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Review

DyP-type peroxidases comprise a novel heme peroxidase family

Y. Sugano

R1 – 30 Chemical Resources Laboratory, Tokyo Institute of Technology, 4259 Nagatsuta, Midori-ku, Yokohama 226-8503, (Japan), Fax: +81-45-924-5976, e-mail: ysugano@res.titech.ac.jp

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Abstract. Dye-decolorizing peroxidase (DyP) is produced by a basidiomycete (*Thanatephorus cucumeris* Dec 1) and is a member of a novel heme peroxidase family (DyP-type peroxidase family) that appears to be distinct from general peroxidases. Thus far, 80 putative members of this family have been registered in the PeroxiBase database (http://peroxibase.isb-sib.ch/) and more than 400 homologous proteins have been detected via PSI-BLAST search. Although few studies have characterized the function and structure of these proteins, they appear to be bifunctional enzymes with hydrolase or oxygenase, as well as typical peroxidase activities. DyP-type peroxidase family suggests an ancient root compared with other general peroxidases because of their widespread distribution in the living world. In this review, firstly, an outline of the characteristics of DyP from *T. cucumeris* is presented and then interesting characteristics of the DyP-type peroxidase family are discussed.

Keywords. DyP, heme peroxidase, molecular evolution, bifunctional enzyme, peroxygenase P450, chloroperoxidase.

Introduction

The structure of heme peroxidases (peroxidases containing prosthetic heme groups) has been well characterized [1]. These enzymes can be divided into two large groups (plant peroxidases and animal peroxidases). Fifteen years ago, Welinder introduced the concept of a plant peroxidase superfamily [2], wherein prokaryotes including mitochondria, fungal, and plant peroxidases are divided into classes I, II, and III, respectively, based on primary structural homology. For example, yeast cytochrome *c* peroxidase [3] and chloroplast ascorbate peroxidase [4] are class I peroxidases, whereas class II enzymes include lignin peroxidase (LiP) [5], manganese peroxidase (MnP) [6], and versatile peroxidase [7, 8]. Similarly, horseradish peroxidase (HRP) [9] and barley grain peroxidase (BGP) [10] are class III peroxidases. This classification system has proven useful for characterizing the majority of known peroxidases, with the exception of chloroperoxidase [11], which was isolated from a fungus (*Caldariomyces fumago*) and which lacks primary structural homology with other peroxidases [12]. In contrast to plant peroxidases, most enzymes isolated from mammals and other animals have not yet been classified. However, several animal peroxidases have been studied with regards to roles in disease and aging [13–15]. Recently, evolutionary relationships among mammalian heme peroxidases have been proposed by advanced phylogenetic analysis [16]. Several studies have examined the properties of heme peroxidases and numerous reviews on this topic have been published. A new category of peroxidases, known as the dye-decolorizing peroxidase-type (DyP-type) peroxidase family, was recently proposed [17]. DyP-type peroxidases have been included in databases such as PeroxiBase (http://peroxibase.isb-sib.ch/) and Pfam [18], and are not

considered members of the plant or animal peroxidase superfamilies because of their specific primary and tertiary structures and unique reaction characteristics. This review focuses on the DyP-type peroxidase family, one of the most novel and intriguing families of heme peroxidases. Unless otherwise stated, amino acid residues are displayed with universal one-letter notation combined with their location numbers of mature protein sequences (e.g., D171) throughout the text.

Discovery of DyP

In 1995, the basidiomycete *Thanatephorus cucumeris* Dec 1 (formerly *Geotrichum candidum* Dec 1) was reported to be able to decolorize 18 types of reactive, acidic, and dispersive dyes, most of which were xenobiotics [19]. When crude extracellular enzyme solutions were prepared via ammonium sulfate precipitation and dialysis, the decolorization activity exceeded 100-fold that observed in Dec 1 culture broth. This decolorization appeared to depend on the interactions of extracellular enzymes with H_2O_2, indicating important roles for peroxidases. In 1999, DyP was purified and characterized from *T. cucumeris* Dec 1 as a glycosylated enzyme with a molecular mass of 60 kDa [20]. The absorption spectrum of DyP included a Soret band at 406 nm (indicating that DyP was a hemoprotein) and the $Na_2S_2O_4$-reduced form of this enzyme revealed a peak at 556 nm (indicating the presence of a prosthetic protoheme group). These characteristics, with the exception of molecular mass, suggested that DyP was a typical heme peroxidase. However, the substrate specificity of DyP did not resemble that of known classical peroxidases. For instance, DyP exhibited higher decolorizing activities toward anthraquinone dyes than toward azo dyes and different degradation spectra toward phenolic compounds such as 2,6-dimethoxy-phenol, guaiacol, and veratryl alcohol. These properties suggested that DyP was a novel type of peroxidase that differed from general peroxidases [1, 3–10]. Thus, DyP remains the first and representative peroxidase of the DyP-type peroxidase family. Unless otherwise specified, I will hereinafter refer to the DyP enzyme of *Thanatephorus cucumeris* as DyP.

