DyP, a Unique Dye-decolorizing Peroxidase, Represents a Novel Heme Peroxidase Family

ASP171 REPLACES THE DISTAL HISTIDINE OF CLASSICAL PEROXIDASES

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Peroxidases have been systematically researched for more than 70 years. Fifteen years ago, Welinder (1) proposed the concept of a “plant peroxidase superfamily” comprising classes I, II, and III, based on primary sequence alignments and isolation from prokaryotes, fungi, and plants, respectively. Using this strategy, yeast cytochrome c peroxidase (2) and chloroplast ascorbate peroxidase (3) were classified as class I peroxidases on account of their prokaryotic source. Representatives of class II include lignin peroxidase (LiP) (4), manganese peroxidase (MnP) (5), and versatile peroxidase (6, 7), whereas class III contains horseradish peroxidase (HRP) (8) and barley grain peroxidase (BGP) (9). This classification has been widely applied to most known peroxidases, with the exception of chloroperoxidase (CPO) isolated from the fungus, Caldariomyces fumago, which lacks primary structural homology with other peroxidases (10, 11). However, in contrast to the plant peroxidases, those from animals, including mammals, are yet to be categorized. To date, most of these enzymes have been grouped into the plant or animal peroxidase superfamily (12).

So far, we isolated and characterized a novel extracellular peroxidase, DyP, from the fungus Thanatephorus cucumeris Dec 1 (13–16). DyP, a glycoprotein having one heme as a cofactor, has a molecular mass of 58 kDa and requires H2O2 for all enzyme reactions, indicating that it functions as a peroxidase. DyP has several characteristics that distinguish it from all other peroxidases, including a particularly wide substrate specificity, a lack of homology to most other peroxidases, and the ability to function well under much lower pH conditions (3–3.2) compared with the other plant peroxidases (13, 15). In terms of substrate specificity, DyP degrades the typical peroxidase substrates, but also degrades hydroxyl-free anthraquinone, which is not a substrate of other peroxidases (7, 13, 15). This is very important characteristic. Many synthetic dyes are derived from anthraquinone compounds. Wastewater containing synthetic dyes (dye-contaminated water) is blamed for multiple environmental problems because of its recalcitrant and xenobiotic characteristic but there is little effective treatment toward the wastewater (17). In contrast, DyP is a promising enzyme for the treatment of the dye-contaminated water because it degrades those dyes effectively (13, 15, 18). The amino acid sequence of DyP shows no homology to any other peroxidase registered in the DNA Data Bank of Japan (DDBJ) (16), indicating it does not belong to plant peroxidase superfamily. A recent report, however, described the peroxide-dependent phenol oxidase from Termitomyces albuminus (TAP), which is 55% homologous to DyP at the primary structure level (19). Moreover, cpop21, a hypothetical peroxidase from Polyporaceae sp. (DDBJ registration code U77073), also shows 55% homology to DyP. Interestingly, these three proteins show no sequence homology to any other registered proteins. Additionally, YcdB hemoprotein from Escherichia coli reportedly shows DyP-like peroxidase activity under acidic conditions and shows partial C-terminal homology to DyP but its detail has been unclear (20).

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2 The abbreviations used are: LiP, lignin peroxidase; MnP, manganese peroxidase; HRP, horseradish peroxidase; BGP, barley grain peroxidase; CPO, chloroperoxidase; TAP, peroxide-dependent phenol oxidase from T. albuminus; cpop21, hypothetical peroxidase from Polyporaceae sp.
Previously, we performed extensive studies to describe the unique characteristics and industrial merits of DyP (7, 15, 18, 21). Here we present the crystal structure of DyP, along with spectrophotometric analyses. Our data suggest that DyP is a clear outlier from the plant peroxidase superfamily. We propose that DyP is a member of a novel peroxidase family, along with TAP, cpop21, and another hypothetical peroxidase. This means that the number of peroxidase families should be expanded.

**MATERIALS AND METHODS**

**Enzyme**—We purified DyP by the method described in our previous report (15). The enzyme activity was defined as the amount of enzyme capable of decolorizing (decreasing absorbance at 623 nm) 1 μmol of 1-amino-2-sulfonyl-4-aminomethyl-9,10-anthraquinone sodium salt at 30 °C for 1 min. The purified DyP was deglycosylated with an endo- β-N-glycosidase (Hampton Research) for crystallization and x-ray analysis (22).

