An integrated view of redox and catalytic properties of B-type PpDyP from *Pseudomonas putida* MET94 and its distal variants

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**A B S T R A C T**

PpDyP from *Pseudomonas putida* MET94 is an extremely versatile B-type dye-decolourising peroxidase (DyP) capable of efficient oxidation of a wide range of anthraquinonic and azo dyes, phenolic substrates, the non-phenolic veratryl alcohol and even manganese and ferrous ions. In reaction with H₂O₂ it forms a stable Compound I at a rate of (1.4 ± 0.3) × 10⁷ M⁻¹ s⁻¹, comparable to those of classical peroxidases and other DyPs. We provide the first report of standard redox potential (E°) of the Compound I/Native redox couple in a DyP-type peroxidase. The value of E° (Native) = 1.10 ± 0.04 (V) is similar to those found in peroxidases from the mammalian superfamily but higher than in peroxidases from the plant superfamily. Site-directed mutagenesis has been used to investigate the role of conserved distal residues, i.e. to replace aspartate 132 by asparagine, and arginine 214 and asparagine 136 by leucine. The structural, redox and catalytic properties of variants are addressed by spectroscopic, electrochemical and kinetic measurements. Our data point to the importance of the distal arginine in the catalytic mechanism of PpDyP, as also observed in DyPB from *Rhodococcus jostii* RHA1 but not in DyPs from the A and D subfamilies. This work reinforces the idea of existence of mechanistic variations among members of the different sub-families of DyPs with direct implications for their enzymatic properties and potential for biotechnological applications.

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**Introduction**

Dye-decolourising peroxidases (DyPs) are novel haem peroxidases, prominent in bacteria, that have primary sequence, structural and apparently mechanistic features unrelated to those of other known peroxidases [1–3]. Haem peroxidases oxidize a wide range of structurally different substrates, including indoles, phenols, aromatic amines, lignin, manganese, halide ions and other proteins such as cytochrome c [4]. On the basis of sequence similarity, the super-family of fungal, plant and bacterial peroxidases has been classified into: Class I (intracellular prokaryotic peroxidases); Class II (secretory fungal peroxidases involved in the degradation of lignin) and Class III (secretory peroxidases from algae and plants known to participate in a variety of processes from germination to senescence and protection against pathogens) [1]. The peroxidase-cytochrome superfamily evolved independently. It consists of soluble mammalian peroxidases that are effective antimicrobial oxidants and take part in the innate immune system [5]. DyPs are classified into 4 phylogenetically distinct classes; class A contains bacterial enzymes with a Tat-dependent signal sequence, which indicates that they function outside the cytoplasm, in the periplasm of Gram (−) bacteria or even extracellularly, as is the case of *Bacillus subtilis* BsDyP [6]. Classes B and C comprise bacterial (putative) cytoplasmic enzymes presumably involved in intracellular metabolism, while fungal DyPs belong to Class D. DyPs successfully degrade a wide range of substrates including high-redox potential anthraquinone-based and azo dyes, and non-phenolic lignin units in the absence of redox mediators. The physiological function of the majority of DyPs is at present unclear. Emerging evidence indicates that these enzymes can be regarded as the bacterial equivalents of fungal lignin-degrading peroxidases [7–11]. This has an enormous biotechnological potential since lignin is the most abundant aromatic polymer in Nature, the second most abundant raw material next to cellulose [11] and a key renewable source of bulk and fine-chemicals.
The midpoint redox potential ($E^0$) of the Fe$^{3+}$/Fe$^{2+}$ redox couple in haem peroxidases can vary over a very wide range (−270 mV for HRP to +29 mV for myeloperoxidase) and it is likely that peroxidases have reduction potentials that are specific for their physiological catalytic activity [4]. Peroxidases are considered to be the enzymes with the highest oxidation potential found in nature, according to the standard redox potential of the reaction: $\text{H}_2\text{O}_2 + 2\text{H}^+ + 2e^- \rightarrow 2\text{H}_2\text{O}$ ($E^0$ = 1.32 V) [12] and this is another property of these enzymes which is of interest for biotechnological applications. Understanding the molecular factors that modulate the redox potentials of haem peroxidases allows them to be engineered for increased electron transfer efficiency, enhanced substrate specificity and the ability to oxidize artificial substrates. Site directed mutagenesis is a powerful tool for probing protein matrix residues that affect and fine-tune redox properties of haem peroxidases. At the same time site-directed mutagenesis has proved to be the most useful approach for identifying the catalytic determinants of enzyme activity. It is generally accepted that an acid-base mechanism underlies the formation of the catalytic intermediate Compound I in haem peroxidases and the roles of distal pocket residues in this process have been the subject of intensive research. In classical peroxidases the conserved distal histidine (replaced by aspartate in DyPs) is the acid-base catalyst that promotes the heterolytic cleavage of hydrogen peroxide while other residues, such as the distal arginine and asparagine, participate in a range of roles [13]. Recent studies point toward major differences between the proposed catalytic mechanism of DyPs and other peroxidases, in particular in the role of the conserved distal residues of the haem pocket [11,14–16].