Gene cloning and overall characteristics of DyP

T. cucumeris Dec 1 produces other DyPs in addition to the first isolated DyP. These enzymes possess similar p*I* values but slightly different molecular masses, indicating that they exist as isozymes [20]. In general, peroxidases have isozymes or members of a multiple gene family [21–25]. Recently, it was shown that *T. cucumeris* produces several DyP isozymes [26]; however, these isozymes have proven difficult to isolate from each other [20]. Characterizing the properties of DyP in detail required an individual *dyp* gene to be cloned from several isozymes. One *dyp* gene was cloned and the primary sequence analyzed [27]. According to the classification of Welinder, DyP has been considered a class II peroxidase because *T. cucumeris* is a basidiomycete (i.e., a fungus). However, the primary structure of the cloned DyP differed from those of other plant peroxidase superfamilies, including class II peroxidases [27]. Generally, heme peroxidases belonging to the plant peroxidase superfamily possess a highly conserved motif (R-X-X-F/W-H, in which R and H are essential arginine and distal histidine, respectively). The tertiary structure of MnP, which is the representative member of the plant peroxidase superfamily, is shown in Figure 1. This conserved motif, which is considered essential for heme peroxidase activity, was not identified in DyP. Until recently, DyP was the only known peroxidase of this type and was therefore the subject of limited research. However, recent bioinformatic analyses suggest that DyP and similar enzymes comprise a novel category of heme peroxidases. Why is there no typical conserved motif in DyP and similar proteins? In general, fungi are considered to be taxonomically close to plants. However, Wainright et al. performed a phylogenetic analysis using rRNA and reported that animal and fungal lineages share a more recent ancestor than either do with the plant lineage [28]. If *T. cucumeris* is more closely related to animals than to plants, it is not surprising that conserved motifs of the plant peroxidase superfamily are not retained in DyP–type peroxidases. Actually, as mentioned in a later section, the DyP-type peroxidase family may be ubiquitously distributed in the living world.

Recombinant expression of DyP

Large yields of DyP are needed to enable research on this enzyme, as *T. cucumeris* Dec 1 produces only small amounts of enzyme. To increase enzyme yield, the cloned *dyp* gene must be heterologously expressed. DyP is a glycoprotein and is thus more appropriately expressed in eukaryotes than prokaryotes. Therefore, the cloned *dyp* gene was integrated into the chromosome of *A. oryzae* and highly expressed [29, 30, 31]. Successful studies of the recombinant DyP (rDyP) have greatly advanced knowledge of DyP-type peroxidases. In this process, exogenous heme (e.g., hemin), which is generally required for heterologous

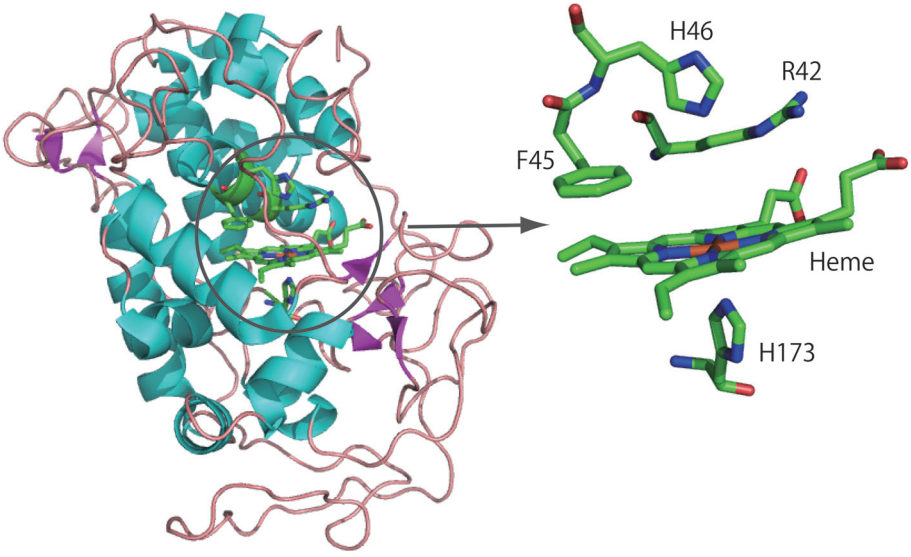

Figure 1. Tertiary structure of MnP of the representative plant peroxidase superfamily. Whole structure of MnP (PDB 1mnp) is displayed by PyMol software (http://pymol.org/). The α-helices and β-sheets are shown in blue and violet, respectively. The magnification of the heme, the conserved motif (R42-X-X-F45-H46) and a histidine as the heme ligand (H173) is indicated by the arrow.

peroxidase expression [32–34], was not necessary for rDyP expression. More surprisingly, DyP-type peroxidases other than DyP were heterologously expressed and formed apo-enzymes in the absence of hemin. Moreover, DyP was a monomer, whereas other DyP-type peroxidases formed a variety of complexes, ranging from monomers to hexamers. The characteristics of these tertiary and quaternary structures are discussed later in further detail. Recombinant DyP expressed in *Escherichia coli* [35] possessed similar enzymatic activity to – but a smaller molecular mass than – native DyP, indicating the absence of sugar modification [35]. Furthermore, the thermostability of recombinant *E. coli*-expressed DyP was inferior to that of *A. oryzae*-expressed DyP, suggesting that oligosaccharide modification contributed to thermostability.

Relationship between DyP and other DyP-type peroxidases

Genetic cloning experiments and heterologous expression of DyP revealed that this enzyme is a unique peroxidase [27, 29]. Here, the most recent findings regarding DyP-type peroxidases are summarized. Thanks to DNA sequencing technology and bioinformatic analyses, several groups have obtained nucleotide sequence data on genes and have registered this information in several databases. Most of them have been automatically translated to amino acid sequences (apparent proteins). However, the functions of many of these proteins remain unknown. Among those databases, PeroxiBase (http://peroxibase.isb-sib.ch/) is the most reliable and dispassionate database of peroxidase and related proteins. For instance, 80 members of DyP-type peroxidases have been registered in PeroxiBase. On the other hand, only 49 members of the haloperoxidase family have been included in the database, indicating that the DyP-type peroxidase family is a large and independent group of heme peroxidases. However, limited amino acid sequence data are available for members of the DyP-type peroxidase family, as most protein sequences were generated via automatic translation, without functional research. A PSI-BLAST search [36] revealed more than 400 proteins sharing homology with DyP; however, only seven proteins were reported to be DyP-type peroxidases [37–42]. Consequently, primary bioinformatics programs, such as PSI-BLAST and FASTA, have proven useful but are not perfect tools.