**Crystallization of DyP**—Crystallization was performed as reported previously (22). Briefly, crystals were grown by the batch method at 283 K in crystallization solution containing 0.89 M ammonium sulfate, 0.92 M sodium chloride, and 15 mg/ml DyP (pH 4.2).

**Phase Determination and Model Building**—Phases were determined using the multiple isomorphous replacement with anomalous scattering method. We collected one native data set and three derivative data sets (K2PtCl4, Hg(OAc)2, and KAu(CN)4) at KEK BL6A and KEK BL18B, as described in the previously published crystallization paper (22). Initial multiple isomorphous replacement with anomalous scattering phases were obtained at 2.96 Å using the MLPHARE computer program (23), and non-crystallographic symmetry averaging and solvent flattening were performed. When electron density maps were calculated using the P6322 space group, secondary structure elements were recognizable. The chain was traced manually using the O program (24). After several cycles of manual building and CNS refinement (25), Rfactor and Rfree converged to 21.4 and 25.5% at 2.96 Å. The root mean square deviations taken as the bond length and the bond angles were 0.018 and 1.863, respectively. The Ramachandran plot showed that the most favored and additional allowed regions were 83.4 and 15.2%, respectively. Only Arg315 was located in the disallowed region. The final model included two DyP molecules consisting of residues 4 to 442, two hemes and 11 water molecules. The three N-terminal-most residues were missing from the electron density. The tertiary structure was generated using the PyMol software (pymol.sourceforge.net), and structural homology between DyP and other proteins registered in the Protein Data Bank (PDB) were searched using the DALI server (www.embl-heidelberg.de/dali/) and evaluated based on Z-score values.

**Electronic Absorption Spectra**—All spectra were recorded on a Shimadzu UV-2400 PC spectrophotometer (Shimadzu Co., Kyoto, Japan) at 30 °C with a spectral bandwidth of 2.0 nm, using cuvettes of 1-cm light path. The oxidation states of DyP and its mutant were prepared by adding 1 or 2 eq of H2O2 to the enzyme in 20 mM citrate buffer (pH 3.2), and spectral changes were recorded.

**RESULTS**

**Full Structure of DyP**—Experimental data are summarized in Table 1, and the structure of DyP is shown in Fig. 1A. Our results revealed that DyP contains one heme with an iron at the center of the molecule. The full structure has dimensions of ~62 × 66 × 48 Å³. The size and heme existence are consistent with other peroxidase structures. Of 442 total residues in DyP, 192 residues form 18 α-helices and 15 β-strands. One unique motif found in the secondary structure of DyP is two sets of anti-parallel β-sheets (β1 and β4, β2 and β3) located between two α-helices (α3 and α8) above the distal area of the heme (see topology in Fig. 1A). This motif has not been identified in any other registered protein. Consistent with the structure of other peroxidases, the fifth ligand of the heme iron of DyP is histidine. However, DyP lacks the distal histidine that is conserved among all other members of the plant peroxidase superfamily (1).

**Specific Observation of DyP**—The primary structure of DyP shows homology to TAP (19) and cpop21 (a hypothetical peroxidase registered in the DDBJ) only. DyP has more β-strands than other peroxidases, including LiP, MnP, and HRP (26–28). Focusing on the heme and its surrounding region (Fig. 1B), we observed that DyP has His308 as the fifth ligand of its heme corresponding to the conserved proximal histidine in other members of the plant peroxidase superfamily. The position of His308 in DyP is biased to the C-terminal region, whereas the corresponding histidines are located in the middle regions of all other known peroxidases (1). The proximal histidine of plant peroxidases possibly forms a strong hydrogen bond with the carboxylate of a nearby aspartic acid (2). However, in DyP, Glu391 appears to form a hydrogen bond with His308. Interest-
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Arg$^{329}$ in DyP are conserved in cpop21 and TAP. Furthermore, the tertiary structure of a hypothetical peroxidase from Bacteroides thetaiotaomicron, VPI-5482, which is unpublished but registered in the PDB as 2GVK, shows moderate homology (Z-score = 25.9) to that of DyP (Fig. 1C), even though the two proteins share no primary sequence homology. The registration title of PDB 2GVK is suggestive of a heme peroxidase, but its function is currently unknown, and no heme has been assigned as yet. However, the residues corresponding to His$^{308}$ and Asp$^{171}$ of DyP are well conserved in PDB 2GVK (Fig. 1C, magnification). Interestingly, Glu$^{391}$ of DyP corresponds to Asp$^{289}$ of PDB 2GVK. The carboxylate sites of these residues are almost identical, suggesting that they both form hydrogen bonds with proximal histidines. Moreover, Arg$^{245}$ of PDB 2GVK, possibly corresponding to Arg$^{329}$ of DyP, appears very bent compared with other conserved residues. This distortion of Arg$^{245}$ may affect the activity of PDB 2GVK.