Recently, we have characterized two recombinant bacterial DyPs belonging to subfamilies A and B, BsDyP from B. subtilis and PpDyP from Pseudomonas putida MET94, respectively [17–19]. We demonstrated that these DyPs have distinct active site structures, (i.e. haem coordination), redox properties, substrate specificity and thermal and chemical stability, and thus have different potential for biotechnological applications. Here we provide details on the formation of catalytic intermediates of PpDyP from P. putida MET94 using fast kinetics and the redox potential of the catalytic determinants of enzyme activity. It is generally accepted that an acid-base mechanism underlies the formation of the catalytic intermediate Compound I in haem peroxidases and the roles of distal pocket residues in this process have been the subject of intensive research. In classical peroxidases the conserved distal histidine (replaced by aspartate in DyPs) is the acid-base catalyst that promotes the heterolytic cleavage of hydrogen peroxide while other residues, such as the distal arginine and asparagine, participate in a range of roles [13]. Recent studies point toward major differences between the proposed catalytic mechanism of DyPs and other peroxidases, in particular in the role of the conserved distal residues of the haem pocket [11,14–16].

One of the major techniques employed site directed mutagenesis to investigate the role of conserved distal residues (Fig. 1) in the peroxidative cycle of PpDyP, and used spectroscopic, kinetic and electrochemical approaches to characterize the variants of PpDyP.

Materials and methods

Stopped-flow experiments

The transient kinetics of the reaction between wild type (WT) PpDyP [17] and hydrogen peroxide ($\text{H}_2\text{O}_2$) were studied by mixing protein solutions with $\text{H}_2\text{O}_2$ at pH 7 in 0.1 M sodium phosphate buffer in a Hi-Tech SF-61DX2 stopped-flow apparatus coupled to a diode array for signal detection. A circulating water bath was used to maintain the temperature of the reactant syringes and the mixing cell at 25 °C. Typically, 2 µM protein solutions (final concentration) were mixed with $\text{H}_2\text{O}_2$ solutions of different concentrations (0–50 µM final concentration) and UV–visible spectra (350–700 nm) were recorded on different time scales (usually 0.75 s and 7.5 s). The concentration of the protein solutions was determined from the absorbance at $\lambda = 280$ nm using the extinction coefficient $\varepsilon_{280nm} = 34.850$ M$^{-1}$ cm$^{-1}$, calculated from the protein sequence using the ExPASy Bioinformatics Resource Portal (http://web.expasy.org) [20]. The $\text{H}_2\text{O}_2$ solutions were prepared from successive dilutions of a stock $\text{H}_2\text{O}_2$ solution, the concentration of which was determined from the absorbance measured at $\lambda = 240$ nm ($\varepsilon_{240nm} = 39.4$ M$^{-1}$ cm$^{-1}$) [21].

Analysis of transient kinetic data

Time dependent spectra obtained for the reaction between PpDyP and hydrogen peroxide display good isosbestic points, indicating that there are no intermediates involved. Thus, the reaction can be described by Scheme 1, where N is the PpDyP native species and Cpd I represents Compound I.

For the determination of the reduction potential of the couple $E^0_{\text{Cpd I/N}}$ the time dependent spectra obtained when the reaction appeared to have reached equilibrium were deconvoluted, using reference spectra for N and for Cpd I species to obtain the mole fraction of each species. The reference spectrum for N was calculated as the average of all spectra acquired after mixing PpDyP with buffer. The reference spectrum for Cpd I was calculated as the average of the last few spectra obtained at the longest times with a large excess of hydrogen peroxide present, assuming that under these conditions the reaction is effectively complete. It is important to note that the definition of $E^0_{\text{Cpd I/N}}$ necessarily assumes that the reaction is thermodynamically reversible.

The time dependence of the concentrations of N and Cpd I, for each $\text{H}_2\text{O}_2$ concentration, was fitted to the kinetic model presented in Scheme 1 using Berkeley Madonna software package (version 8.3.18 for Windows, Kagi Shareware, Berkeley, CA) to obtain the values of the rate constants $k_{1f}$ and $k_{1r}$. Both forward and reverse rate constants are needed to fit the data obtained because the reactions reach equilibria with some native protein still present.

The Gibbs free energy of the reaction can be related to the difference of the reduction potentials of the electron acceptor pair ($E^0_{\text{H}_2\text{O}_2}$) and the electron donor pair ($E^0_{\text{Cpd I/N}}$), according to Eq. (1):

$$\Delta G^0 = -nF(E^0_{\text{H}_2\text{O}_2} - E^0_{\text{Cpd I/N}})$$

(1)

where $F$ is the Faraday constant and $n$ is the number of electrons transferred in the reaction ($n = 2$). Since the reduction potential of

$$\text{N} + \text{H}_2\text{O}_2 \xrightarrow{k_{1f}} \text{Cpd I} \xrightarrow{k_{1r}}$$

Scheme 1. Kinetic model of the reaction of PpDyP with $\text{H}_2\text{O}_2$ at pH 7.
the pair H₂O₂/H₂O at pH 7 is known (E°(H₂O₂/H₂O) = 1.32 V) [12], it is possible to calculate the reduction potential of the couple Cpd I/N using Eq. (2), which is derived from Eq. (1) and from the relation between the Gibbs free energy and the equilibrium constant:

\[ E_{\text{red}}^{\text{Cpd I/N}} = E_{\text{red}}^{\text{H₂O₂/H₂O}} - \frac{RT}{2F} \ln K_{\text{eq}} \]  

(2)