Moreover, structural data of DyP-type peroxidases are rather fewer than functional data. Thus, I will discuss the known characteristics of these enzymes with regard to published functional and structural data. In the following section, current findings on DyP-type peroxidases will be discussed.

Primary structural analysis

Four primary structures of DyP-type peroxidases and one hypothetical peroxidase have been characterized and their sequence homologies are shown in Figure 2 [37–39]. The 57,256 Da *Po*DyP produced by *Pleurotus ostreatus* was cloned from a cDNA library and is composed of 516 amino acid residues. The *Po*DyP and DyP are similar in primary structure [39]. However, only genetic information is currently available for *Po*DyP, and further research is needed to elucidate the essential characteristics of this enzyme. On the other

hand, the MsP1 and MsP2 from *Marasmius scorodonius* have been characterized in detail [38] and been shown to be extracellular peroxidases that form homodimers. Interestingly, both MsP1 and MsP2 possess O_2–dependent β-carotene–degrading activity in the absence of H_2O_2 [38, 43]. This indicates that these enzymes possess oxidase or oxygenase activity; however, it is unclear that they possess peroxidase activity because no peroxidase activities were shown in the research. If these assumptions are correct, DyP-type peroxidases may be versatile functional enzymes, as described in a later section. The quaternary structures of MsP1 and MsP2 are similar to those of other DyP-type peroxidases, with the exception of DyP and a hydrogen peroxide-dependent phenol oxidase (i.e., TAP). TAP is a 67 kDa extracellular monomeric enzyme produced by *Termitomyces albuminosus* [37]. The deduced amino acid sequence is 56 % identical to DyP, the highest homology observed among members of the DyP-type peroxidase family. Overall, the characteristics of TAP most closely resemble those of DyP, indicating that these proteins are phylogenetically related. The cpop21 protein is registered as a hypothetical peroxidase in the Genbank, EMBL, and DDBJ databases, and the primary sequence is highly similar to that of DyP (Fig. 2). H164 or H166 found in DyP might function as ligand of the heme (i.e., proximal histidine). This is because the proximal histidine found in the plant peroxidase superfamily is conserved near the 170th residue from the N-terminus of the mature sequence [2]. As shown in Figure 2, all sequences, with the exception of *Pleurotus ostreatus,* contain conserved histidine residues (i.e., H164 and H166). Interestingly, the DyP produced by *Pleurotus ostreatus* contains a H164K replacement. Initially, H164 was designated as the proximal histidine [44]; however, further structural analyses demonstrated that this residue was not a ligand of heme [17]. When the H164 residue was replaced with A164, both DyP activity and heme binding were lost, indicating that this residue is important for heme binding or conformational maintenance [35].

Tertiary structural and phylogenetic analyses

In the previous section, findings on the primary structural homologies of DyP-type peroxidases were discussed; however, these studies were unable to determine the proximal histidine. Thus, tertiary structural analyses were necessary to characterize DyP-type peroxidases in depth. The first solved DyP-type peroxidase structure was that of DyP from *T. cucumeris* (PDB code 2d3q) [17]. Based on the structure of this enzyme, secondary structural predictions were performed using the DALI server [45]. These analyses revealed two highly homologous proteins (i.e., Z-score > 20), including TyrA (PDB code 2hag, 2iiz) and *Bt*DyP (PDB code 2gvk) [40, 41]. Whereas the primary sequence homology between those two proteins and DyP is very low (Fig. 3), their overall tertiary structures were homologous to DyP (Fig. 4A). These findings indicate that primary sequence alignments are insufficient for determining the homology of actual protein structures. In other words, general homology searches (e.g., using PSI-BLAST, BLAST, and FASTA) [46] are likely to overlook tertiary structure homology in various proteins. Moreover, several conserved regions were observed in both the primary homologous proteins (Fig. 2) and tertiary homologous proteins (Fig. 3). In particular, the D171, H308, and R329 were later found to play important roles in the specific functions of DyP-type peroxidase family members.

A unique motif was identified in the secondary structure of DyP, within two sets of anti-parallel β-sheets located between α-helices above the distal area of the heme. Although this motif has not been identified in any other registered proteins, the β-barrel fold is ferredoxin-like and is distinct from motifs found in all other general heme peroxidases. The *Bt*DyP from *Bacteroides thetaiotaomicron* is the second reported tertiary structure found for a DyP-type peroxidase. *Bt*DyP consists of an N-terminal domain and a C-terminal domain, which are likely related by duplication of an ancestral gene, as inferred from the conserved topology of the domains. Unfortunately, the Reinheitzahl (Rz) value was very low (approximately 0.18), suggesting low levels of heme in the expressed protein. Previous studies have reported that the characteristic electron density of heme is not seen in the *Bt*DyP structure. Thus, the crystal structure of *Bt*DyP is considered an apo-structure, without heme. However, a putative heme binding site was predicted and superimposed on the protein, based on the high-resolution crystal structure of heme-bound horseradish peroxidase. The TyrA protein from *Shewanella oneidensis* was the third tertiary structure published for DyP-type peroxidases. This protein also contains an N-terminal distal domain and a C-terminal proximal domain. Heterologously expressed TyrA is also considered an apo-structure, without heme. However, the tertiary structure of a heme-bound TyrA protein was resolved using hemin chloride and compared with the apo form of TyrA. The most apparent changes between the apo and holo forms involved side-chain orientations (i.e., at E154, V228, and N229). Zubieta and colleagues proposed that the alternate conformation of TyrA generated by