Characteristics of the Absorption Spectra of DyP—The absorption maxima of resting DyP were 406, 506, and 636 nm, corresponding to Soret, β- and charge transfer bands, respectively (Fig. 2A). Upon the addition of 1 eq of H$_2$O$_2$ to the resting enzyme, the Soret absorption band broadened and its intensity decreased to 52% that of the resting enzyme after 0.2 min. The visible region showed broad absorption with peaks at 530, 556, 615, and 644 nm (Fig. 2A, inset). When 2 eq of H$_2$O$_2$ were added to the resting enzyme, the spectral patterns and time courses were similar to those observed with 1 eq of H$_2$O$_2$. In both cases, the intermediates spontaneously returned to those of resting DyP after 20 min.

Characteristics of D171N Mutant DyP—The resting states (no H$_2$O$_2$) spectra of the D171N mutant and native DyP were identical (Fig. 2), indicating that the mutant retains a similar structure to the wild-type protein. The addition of H$_2$O$_2$ to the D171N mutant yielded no spectral changes relative to the resting state (Fig. 2B). The enzyme activity of the D171N mutant for 1-amino-2-sulfonyl-4-aminomethyl-9,10-anthra-

![FIGURE 1. A, full structure of DyP. The α-helices and β-sheets are shown in light blue and violet, respectively. The proximal His and heme are shown in dark blue and green, respectively. B, the heme and its surrounding residues important for the peroxidase activity of DyP. C, tertiary structural alignment of DyP and the hypothetical peroxidase, 2GVK. DyP and 2GVK are shown in blue and yellow, respectively. The heme and His$^{308}$ of DyP are shown in light blue. Data on the heme position of 2GVK are not currently available through the PDB. The magnification of the heme and its critical residues is indicated by the arrow. Two possible phases of the Asp$^{157}$ residue of 2GVK, which corresponds to Asp$^{171}$ of DyP, are shown.](image-url)
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**DISCUSSION**

Because DyP oxidizes the typical peroxidase substrate, guaiacol, using \( \text{H}_2\text{O}_2 \) as the electron acceptor, it is classified as a peroxidase (Enzyme Commission number EC 1.11.1; Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (www.chem.qmul.ac.uk/iubmb/enzyme)). According to the classification scheme of Welinder, DyP does not belong to the plant peroxidase superfamily. Moreover, the overall structure of DyP differs from that of almost all other peroxidases. Therefore, DyP is regarded as an exceptional peroxidase, similar to CPO. However, our structural and spectrophotometric analyses clearly indicate that DyP is a part of a novel peroxidase family.

The spectral characteristics of DyP at the resting state and in the presence of 1 eq of \( \text{H}_2\text{O}_2 \) are similar to those of other peroxidases (12, 29, 30), implying that the primary reaction state of DyP is the same \( \text{Fe}^{4+} \) oxoferryl center and a porphyrin-based cation radical intermediate (compound I) generated by other peroxidases. In contrast, the spectra of DyP in the presence of 2 eq of \( \text{H}_2\text{O}_2 \) differ from those of other peroxidases, with DyP failing to show the red-shift of the Soret band seen in other peroxidases as shown in Table 2. Moreover, the time courses of the spectral changes and absorption maxima are similar for DyP in the presence of 1 and 2 eq of \( \text{H}_2\text{O}_2 \). Interestingly, DyP spontaneously returns to its resting state within 20 min, whereas compound I of LiP spontaneously converts to an oxoferryl intermediate (compound II) within 1 min (29). In general plant peroxidases, such as LiP, typical compound II absorbance features are observed upon the addition of 2 eq of \( \text{H}_2\text{O}_2 \) (Table 2). However, we could not detect compound II of DyP under these experimental conditions. The differences may indicate that the full catalytic pathway of DyP is distinct from that of other peroxidases. Further studies are required to confirm whether the DyP catalytic pathway is completed via compound II.