The equilibrium constant may be obtained directly from the ratio of the concentrations of the reagents after the system had effectively reached equilibrium (Eq. (3)), as described by Furtmüller et al. [22,23]:

\[ K_{\text{eq}} = \frac{[\text{Cpd I}]_{\text{eq}}}{([\text{H₂O₂}]-[\text{Cpd I}]_{\text{eq}}) ([\text{N}] - [\text{Cpd I}]_{\text{eq}})} \]  

(3)

where [Cpd I]eq is the concentration of Cpd I, obtained from spectral deconvolution, and [N] and [H₂O₂] are the concentrations of PpDyP and hydrogen peroxide used in the experiment. Alternatively, even if the reaction does not reach equilibrium, the ratio of the fitted rate constants (Eq. (4)) can be obtained:

\[ K_{\text{eq}} = \frac{k_{\text{f}}}{k_{\text{r}}} \]  

(4)

Construction, overproduction and purification of PpDyP variants

Single amino acid substitutions were created from the QuickChange site-directed mutagenesis kit (Stratagene). Plasmid pRC-1 (containing the WT PpDyP sequence) was used as template [17]. The primers, forward 5’GAAGACGGCCAGCAGACTGCGGACAGCAAGAGGCGGACGGAGAGGAGGCGGTCTCGGGGTCCCTGGTCTTCC’ were used to create the N136L mutation; forward 5’GGAGTACCGCGATCTCCGCGGCGGAGAGGAGGAGGCGACGGAGAGGAGGACGAGGTGCGGTTCTGGTCTTCC’ were used to create the R214L mutation. The presence of the desired mutations in the resulting plasmids and the absence of unwanted mutations in other regions of the insert were confirmed by DNA sequence analysis. The plasmids containing the ppDyP genes with the desired mutations were transformed into Escherichia coli Tuner strain, in which the recombinant PpDyP variants were produced under the control of the T7lac promoter. Cell growth, disruption and protein purification were undertaken as previously described [17]. The protein concentration was determined by the Bradford assay with bovine serum albumin as standard. Purified enzymes were stored at −20 °C until use.

Static light scattering

Static light scattering was measured using a Cary Eclipse spectrophotometer at 360 nm as excitation and emission wavelengths. WT enzyme aggregation was monitored at concentrations between 0.01 and 2 μM in sodium acetate buffer, pH 4.3. Aggregation of 2 μM wild-type and variants was monitored at pH values between 3 and 8 in Britton–Robinson buffer (100 mM phosphoric acid, 100 mM boric acid, and 100 mM acetic acid mixed with NaOH to the desired pH in the range of 2–10). The pl values were predicted using the ExpASy Bioinformatics Resource Portal (http://web.expasy.org) [20].

Spectroscopic analysis

The UV–visible absorption spectra of purified enzymes were recorded on a Nicolet Evolution 300 spectrophotometer (Thermo Industries, Waltham, MA, USA). The Reinheitszahl values were determined by the ratio between absorbance at the wavelength of the Soret band and the absorbance at 280 nm (A280nm/A420nm). The haem content was determined by the pyridine ferro-ferrihaemochrome method using an extinction coefficient of pyridine haemochrome (R) – extinction coefficient of pyridine hemichrome (O) (c=O) of 28.32 mM⁻¹ cm⁻¹ at 556 nm [24]. Room temperature resonance Raman (RR) experiments were carried out in a rotating cuvette (from Hellma) containing ~80 μL of 10–50 μM sample (50 mM Tris–HCl, pH 7.6) to prevent prolonged exposure of individual enzyme molecules to laser irradiation. A confocal microscope, equipped with an Olympus 20× objective lens (working distance of 21 μm, numeric aperture of 0.35) was used for laser focusing onto the sample and light collection in a backscattering geometry. The microscope was coupled to a Raman spectrometer (Jobin Yvon U1000), equipped with 1200 l/mm grating and a liquid nitrogen cooled CCD detector. The 413 nm line from a Kr⁺ laser (Coherent Innova 302) was used as the excitation source. The laser power was set to 7 mW; 10 spectra, measured with 60 s accumulation times were co-added in each measurement. All spectra were subjected to polynomial baseline subtraction and component analysis as described previously, using in-house software [19,20].

Cyclic voltammetry

Electrochemical measurements were carried out in a three-electrode cell containing a working pyrolytic graphite electrode (basal plane), a platinum wire counter electrode and an Ag/AgCl reference electrode. The supporting electrolyte, 0.1 M KCl in 50 mM Tris–HCl pH 7.6 was thoroughly purged with argon before each experiment. The cyclic voltammetry measurements were performed using a Princeton Applied Research 263A potentiostat. The potential was cycled between 0 and −0.7 V vs. Ag/AgCl at scan rates between 20 and 150 mV·s⁻¹. Prior to each experiment, the working electrode was polished with alumina slurry (0.3 μm particle size), thoroughly washed with water, ultrasonicated for 5 min and dried with compressed air. A small volume of the protein solution (~10 μL of 5–10 μM protein) was entrapped between the electrode surface and a dialysis membrane (MWCO 3000, Spectra/Por), which was fixed around the electrode body by a rubber o-ring [25].