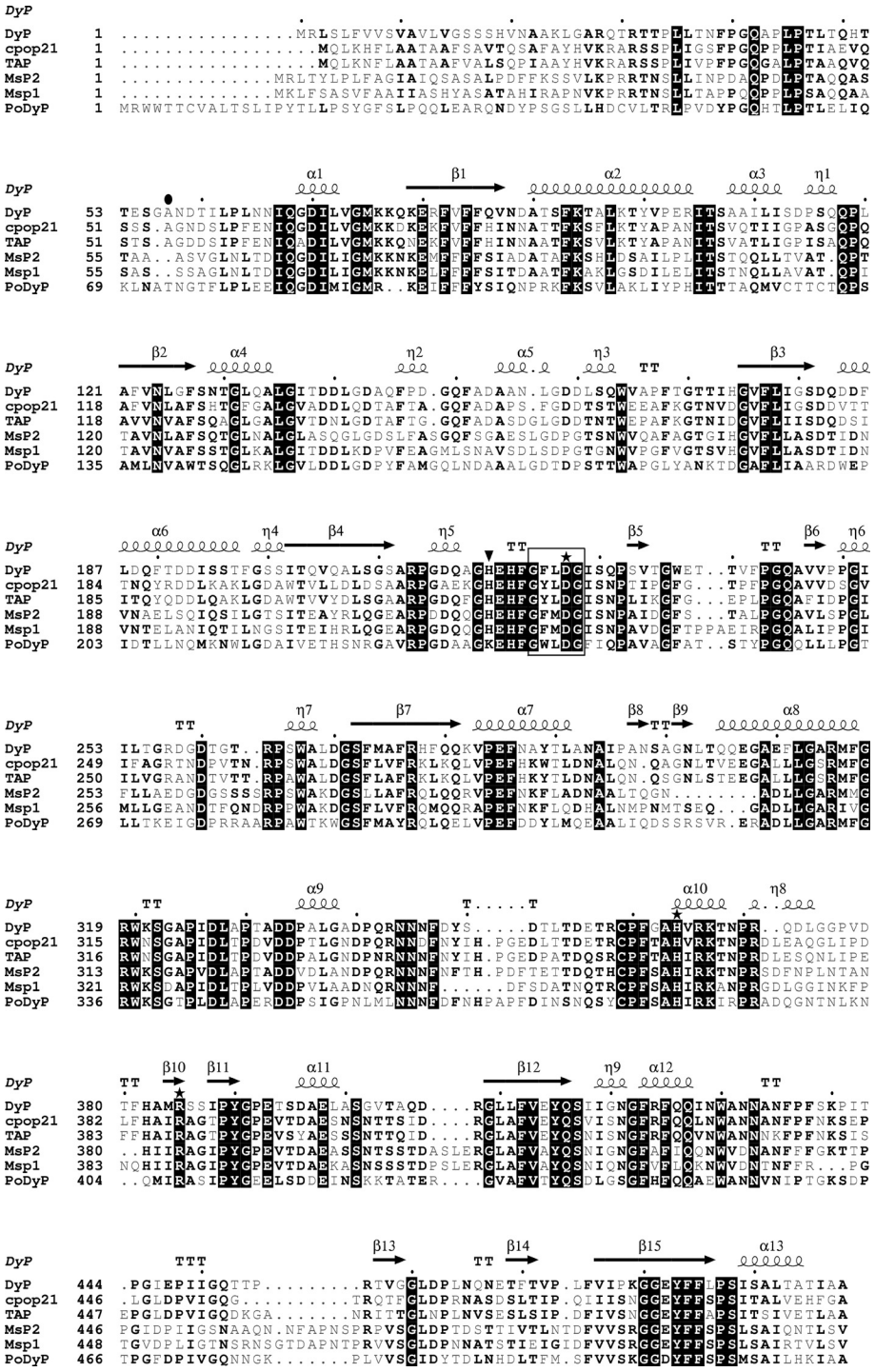

Figure 2. Multiple sequence alignments of DyP and DyP-type peroxidases, including a hypothetical peroxidase. In this figure, all sequences are displayed as not mature sequences but full length sequences before processing. The sequence alignments were performed using Clustal W [104] and ESPript [105]. The secondary structure of DyP is shown in the top column. Abbreviations: α, α-helix; β, β-sheet; η, 3_{10}-helix; TTT, α-turn; TT, β-turn. Perfect match residues are displayed in white on black. Similar amino acid residues (i.e., in four of six proteins) are displayed as bold, black typeface. The N-terminal residue of mature DyP is displayed as a closed circle. The D171, H308 and R329 residues of DyP are displayed as stars. The H164 residue of DyP is displayed as an inverted triangle. The GXXDG motif is boxed. The DyP sequence was obtained from DDBJ AB013135, while the cpop21, TAP, MsP2, MsP1 and PoDyP sequences were obtained from UniProt (i.e., accession numbers P87212, Q8NKF3, B0BK72, B0BK71 and Q0VTU1, respectively). Similar amino acid groups were classified in ESPript, with H, K and R defined as polar positive; D and E defined as polar negative; S, T, N and Q defined as polar neutral; A, V, L, I and M defined as non-polar aliphatic; F, Y and W defined as non polar aromatic; P and G; and C.

the E154 is necessary to avoid steric clashes with the heme propionate. In addition, the N229 was proposed to be a rotamer that forms a hydrogen bond with one of the heme propionate groups. However, these hypotheses have not yet been experimentally confirmed. Although reconstituted TyrA exhibited RB5 decolorizing activity, the specific activity and k_{cat} values were approximately 1000-fold and 50-fold lower, respectively, than those of DyP. These findings suggest that the peroxidase activity of TyrA may be higher in the presence of the physiological substrate under optimized conditions.