In members of the plant peroxidase superfamily, distal His and essential Arg residues act as proton acceptors from \( \text{H}_2\text{O}_2 \) and charge stabilizers, respectively (2, 31). In particular, distal His is known to be indispensable for the formation of compound I. However, DyP has no distal histidine. Similarly, CPO lacks distal histidine. Instead, histidine at this position is replaced with glutamic acid (11). However, a histidine existing near the heme distal area of CPO has been postulated to be indirectly involved in modulating the acidity/basicity of the carbonyl group of glutamic acid. According to the proposal, the carboxylate accepts a proton from the peroxide and donates a proton to the leaving hydroxyl group, following which the \( \text{Fe}^{3+} \).o species oxidizes porphyrin and iron to produce compound I. In the case of DyP, we propose that Asp\(^{171} \) substitutes for the distal histidine, because the charged group is sufficiently close to the peroxide-binding site to have a direct catalytic role. It is reasonable to assume that hydrogen peroxide is arranged between Asp\(^{171} \) and Arg\(^{299} \), based on the structure and electron density map (Figs. 1B and 3). Specifically, Asp\(^{171} \) acts as the acid-base catalyst, analogous to glutamic acid of CPO. However, DyP has no histidine in the heme distal area, distinct from CPO. The role of Asp\(^{171} \) just transfers a proton from one peroxide oxygen atom to another. It may be that one more proton gets involved from solvent, probably by protonating the oxoferryl oxygen atom depending on its \( pK_{a} \). This is the reason why DyP has no histidine to modulate the acidity/basicity of the carbonyl group of Asp\(^{171} \). One more noteworthy point is that \( pK_{a} \) (3.9) of the side chain of aspartic acid is lower than that of histidine (6.0). Therefore, DyP has a low pH optimum (3.2) that would be consistent with an aspartic acid and not histidine.

**TABLE 2**

Absorption maxima of oxidized intermediates of several peroxidases

| Name  | Resting | Compound I | Compound II* | Ref.          |
|-------|---------|------------|--------------|---------------|
| DyP   | 406\(^a\), 506, 636 | 401, 530, 556, 615, 644(sh)\(^c\) | 399, 529, 555, 615, 644(sh) | This work     |
| LiP   | 408, 496, 630 | 408, 550, 608, 650 | 420, 525, 556 | 29            |
| MnP   | 406, 502, 632 | 407, 558, 617, 650 | 420, 528, 555 | 30            |
| HRP   | 403, 498, 640 | 400, 525(sh), 577, 622(sh), 651 | 420, 527, 555 | 12            |

\(^a\) In case of DyP, these absorption maxima are the same as those of compound I.

\(^b\) Bold type face wavelength corresponds to the Soret band.

\(^c\) sh, shoulder peak.
serving as the acid-base catalyst. This characteristic is similar to that of CPO.

Our hypothesis is further supported by spectrophotometric data and the finding that activity of the D171N mutant DyP is drastically decreased relative to that of wild-type DyP, although their proteins have similar structures (Fig. 2). Moreover, the D171N mutant shows no spectral changes following the addition of H2O2, further indicating that the mutant protein does not form compound I. Based on these findings, we have proposed a scheme for the catalytic process of DyP in Fig. 4. In our model, Asp171 and Arg329 act as the proton acceptor and charge stabilizer, respectively. Glu391 stabilizes Fe5+ and Fe4+ by forming a hydrogen bond with His308. At the resting state of DyP, the sixth ligand of the heme is absent. When H2O2 is added, it forms a complex (stage 2). From stages 2 to 4, the following processes may occur sequentially: Asp171 works as an acid-base catalyst to take the proton off the iron-linked oxygen peroxide and deliver it to the distal peroxide oxygen atom. The peroxide O–O bond undergoes heterolytic fission, releasing water. The remaining oxygen atom has 6 valence electrons, indicating a very electron-hungry atom. Therefore, one electron is removed from iron, and another electron is probably removed from porphyrin, as in general peroxidases. Finally, compound I is generated, as shown in stage 4. In fact, the absorbance maxima of compound I of DyP are similar to those of other representative plant peroxidases (Table 2).