Steady-state kinetic assays

The enzymatic activity of PpDyP and variants was monitored using either a Nicolet Evolution 300 spectrophotometer (Thermo Industries, Madison, USA), or a Synergy2 microplate reader (BioTek, Vermont, USA). All enzymatic measurements were performed at least in triplicate. The pH dependence was measured by monitoring the oxidation of 1 mM 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS) at 420 nm (ε420nm = 36,000 M⁻¹ cm⁻¹) in the presence of 0.2 mM H₂O₂ at 25 °C in Britton–Robinson buffer. The apparent steady-state kinetic parameters for H₂O₂ (0.0125–2.25 mM for WT, 0.005–15 mM for D132N, 0.005–12 mM for R214L and 0.005–5 mM for N136L) were performed at 25 °C, in 100 mM sodium acetate or phosphate buffer at the optimal pH (4.3 for WT, 7.4 for D132N, 3.6 for R214L and 5.6 for N136L) using ABTS as reducing substrate (10 mM for WT and 1 mM for D132N, R214L and N136L) and adequate amounts of the respective enzymes. The apparent steady-state kinetic parameters were measured for ABTS (0.1–14 mM for WT and 0.005–5 mM for D132N, R214L and N136L with 0.2 mM H₂O₂ for WT, 20 mM H₂O₂ for D132N, 6 mM H₂O₂ for R214L and 4 mM H₂O₂ for N136L) at the optimal pH for each variant and adequate amounts of the respective enzyme. Initial rates were obtained from the slope of the tangent to the initial data points. The apparent kinetic parameters kcat and km were then fitted directly using the Michaelis–Menten equation (Origin-Lab software, Northampton, MA, USA).
The kinetic parameters for H2O2 reduction using the wild-type enzyme were obtained using the equation \( v = \frac{V_{\text{max}}}{1 + K_m/[S]} + \frac{[S]/K_i}{1 + [S]/K_i} \) (Origin-Lab software, Northampton, MA, USA).

**Results and discussion**

We have shown in previous work that PpDyP has the ability to oxidize a large range of substrates with high efficiency, e.g. anthraquinonic and azo dyes, phenolic compounds, such as guaiacol, syringaldehyde or acetoxychryzone, and high redox potential non-phenolic veratryl alcohol, also showing reasonable activity towards manganese and ferrous ions [17]. In the reaction with H2O2 it forms a stable Cpd I [17]. In this work we aim to gain detailed insights into the Cpd I formation and its redox properties, and investigate the effect of mutations of conserved distal residues on the structural, catalytic and kinetic properties of PpDyP.

**Stopped-flow kinetic data and standard reduction potential of Compound I/Native PpDyP couple**

The available information on the redox properties of Cpd I in haem peroxidases is still fairly limited due to its high reactivity. In particular, there are no reports on \( E_{\text{red}} \) in DyPs. In order to fill this gap, the transient kinetic parameters of PpDyP and hydrogen peroxide were measured at pH 7.0 using the stopped flow apparatus. Upon addition of increasing concentrations of H2O2 (0.5-50 \( \mu \)M) to 2 \( \mu \)M ferric PpDyP, the intensity of the Soret band decreased with no apparent shifts in the wavelength maximum or Q and CT bands (Fig. 2A), which is indicative of Cpd I formation [3]. These spectral characteristics and the presence of isosbestic points reveal that native PpDyP is directly converted into Cpd I upon reaction with H2O2. After deconvolution of the time dependent spectra using the reference spectra of N and Cpd I species (vide supra, Section “Materials and methods”) the respective molar fractions were converted into concentrations and plotted as a function of time (Fig. 2B). The second order rate constant for Cpd I formation was calculated by fitting the time dependence of the concentrations of N and Cpd I to the kinetic model in Scheme 1. Equilibrium constants \( (K_{eq}) \) were calculated using Eq. (3) with the equilibrium concentrations of [Cpd I]/[N] obtained from spectral deconvolution (data in Table 1) and also from the ratio of forward and reverse rate constants (data in Supplementary materials) using Eq. (4). The \( E_{\text{red}} \) were then calculated according to the Eq. (2). The value of \( E_{\text{red}} \) was found to be 1.10 ± 0.04 V, with the two methods for obtaining \( K_{eq} \) in good agreement. Determination of \( E_{\text{red}} \) is facilitated by the straightforward reaction of PpDyP with H2O2 which stops at Cpd I, which is reasonably stable. The value determined here for PpDyP is comparable to the standard redox potential of Compound II/Fe3+ (generally assumed to be in the same range as Cpd I/N) recently determined for several fungal DyPs, which fall between 1.10 and 1.20 V [26]. It is also similar to the \( E_{\text{red}} \) measured in mammalian proteins belonging to the peroxidase–cyclooxygenase superfamily such as eosinophil peroxidase (1.10 V) [27], human myeloperoxidase (1.16 V) [22,27] and lactoperoxidase (1.09) [23]. When compared with peroxidases from the plant superfamily, e.g. APX [28], ARP [29], HRP [4], or CCP [30], PpDyP presents around 0.20 V higher redox potential for the couple Cpd I/N. Since Cpd I participates directly in the substrate oxidation reaction, the high \( E_{\text{red}} \) value of PpDyP is in accordance with its ability to oxidize veratryl alcohol to veratryl aldehyde (1.36 V), Mn2+ to Mn3+ (1.51 V), or high redox potential synthetic dyes [17]. PpDyP shows aggregation in a concentration and pH-dependent manner (Fig. 3) which prevents measuring Cpd I formation at lower pH values, in particular at 4.3, the optimal pH for activity [17]. Temperature-dependent aggregation was also previously