Additionally, two moderately homologous proteins (i.e., $10 < \text{Z-score} < 20$), including TT1485 (PDB code 1vdh) and putative chlorite disumutase (PDB code 1t0t) were detected via the secondary structural

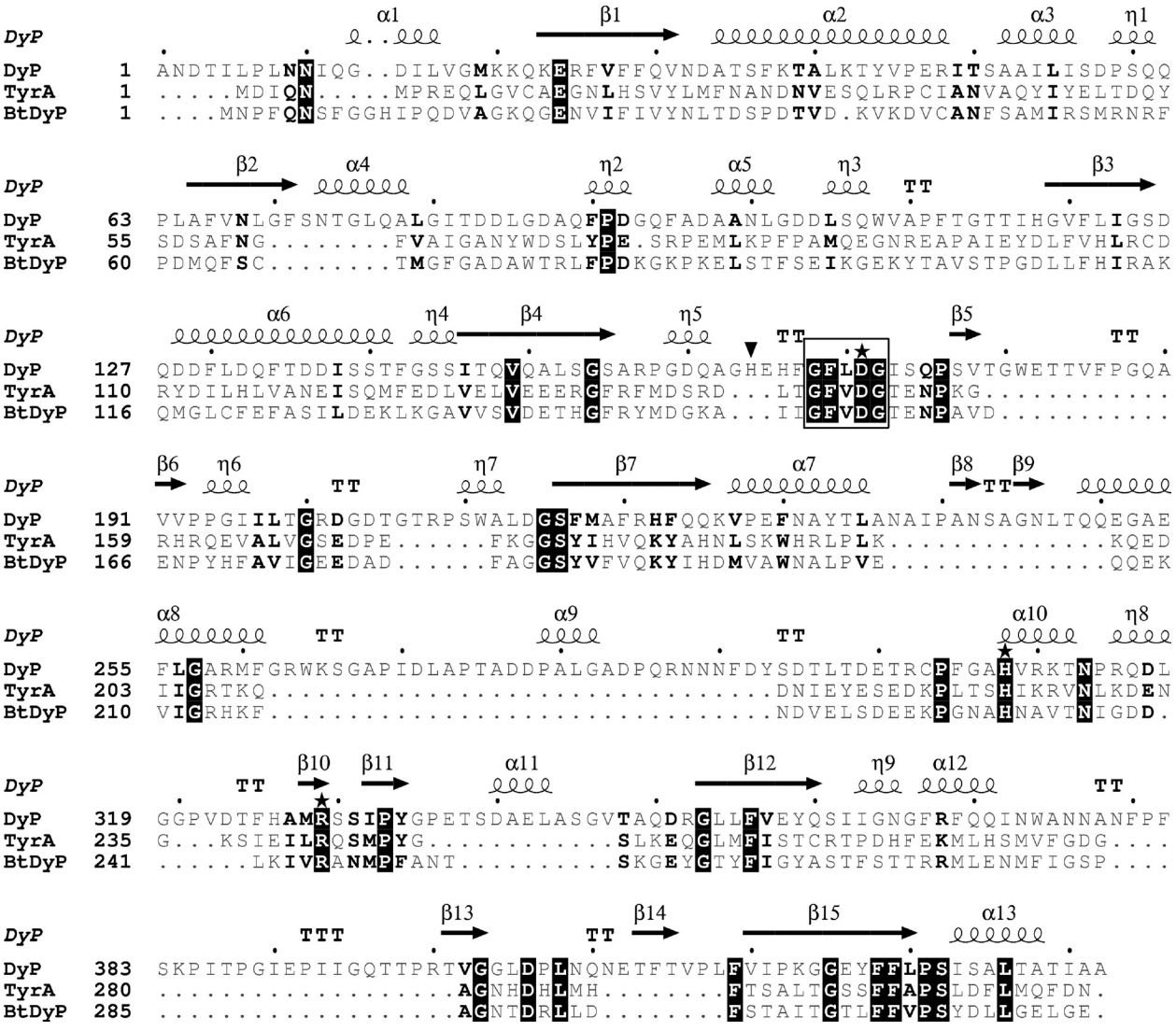

Figure 3. Multiple sequence alignments of tertiary homologous proteins with mature DyP. The main abbreviation and symbols were defined as in Figure 2. The sequences of TyrA and *Bt*DyP corresponded to 2hag and 2gvk in PDB accession numbers, respectively. Although the N-terminal residues of PDB 2hag and 2gvk are glycines (G), G was reported to be the remaining residue (i.e., with zero indicating the residue number in the original report [40]) of the expression tag. Therefore, the N-terminal G has been omitted from this figure. The alignment indicates that DyP contains several more insertion sequences than TyrA and *Bt*DyP.

prediction analysis described above. The TT1485 was identified in *Thermus thermophilus* HB8 and was thought to function as a novel heme peroxidase that detoxified H_2O_2 within the cell [47]. However, no experimental data have demonstrated such activity other than oxygen generation in the presence of H_2O_2. Similarly, the functional characteristics of the 1t0t protein remain unknown and no further study has examined this protein. Therefore, it remains unclear whether TT1485 and 1t0t comprise members of a new subfamily of DyP-type peroxidase proteins.