Notably, the residues corresponding to His308, Asp171, and Arg329 in DyP are conserved in the two DyP-like proteins, cpop21 and TAP. On the other hand, whereas the important residue (Asp171) of DyP resembles that (Glu) of CPO, as mentioned above, CPO has cysteine, but not histidine as the proximal ligand of the heme, and has no essential arginine at the heme distal area. In contrast to CPO, a unique peroxidase, DyP appears to form a new subfamily with at least TAP and cpop21. A fourth potential member of this subfamily, PDB 2GVK from B. thetaiotaomicron VPI-5482, shows moderate homology to DyP at the tertiary structure level (Fig. 1C). Interestingly, despite no sequence similarities between PDB 2GVK and DyP, the residues corresponding to His308 and Asp171 of DyP are conserved (Fig. 1C). It appears that these two residues are critical for the catalytic activity of this type of protein. Additionally, in plant peroxidases, an aspartic acid near the proximal histidine is well conserved, forming a strong hydrogen bond with the proximal histidine. The histidine functions as an imidazolate (Im-) in this bonding. This characteristic is important for peroxidase activity, as it stabilizes the high oxidation states, such as Fe5+ or Fe4+ (2, 12, 31). From this viewpoint, it is important to note that Asp390 is also conserved in PDB 2GVK. However, the corresponding residue in DyP is not Asp, but Glu391. Although this is a rare case, Goodin and McRee (31) showed that a mutant cytochrome c peroxidase with aspartic acid altered to glutamic acid retained 41% of the activity of the wild-type protein, indicating that glutamic acid is a probable substitute for aspartic acid. PDB 2GVK is listed as a heme peroxidase from a bacterium, but it shows no homology to other

![Figure 3: Stereo view of 2Fe - F, omit electron density map around heme region. The map was contoured at 2a. Asp171, Arg329, and heme are shown for reference.](image)

![Figure 4: Schematic diagram of the proposed formation of compound I by DyP. Asp171 and Arg329 function as the proton acceptor and charge stabilizer, respectively. Glu391 stabilizes Fe5+ and Fe4+ by forming a hydrogen bond with His308. Stages 1 and 2 represent the resting state and the Fe3+ - H2O2-DyP complex, respectively. From stages 2 to 4, the following pathway would be feasible: Asp171 acts as an acid-base catalyst, taking the proton off the iron-linked oxygen atom peroxide and delivering it to the distal peroxide oxygen atom. The peroxide O–O bond undergoes heterolytic fission, following which 1 electron is moved from iron and another electron is transferred from porphyrin to the remaining oxygen atom. Finally, the Fe5+ oxoferryl center and a porphyrin-based cation radical intermediate (compound I) are generated, as shown in stage 4. The dotted lines represent probable hydrogen bonds.](image)
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known peroxidase. Based on the present findings, we propose that DyP, 2GVK, TAP, and cpop21 form a novel peroxidase family.

To examine this association in more detail, we have analyzed the tertiary structural homologies among the various plant peroxidases. The results are of particular interest, because the Welinder classification scheme does not include tertiary structural information. Full protein structures of CcP, MnP, and BGP (representative of the class I, II, and III peroxidases, respectively) are similar (supplemental Fig. S1, all Z-scores > 20), and show conservation of heme, essential arginine, distal histidine, and proximal histidine sites. This result is reasonable compared with some previous research (26–28, 32, 33). Moreover, as well known, catalase-peroxidases (34), which are not typical peroxidases and defined as catalases (EC number 1.11.1.6), are structurally homologous to the peroxidases (Z-scores > 20). Based on the present data, we have constructed a new phylogenetic tree using the ClustalX program to apply the neighboring-joining method combined with bootstrap calculations (35, 36). As shown in Fig. 5, division into classes I, II, and III appears to be phylogenetically reasonable and appropriate with the exception of CPO, which is an outlier. On the other hand, DyP, cpop21, and TAP form an apparently independent group from the plant peroxidase superfamily. Thus, our phylogenetic and tertiary structural analyses clearly reveal that DyP, TAP, and cpop21 form a separate family from the plant peroxidase superfamily, possibly including PDB 2GVK, which shows tertiary structural homology but no sequence similarity to DyP-like proteins.

In conclusion, we provide structural and spectrophotometric data showing that DyP has unique structural features, and a different residue, Asp^{171}, contributes to the catalytic process in lieu of the distal histidine of other plant peroxidases. This means that DyP works well at lower pH than other plant peroxidases depending on the difference of $pK_a$ between aspartic acid and histidine. Our results strongly indicate that DyP is a novel peroxidase (EC 1.11.1.10) that forms an independent family separate from the classical peroxidase groups, which we designate “DyP-type peroxidase family.” As the only member of the family that has been characterized in detail, DyP is the leading enzyme for advancing functional research on this new class of heme peroxidases.

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