**Table 1**

<table>
<thead>
<tr>
<th>H2O2 (( \mu )M)</th>
<th>Native PpDyP (( \mu )M)</th>
<th>Compound I (( \mu )M)</th>
<th>( K_{eq} )</th>
<th>( E^{\circ} ) (Compound I/Fe3+) (V)</th>
</tr>
</thead>
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<tr>
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<tr>
<td>1.5</td>
<td>0.44</td>
<td>1.36</td>
<td>2.30 x 10^5</td>
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<tr>
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<td>2.73 x 10^5</td>
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<td>6.72 x 10^5</td>
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</tr>
<tr>
<td>50</td>
<td>0</td>
<td>1.80</td>
<td>5.56 x 10^5</td>
<td>1.03</td>
</tr>
</tbody>
</table>

**Fig. 2.** Stopped-flow analysis of the reaction of PpDyP with H2O2 at pH 7.0. (A) Electronic absorption spectral changes upon mixing of 2 \( \mu \)M ferric PpDyP with 5 \( \mu \)M hydrogen peroxide in the stopped-flow apparatus. The arrow shows the direction of the absorbance change with time. The first spectrum was measured 0.000375 s after mixing, with subsequent spectra at 0.023, 0.053, 0.083, 0.123, 0.183, 0.363 and 0.753 s. The reaction was carried out in 100 mM phosphate buffer, pH 7 at 25 °C. (B) Time evolution of the concentration of the native enzyme (filled symbols) and Compound I (open symbols), and simulations (solid lines), obtained by using the software Berkeley Madonna after deconvolution of the time dependent spectra.
reported for PpDyP [17]. Nevertheless, the standard redox potential determined here allows comparison with the $E_{1/2}$ values reported in the literature for other peroxidases at this neutral pH.

Biochemical and UV–Vis characterization of PpDyP mutants

Replacement of Asp132 by asparagine and Asn136 and Arg214 by leucine (Fig. 1) resulted in variants showing a similar chromatographic pattern during purification as the WT PpDyP. The Reinheitszahl values are ~1.5 for WT and D132N, ~1.6 for N136L, and ~1.0 for R214L. The haem $b$ content in the variants was ~1 mol of haem per mole of protein, which is similar to the WT (Table 2). The overall electronic absorption features of D132N and N136L variants (Fig. 4B and D) were assessed by UV–Vis spectroscopy at pH 7 (Fig. 3). Characteristic Soret bands are at ~400 nm, Q bands, at ~500 and ~540 nm and a CT band at ~640 nm, as observed in the WT enzyme (Fig. 4A). Small differences in the spectra indicate subtle perturbations of the active sites. The R214L variant shows the Soret band height reduced by ~60% and red-shifted to 414 nm, upshifted Q bands (537 and 557 nm) and largely attenuated CT bands which are all indicative of predominantly low spin configuration of the protein [11] (Fig. 4C). The nature of the 6th ligand is not clear at this point. The formation of an intermediate with spectral features of Cpd I (decrease in intensity of the Soret band, disappearance of Q bands and appearance of a band at 600 nm) was observed in all variants upon addition of 1–3 equivalents of hydrogen peroxide (Fig. 4A). Small differences in the spectra indicate subtle perturbations of the active sites. Under the tested conditions Cpd I showed much longer lifetime in the D132N and N136L variants with $t_{1/2}$ of ~77 and 105 h, respectively in comparison with that of the WT enzyme ($t_{1/2} = 1.8$ h). In the R214L variant, spectral changes 1 h after addition of $H_2O_2$ are consistent with protein degradation (Fig. 4C). The Cpd I half-lives obtained at pH 7 for PpDyP WT and variants are much higher than those reported for analogous variants of DyPB WT from R. jostii RHA1 ($t_{1/2}$ of ~540 s and ~0.13 s at low pHs close to the optimal for enzymatic activity [11]), indicating a remarkably stable enzyme/radical species. PpDyP variants show aggregation at a concentration and pH-dependent manner (results not shown and Fig. 3B) as in the case of the WT, which prevents measuring Cpd I formation at lower pH values.

Resonance Raman spectroscopy

In order to provide a site for hydrogen peroxide binding, the active site of resting peroxidases typically has either a vacant sixth axial position, giving a 5-coordinated ferric haem iron, or it carries a loosely bound water molecule, yielding a 6-coordinated high spin (6cHS) configuration. RR spectroscopy is recognized as a powerful approach for characterization of heterogeneous spin population in haem peroxidases [31,32]. Except for our previous work [19,20], the configuration that the haem iron in DyP-type peroxidases adopt in solution has been only addressed by UV–Vis absorption spectroscopy, which is insufficiently sensitive to fully characterize haem configurations. As in classical peroxidases, the active site of DyPs adopt several configurations in the resting state, including high spin (HS) and low spin (LS), and in some cases the quantum mechanically admixed spin state (QS), which was previously observed only in the class III plant secretory peroxidases and catalase-peroxidases [18,19,31–34]; the spin population distribution is sensitive to pH, temperature, and solution vs. crystal state [31–34].