Although DyP is a monomer, the *Bt*DyP, TyrA, and TT1485 proteins are a hexamer, a dimer, and a pentamer, respectively. Sequence alignments demonstrate that some of the residues involved in oligomerization in *Bt*DyP and TyrA are adjacent to insertions in DyP, as shown in Figure 3. These insertions may influence the quaternary structure of the proteins, with insertions favoring a monomeric form. On the other hand, MsP1 and MsP2 are dimers, even though the primary structures of these proteins are homologous to that of DyP (Fig. 2). These findings suggest that insertion sequences do not necessarily affect quaternary structure formation. At the least, the putative active sites and conserved amino acid residues are not restricted to the inter-molecular surface (i.e., the oligomerization interface). Thus, domains important for DyP activity may be expressed in monomers. In fact, DyP and TAP monomers display enzymatic activities. All proteins, with the exception

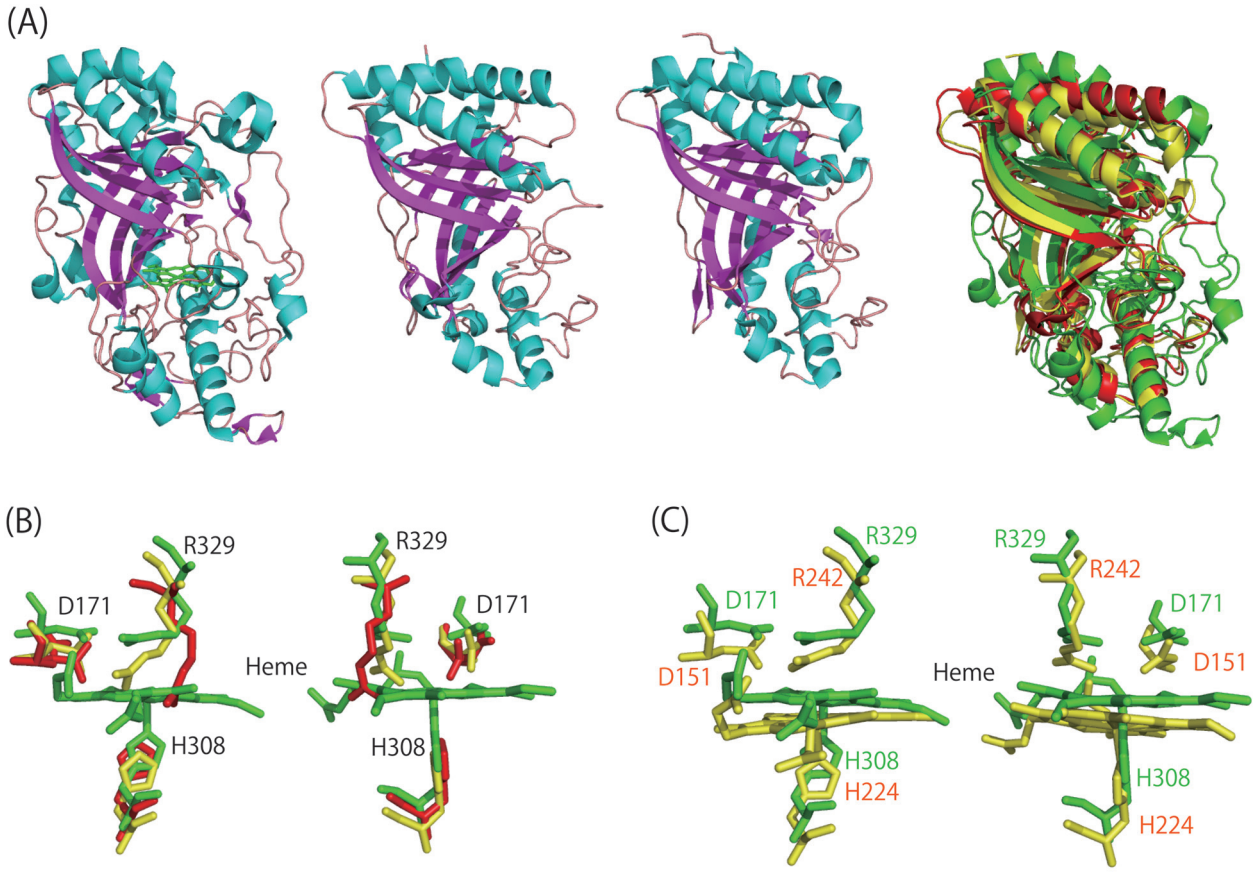

Figure 4. Tertiary structural comparison of all solved DyP-type peroxidases using PyMol software. (A) Whole structures of DyP, *Bt*DyP, TyrA and structural alignments, shown in order from left to right. The α-helices and β-sheets are shown in blue and violet, respectively. In the structural alignment, DyP, *Bt*DyP and TyrA are shown in green, red and yellow, respectively. (B) Structural alignment of the region surrounding heme. With the exception of DyP, the proteins are shown as apo-forms without heme. Colors were used as described in A. The DyP residue numbers are shown. Views from two different directions emphasize the angles of D and R, respectively. (C) Structural alignment of the region surrounding heme (i.e., between DyP (green) and reconstituted TyrA (yellow)). Residues of DyP and reconstituted TyrA are shown in green and orange, respectively. Other abbreviations were used as described in B.

of DyP, form apo structures (i.e., structures that contain few or no heme molecules) during heterologous expression. In the next section, I will discuss regions surrounding the bound heme molecules, which are considered important for enzymatic function.

