
<td>5C2I</td>
<td><em>Anabaena</em> sp. PCC 7120</td>
<td>469</td>
<td>7</td>
<td>13</td>
<td>1.5</td>
<td>2.8</td>
<td>4.3</td>
</tr>
<tr>
<td>DyP2</td>
<td>4G2C</td>
<td><em>Amycolatopsis</em> sp. 75iv2</td>
<td>464</td>
<td>5</td>
<td>9</td>
<td>1.1</td>
<td>1.9</td>
<td>3.0</td>
<td>K7N5M8</td>
</tr>
<tr>
<td>SaDyP2</td>
<td></td>
<td><em>Streptomyces avermitilis</em></td>
<td>456</td>
<td>7</td>
<td>10</td>
<td>1.5</td>
<td>2.2</td>
<td>3.7</td>
<td>Q82HB1</td>
</tr>
</tbody>
</table>

Average class C: 1.37 ± 0.25, 2.30 ± 0.43, 3.67 ± 0.63

| D     | AauDyP | 4AU9 | *Auricularia auricula-judae* | 487 | 4 | 7 | 0.8 | 1.4 | 2.3 | I2DBY1 | [34,71] |
|       | DyP    | 2D3Q | *Bjerkandera adusta Dec 1* | 476 | 5 | 6 | 1.1 | 1.3 | 2.3 | Q8WZK8 | [2,12] |
|       | EgIDyP |  | *Exidia glandulosa* | 480 | 4 | 6 | 0.8 | 1.3 | 2.1 | I2DBY2 | [72] |
|       | Ftr-DyP |  | *Funalia trogii (Coriolopsis trogii)* | 484 | 5 | 5 | 1.0 | 1.0 | 2.1 | GenBank: AUW34346.1 | [73] |
|       | GI-DyP |  | *Ganoderma lucidum* | 488 | 5 | 5 | 1.0 | 1.0 | 2.0 | G0X8C9 | [74] |
|       | l. lacteus DyP |  | *Irpex lacteus* | 447 | 6 | 7 | 1.3 | 1.6 | 2.9 | A0A1R7T0P5 | [75] |
|       | MepDyP |  | *Mycena epipterygia* | 505 | 5 | 4 | 1.0 | 0.8 | 1.8 | I2DBY3 | [72] |
|       | Msp1   |  | *Mycetinis scorodonius* | 493 | 4 | 3 | 0.8 | 0.6 | 1.4 | BO8K71 | [76] |
|       | Msp2   |  | *Mycetinis scorodonius* | 491 | 4 | 4 | 0.8 | 0.8 | 1.6 | BO8K72 | [76] |
|       | PoDyP  |  | *Pleuratus ostreatus* | 506 | 9 | 15 | 1.8 | 3.0 | 4.7 | Q0VTV1 | [77] |
|       | PsaDyP |  | *Pleurotus sapidus* | 497 | 9 | 15 | 1.8 | 3.0 | 4.8 | A0A0F7VJ89 | [78] |
|       | TAP    |  | *Termitomyces albuminosus* | 482 | 5 | 11 | 1.0 | 2.3 | 3.3 | Q8NKF3 | [79] |

Average class D: 1.11 ± 0.35, 1.50 ± 0.82, 2.62 ± 1.14

Average class A-D: 1.22 ± 0.50, 1.88 ± 0.89, 3.10 ± 1.02

Exploring the catalytic properties of TfuDyP by site-directed mutagenesis
Table S3. Tyrosine and tryptophan content of prokaryotic and eukaryotic intra/extracellular proteins.

<table>
<thead>
<tr>
<th>Protein origin/location</th>
<th>W (%)</th>
<th>Y (%)</th>
<th>Literature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prokaryote</td>
<td>1.35</td>
<td>2.60</td>
<td>[49]</td>
</tr>
<tr>
<td>Eukaryote</td>
<td>1.14</td>
<td>2.91</td>
<td></td>
</tr>
<tr>
<td>Prokaryote, intracellular</td>
<td>1.11</td>
<td>2.80</td>
<td>[47]</td>
</tr>
<tr>
<td>Prokaryote, extracellular</td>
<td>1.44</td>
<td>4.03</td>
<td></td>
</tr>
<tr>
<td>Eukaryote, intracellular</td>
<td>1.01</td>
<td>3.16</td>
<td></td>
</tr>
<tr>
<td>Eukaryote, extracellular</td>
<td>1.43</td>
<td>3.50</td>
<td></td>
</tr>
<tr>
<td>Intracellular</td>
<td>1.2 ± 0.82</td>
<td>3.1 ± 1.49</td>
<td>[48]</td>
</tr>
<tr>
<td>Extracellular</td>
<td>1.4 ± 1.3</td>
<td>3.6 ± 2.02</td>
<td></td>
</tr>
</tbody>
</table>