As in the case of the WT, in which we observed 6cHS, 5cHS, 6cLS and 5cQS spin states, the high frequency region of the RR spectra of the D132N and N136L variants indicates co-existence of several spin populations in the resting enzymes (note that the R214L mutant was not sufficiently stable to allow for measurements of RR spectra) (Fig. 5). Mutations of Asp132 and Asn136 have a substantial effect on the geometry of the distal side of the active site. This is particularly evident from the $v_{13}/v_{12}$ spin and coordination state marker band region, and the $v_{10}/v_{6}$ region. They are composed of several overlapping bands with different relative ratios in D132N and N136L (Fig. 5). The component analysis of the spectra of the variants allowed us to identify individual species in the RR spectra (Table S1) using the parameters (band frequencies and widths) defined previously for the WT PpDyP and the 6cLS PpDyP-imidazole complex [18,19]. They can be attributed, as in the WT, to 5cQS, 6cHS, 5cHS and 6cLS populations. The relative abundance of these species, determined from the relative band intensity ratios of the well separated $v_{3}$ modes (Fig. 5), is distinctly different among N136L, D132N and the WT (Table 3). While in N136L, the amount of 5cHS, 6cHS and 6cLS species is approximately the same (~30% each), the most abundant species in D132N is the 6cLS (42%), followed by 5cHS (32%) and less populated 6cHS and QS. On the contrary, under the same experimental conditions, at pH 7.6 and RT, the 5cQS species was found to be

Table 2

<table>
<thead>
<tr>
<th>Biochemical and spectroscopic characteristics of WT PpDyP and distal variants.</th>
<th>$\lambda_{\text{max}}$ (nm)</th>
<th>$e$ (mM$^{-1}$ cm$^{-1}$)</th>
<th>Haem content</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>406</td>
<td>40</td>
<td>1.0 ± 0.02</td>
</tr>
<tr>
<td>D132N</td>
<td>408</td>
<td>39</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>R214L</td>
<td>414</td>
<td>6</td>
<td>0.9 ± 0.03</td>
</tr>
<tr>
<td>N136L</td>
<td>409</td>
<td>39</td>
<td>1.0 ± 0.04</td>
</tr>
</tbody>
</table>
largely predominant in the WT PpDyP. A high abundance of 6cLS species in the case of D132N, with the 6th axial coordination site of the haem iron occupied, likely has an impact on H2O2 binding, as also suggested by the $K_m$ (measured at pH 7.4 (Table 5)).

Redox properties of the Fe3+/Fe2+ couple in PpDyP variants

The effect of the distal mutations on midpoint redox potential of the Fe3+/Fe2+ couple ($E_{\text{m}0}^{\text{Fe}2+/\text{Fe}3+}$) was assessed by cyclic voltammetry (Table 4). All mutations result in a significantly higher redox potential than that of the WT ($E_{\text{m}0}^{\text{Fe}2+/\text{Fe}3+}$ = −260 mV). Note that the redox potential of the R214L variant was obtained from a single CV scan, due to the irreversibility of the electrode process related to instability of this variant, also observed by UV–Vis and RR spectroscopies (vide supra). The replacement of arginine and asparagine residues with the nonpolar leucine group increases $E_{\text{m}0}^{\text{Fe}2+/\text{Fe}3+}$ significantly, by 140 and 160 mV, in R214L and N136L, respectively (Table 4), consistent with the destabilization of the Fe3+ haem in a less polar environment. Similarly, replacement of the distal arginine with leucine (R48L) in CCP resulted in an upshifted $E_{\text{m}0}^{\text{Fe}2+/\text{Fe}3+}$ [35]. The substitution of the distal aspartate by the polar but uncharged asparagine resulted in an increase of the redox potential of D132N by 150 mV (Table 4), which was related with an increased accessibility to solvent [4]. Molecular factors that affect haem reduction potentials include the nature of axial haem ligands, electrostatics of the local haem environment, and solvent exposure of the haem group; these factors contribute to the resulting redox potential to different extents in different proteins [4]. The distal Asp, Asn and Arg residues participate in an extensive network of hydrogen bonds connecting the distal and proximal haem sites and influence the polarity of the haem environment in DyPs. At this point and in the absence of data on other DyPs, we can speculate that electrostatics seems to be the dominant factor that modulates $E_{\text{m}0}^{\text{Fe}2+/\text{Fe}3+}$ in PpDyP. This view is supported by the highly flexible haem cavity (vide supra), pointing to the importance of electrostatics and the H-bond network of the second coordination sphere of the active site.

Steady-state kinetic characterization of PpDyP variants

The pH-dependence of activity could be measured for PpDyP WT and variants since the enzyme concentrations used in these experiments are far lower than those that lead to protein aggregation. The effect of pH on the activity of variants (Fig. 6) reveals that D132N and N136L show maximal activity at pH 7.4 and 5.6, an increase compared with the WT ($pH_{\text{opt}} = 4.3$), while R214L variant shows a reduction, with an optimum at pH 3.6. In addition to the effect of the mutations on the optimal pH of the enzymes, the widths of the bell-shaped pH profiles of D132N and N136L variants of PpDyP are surprisingly large (span over 5 pH units). Although the explanation of these variations in the pH profile of PpDyP...
variants is unclear, it is noteworthy that in the absence of Asp the optimal pH shifts toward the pK_a value of Arg (12.48), while when Arg is missing an opposite shift is observed, towards the pK_a value of Asp (3.9), indicating a likely cooperation of these two residues in the acid-base mechanism underlying the hydrogen peroxide binding and splitting in the haem pocket of PpDyP.