Heme-surrounding regions

Structural alignments of the regions surrounding heme molecules are shown in Figure 4B. The DyP, *Bt*DyP, and TyrA proteins contain three conserved residues [i.e., D171(DyP)/D157(*Bt*DyP)/D151(TyrA), R329(DyP)/R245(*Bt*DyP)/R242(TyrA) and H308(DyP)/H231(*Bt*DyP)/H224(TyrA)]. It is noteworthy that the R245 and R242 are absolutely bent compared to the R329. As the R329 is thought to assist with the formation of compound I, this structural bend may contribute to the low activity of TyrA. In particular, the R245 is extremely bent and seems to penetrate the heme molecule. This bend may contribute to the lack of RB5-decolorizing activity by *Bt*DyP. The D171 plays an important role (as an acid/base catalyst) in the formation of compound I [17]. However, the D157 and D151 seem to rotate toward the heme surface, compared with the D171, thereby hampering the formation of the Fe-O intermediate. In the case of PDB 2iiz that had been reconstituted with heme to form an holoprotein, the bend at R242 was drastically improved but the angle of D151 relative to the heme surface was retained (Fig. 4C). Overall, the D171 is important for DyP activity and, along with the G172, has been conserved among all members of the DyP-type peroxidase family. Therefore, I will refer to these residues as the GXXDG motif (Figs. 2 and 3). In addition, the proximal histidine residue (i.e., H308, the fifth ligand of the heme molecule) plays an important role in heme binding. Both H231 and H224 seem reasonable candidates for heme ligands. However, no residues corre-

sponding to D171 or R329 have been identified in TT1485, suggesting the absence of an active site that degrades hydrogen peroxide. The result supports the supposition that TT1485 is most likely a member of the novel superfamily of DyP-type peroxidases.

Phylogenetic analysis of DyP-type peroxidases

Most studies have assessed homology by creating phylogenetic trees based on primary protein sequences. As shown in Figure 5, proteins sharing primary structure homology with DyP form a group (boxed) distinct from the plant peroxidase superfamily (shown in the dotted-line box). Interestingly, proteins sharing tertiary homology with DyP (TyrA and *Bt*DyP) are members of a different group within the phylogenetic tree. Thus, future studies should classify proteins on the basis of tertiary homology to generate more accurate information. Classical heme peroxidases within the DyP-type peroxidase family may require re-categorization when analyzed using a combination of tertiary structural and primary structural methods.

Postulates on other candidate proteins

As previously mentioned, numerous proteins with homology to DyP have been detected via general homology searches. However, most of these proteins are hypothetical or have unknown functions. Thus, appropriate databases are necessary to accurately determine the primary, secondary, and tertiary structures of such proteins. As described in a previous section, one of the most reliable databases is PeroxiBase (http://peroxibase.isb-sib.ch/), in which apparent peroxidases (including putative peroxidases) have been both automatically and manually selected. According to the classification used by PeroxiBase, the DyP-type peroxidase family is further phylogenetically divided into four types (A, B, C, and D). However, TyrA, *Bt*DyP, MsP1, and MsP2 have not yet been registered and will possibly appear in the next updated version. Alignment searches of the Pfam database and conserved domain database (CDD) [48] provide clues as to the characteristics of DyP-type peroxidases. When the amino acid sequence of DyP has been used to search for other DyP-type peroxidases, the 26 members of the corresponding COG2837 family of predicted iron-dependent peroxidases have been detected. According to the alignment search, five of the 26 members were deduced to be putative peroxidases or their precursors [42, 49–54]. One candidate, YcdB, was reported to use a unique secretion mechanism [42]. Twenty-one of the

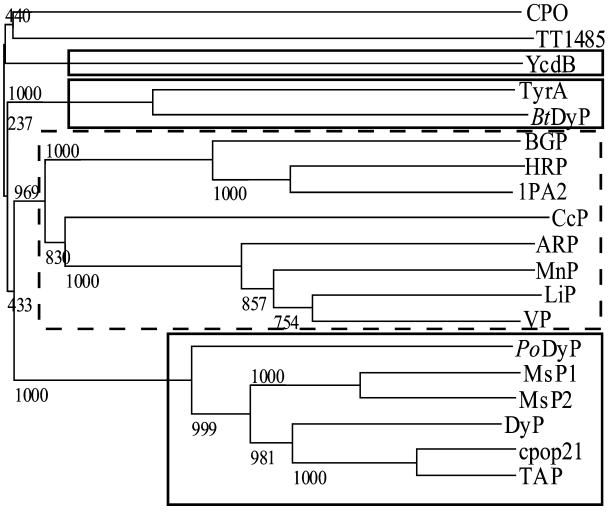

Figure 5. Phylogenetic comparison of probable DyP-type peroxidase family members (i.e., boxed) and classical plant peroxidase superfamily (i.e., dotted-line boxed) members, using the neighbor-joining method [106]. Numbers on the phylogenetic tree indicate bootstrap values for 1000 replicates. DyP, DyP from *Thanatephorus cucumeris* (PDB 2d3q); TyrA (PDB 2iiz) and *Bt*DyP (PDB 2gvk) are from *Shewanella oneidensis* and *Bacteroides thetaiotaomicron* VPI-5482, respectively; TT1485 (PDB 1vdh), a heme binding protein from *Thermus thermophilus*; TAP, peroxide-dependent phenol oxidase from *Termitomyces albuminosus* (UniProt Q8NKF3); cpop21, hypothetical peroxidase from *Polyporaceae* sp. (UniProt P87212); MsP2 and MsP1 are from *Marasmius scorodonius* (UniProt B0BK72 and B0BK71, respectively); *Po*DyP from *Pleurotus ostreatus* (UniProt Q0VTU1); YcdB from *E. coli* K12 (gi2506638); CcP, cytochrome *c* peroxidase from *Saccharomyces cerevisiae* (PDB 1krj); BGP, barley grain peroxidase from *Hordeum vulgare* (PDB 1bgp); 1PA2, *Arabidopsis thaliana* peroxidase A2 (PDB 1pa2); HRP, horseradish peroxidase from *Armoracia rusticana* (PDB 1atj); ARP, *Arthromyces ramosus* peroxidase (PDB 1arp); MnP, manganese peroxidase from *Phanerochaete chrysosporium* (PDB 1mnp); VP, versatile peroxidase from *Pleurotus eryngii* (PDB 2boq); LiP, lignin peroxidase from *Phanerochaete chrysosporium* (PDB 1lga); CPO, chloroperoxidase from *Caldariomyces fumago* (PDB 1cpo).