The catalytic properties of the variants were then determined at the optimal pH using H_2O_2 and ABTS as substrates (Tables 5 and 6 and Figs. 1S and 2S). The replacement of distal Asp, Arg and Asn resulted in variants showing significantly higher Km values than the WT for hydrogen peroxide and elimination of the hydrogen peroxide inhibition observed in the WT (K_i = 0.4 mM, [17], Table 5 and Fig. 1S). The R214L variant shows a 200-fold lower k_cat value than the WT. However, both D132N and N136L variants show 2-fold higher k_cat values than the WT. The resulting k_cat/K_m values reveal similar efficiency of N136L for H_2O_2 to that of the WT, which is 1- to 3-orders of magnitude lower for the D132N and R214L variants. Altogether, these results indicate that both distal Arg and Asp are important in defining an appropriate geometry for hydrogen peroxide binding to the haem, whereas Arg exerts an

![Fig. 5. High frequency region of resonance Raman spectra of ferric D132N and N136L variants and wild type PpDyP (inset). The spectra of 50 μM proteins (50 mM Tris–HCl, pH 7.6) were measured with 413 nm excitation and 7 mW laser power at RT. The experimental spectra of the variants are shown together with their component spectra and the overall fit; the component bands of f_1 region, representing 6cHS, 5cHS, QS and 6cLS populations, are designated.

Table 3
Relative abundance of 5-coordinated high-spin, 5cHS, 6-coordinated high-spin, 6cHS, 6-coordinated low-spin, 6cLS and 5-coordinated quantum mechanically admixed spin state, 5cQS, populations in PpDyP (wild type) and D132N and N136L variants, calculated from the relative contribution of each group, defined in the component analysis of the experimental spectra (31). Resonance Raman (RR) experiments were carried out at RT using ~80 μL of 10–50 μM sample (50 mM Tris–HCl, pH 7.6).

<table>
<thead>
<tr>
<th>%</th>
<th>WT</th>
<th>D132N</th>
<th>R214L</th>
<th>N136L</th>
</tr>
</thead>
<tbody>
<tr>
<td>5cHS</td>
<td>29</td>
<td>32</td>
<td>nd</td>
<td>31</td>
</tr>
<tr>
<td>6cHS</td>
<td>6</td>
<td>10</td>
<td>nd</td>
<td>32</td>
</tr>
<tr>
<td>6cLS</td>
<td>2</td>
<td>42</td>
<td>nd</td>
<td>29</td>
</tr>
<tr>
<td>5cQS</td>
<td>63</td>
<td>16</td>
<td>nd</td>
<td>8</td>
</tr>
</tbody>
</table>

* [18]. nd – not detected.

Table 4
Midpoint redox potentials of PpDyP and its distal variants (vs. SHE). The cyclic voltammetry measurements were performed using ~10 μL of 5–10 μM protein in 50 mM Tris–HCl, pH 7.6.

<table>
<thead>
<tr>
<th></th>
<th>E_0^Fe^{+++}/Fe^{++} (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>-260 ± 10</td>
</tr>
<tr>
<td>D132N</td>
<td>-110 ± 10</td>
</tr>
<tr>
<td>R214L</td>
<td>-120 ± 20</td>
</tr>
<tr>
<td>N136L</td>
<td>-100 ± 10</td>
</tr>
</tbody>
</table>

* [19].

Table 5
Apparent steady-state catalytic parameters of PpDyP and distal variants for hydrogen peroxide. Reactions were performed with H_2O_2 (0.0125–2.25 mM for WT, 0.005–15 mM for D132N, 0.005–12 mM for R214L and 0.005–5 mM for N136L), at 25 °C, in 100 mM sodium acetate or phosphate buffer at the optimal pH for each enzyme (4.3 for WT, 7.4 for D132N, 3.6 for R214L and 5.6 for N136L) using ABTS as reducing substrate (10 mM for WT and 1 mM for D132N, R214L and N136L, Fig. 1S).

<table>
<thead>
<tr>
<th></th>
<th>Km (μM)</th>
<th>k_cat (s^-1)</th>
<th>k_cat/K_m (M^-1 s^-1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H_2O_2 (ABTS)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>79 ± 5</td>
<td>13.7 ± 0.4</td>
<td>2 x 10^5</td>
</tr>
<tr>
<td>D132N</td>
<td>1998 ± 219</td>
<td>25 ± 2</td>
<td>1 x 10^4</td>
</tr>
<tr>
<td>R214L</td>
<td>615 ± 106</td>
<td>0.065 ± 0.001</td>
<td>1 x 10^2</td>
</tr>
<tr>
<td>N136L</td>
<td>334 ± 31</td>
<td>26 ± 1</td>
<td>1 x 10^3</td>
</tr>
</tbody>
</table>

* [17].
essential role in the cleavage of the O−O bond. These results are in line with, but they are not as dramatic as, those reported for the subfamily B DyP from *Rhodococcus jostii* RHA1, in which substitution of distal Arg with Leu leads to the absence of reactivity towards H$_2$O$_2$ [11]. One hypothesis is that the conditions used to measure the catalytic parameters in the latter study were far from the optimal for the Arg/Leu variant, since, as shown here, the optimal pH for the WT is not necessarily identical to those of the variant enzymes. In contrast, in DyPs from subfamilies A or D, such as EfeB/YcdB from *E. coli* [16], TfuDyP from *Thermobifida fusca* [14] and *Bjerkandera adusta* Dec1D [15], distal Asp seems to have a more prominent role in the enzymes reactivity as compared with Arg.

The analysis of the kinetic data for a bulky oxidizing substrate ABTS reveals that the substitution of distal Asp, Arg and Asn, resulted in a significant decrease of the $K_m$ values (Table 6 and Fig. 25). This is particularly evident in the D132N variant which has around 20 times lower $K_m$ for ABTS, resulting in a catalytic efficiency ($k_{cat}/K_m$) which is 25-times higher than in the WT. The N136L mutant shows a slight increase in $k_{cat}$, together with a 7-fold decrease in $K_m$, which results in one order of magnitude higher efficiency in comparison with the WT. Despite the 4-fold decreased $K_m$ of R214L for ABTS, this mutant showed a 300-fold slower oxidation rate and an efficiency of one order of magnitude lower than the WT. Overall, these results reinforce the view of the importance of the arginine residue in the catalytic mechanism of PpDyP, being essential for Cpd I formation and stabilization.

It is generally accepted that the catalytic cycle in DyPs is similar to that of classic peroxidases. In the latter, the conserved distal histidine (His52 in CcP and His42 in HRP) appears to be an obvious candidate for the acid-base catalyst in the formation of the Cpd I, since the substitution of histidine with leucine in both CcP and HRP lowered the rate of Cpd I formation by 5 orders of magnitude [36,37]. However, replacement of arginine in HRP significantly decreases the reactivity towards hydrogen peroxide (1200-fold), while in CcP only 200-fold lower rates were measured, indicating differences in the catalytic mechanisms [36,38]. Therefore it has been proposed that the positively charged guanidinium group of arginine plays a role in the orientation of hydrogen peroxide at the active center, facilitating its binding, and also in promoting the heterolytic cleavage of peroxide by acting as proton donor [39,40]. Hydrogen peroxide binding to the haem iron lowers its pK$_a$ and its deprotonation does not require the presence of strong bases. While most peroxidases rely on a histidine residue, a deprotonated aspartate residue was proposed to be its analog in DyPs [41]. A swing mechanism of Cpd I formation in fungal DyPs was proposed, where the Asp swings toward the haem in the presence of H$_2$O$_2$ and thereupon mediates the rearrangement of a proton and swings back to the initial position after Cpd I formation [42]. Therefore, the aspartate residue was envisaged as being responsible for acid-base catalysis in DyPs. More recent insights demonstrated that this scenario holds only for few DyPs belonging to sub-families A and D [14–16,41]. As previously described for classic peroxidases, distal arginine can also play a key role in catalysis and this is particularly evident in DyPs from sub-family B such as DyP8 from *R. jostii* RHA1 [11] and PpDyP from *P. putida* MET94, where Arg appears to be essential in Cpd I formation and reactivity, possibly through proton transfer and charge stabilization.

A growing body of evidence on DyPs seems to point to a sub-family dependent redox and catalytic properties. Clearly more members of DyP-type peroxidase superfamily need to be thoroughly characterized and more solid kinetic, thermodynamic and structural information provided for the full understanding of the molecular determinants of catalytic mechanisms of DyPs. These insights will allow us to fully exploit the unique properties of DyP-type peroxidases in biotechnological applications.

**Acknowledgments**

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**Appendix A. Supplementary data**

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jabb.2015.03.009.

**References**


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**Table 6.** Apparent steady-state catalytic parameters of PpDyP and distal variants for ABTS. Reactions were performed with ABTS (0.1–14 mM for WT and 0.005–5 mM for D132N, R214L and N136L with 0.2 mM H$_2$O$_2$ for WT, 20 mM H$_2$O$_2$ for D132N, 6 mM H$_2$O$_2$ for R214L and 4 mM H$_2$O$_2$ for N136L, Fig. 25) at 25 °C, in 100 mM sodium acetate or phosphate buffer at the optimal pH for each enzyme (4.3 for WT, 7.4 for D132N, 3.6 for R214L and 5.6 for N136L).

<table>
<thead>
<tr>
<th></th>
<th>$K_m$ (µM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat}/K_m$ (M$^{-1}$ s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABTS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>2500 ± 190</td>
<td>21 ± 1</td>
<td>8 × 10$^3$</td>
</tr>
<tr>
<td>D132N</td>
<td>126 ± 13</td>
<td>23 ± 2</td>
<td>2 × 10$^1$</td>
</tr>
<tr>
<td>R214L</td>
<td>566 ± 75</td>
<td>0.07 ± 0.01</td>
<td>2 × 10$^2$</td>
</tr>
<tr>
<td>N136L</td>
<td>347 ± 88</td>
<td>34 ± 7</td>
<td>10 × 10$^4$</td>
</tr>
</tbody>
</table>

* [17].

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**Fig. 6.** pH activity profile of PpDyP wild-type (filled squares), D132N (filled circles), R214L (open circles), and N136L (open squares).