26 members were hypothetical (i.e., uncharacterized) proteins, because most of these proteins were automatically translated during genomic sequencing. Interestingly, the H308 and R329 were perfectly conserved among all sequences. The D171 was also conserved in 25 of 26 sequences. In the exception, a hypothetical protein [55], the aspartic acid was replaced with glutamic acid; however, this replacement is unlikely to have a significant effect because both aspartic acid and glutamic acid contain carboxyl groups on their side chains. Overall, this conserved domain analysis supports the notion that these residues are important for the function of DyP-type peroxidases. In contrast, the H164 and H166 were not conserved among the 26 sequences, although the H164 is known to play an important role in DyP folding and heme binding [35]. YcdB shares partial primary homology with DyP, but this level of homology may

be insignificant from the viewpoint of the entire primary sequence.

YcdB and secretion mechanisms

YcdB is a dimeric protein that is produced by *E. coli* and contains a heme cofactor [42]. It is the only protein detected in a CDD alignment search for DyP and exhibits peroxidase activity at low pH, similar to DyP. Notably, YcdB is thought to use the twin-arginine translocation (TAT) secretion system (a recently described secretion pathway that differs from that of the general *sec* system) [56, 57], which delivers folded proteins across biological membranes [58]. Thus, heme molecules may be folded in the cytoplasm and translocated to the periplasm. Together, these data suggest that YcdB may function as a periplasmic peroxidase at low pH. In contrast, DyP is thought to use the general *sec* system of protein secretion [59–61]. The difference in transport systems used by DyP and YcdB suggests that the DyP-type peroxidase family should be further classified to subfamilies, according to the secretion system employed.

Structural analyses of each dimension (primary, secondary, tertiary and quaternary) have provided useful information about DyP-type peroxidases, while also introducing many puzzling conundrums.

Substrate specificity and the reaction mechanisms of DyP

As mentioned above, DyP-type peroxidases possess unique structures that differ from those of general peroxidases. These unique structures may reflect the catalytic mechanisms used by DyP-type peroxidases. To date, limited research has focused on the DyP-type peroxidase family, and the catalytic cycles or pathways used by these proteins have not yet been elucidated. In the following sections, several possible mechanisms will be proposed by which DyP-type peroxidases degrade substrates and catalyze changes in anthraquinone compounds, based on current knowledge of the DyP protein produced by *T. cucumeris*.

Degradation of Reactive Blue 5 by DyP

Fungal peroxidases, such as LiP, MnP, and VP, have been thoroughly studied and several reaction mechanisms have been proposed [62–64]. These enzymes, like DyP, exhibit wide substrate specificity. Many studies have reported azo degradation by fungal peroxidases [8, 65–73], whereas few studies have reported anthraquinone degradation by peroxidases [74–76]. On the other hand, DyP degrades several anthraquinone dyes, including Reactive Blue 5 (RB5, a synthetic dye and xenobiotic) [20, 29]. The reaction products of RB5 treated with DyP have been analyzed using electrospray ion mass spectrometry (ESI-MS), and nuclear magnetic resonance, and a reasonable degradation pathway has been proposed [77]. As shown in Figure 6, four reaction products were detected (i.e. phthalic acid, product 2, product 3, and 2,2'-disulfonyl azobenzene). It is reasonable that the reaction products except phthalic acid were generated by usual peroxidase reaction. On the other hand, it is interesting that phthalic acid was generated during this degradation cascade. The anthraquinone frame is not degraded during general peroxidase reactions because the frame does not have a hydrogen molecule suitable for radicalization by peroxidase as mentioned above. However, some anthracyclines possess hydroquinone moieties that offer potential peroxidase targets. For instance, lactoperoxidase is thought to oxidize hydroquinone-containing anthracyclines in the presence of H_2O_2 and NO_2^- [74], and ferrylmyoglobin reportedly promotes oxidative degradation of hydroquinone-containing anthracyclines [78]. In addition, horseradish peroxidase (HRP) indirectly degrades anthracycline by acting on hydroquinone [75]. These findings support the notion that peroxidases can easily convert hydroquinones to hydroxyl radicals, which may then be used in general peroxidase reactions [76, 79, 80]. In other words, it is likely difficult for peroxidases to degrade hydroquinone-free anthraquinones and no such degradations have been reported to date. Instead, recent studies suggest that laccase (a type of oxidase that does not depend on H_2O_2) plays an important role in the decolorization of anthraquinone dyes [81–84]. However, the specific decolorization mechanism remains unclear. There has only a single report on the crude enzymatic degradation of an anthraquinone compound [85]. The authors hypothesized that questin, an anthraquinone compound, was oxidized to a lactone intermediate by an oxygenase, generating NADPH and O_2, via a Baeyer-Villiger type mechanism. The resulting lactone intermediate was then hydrolyzed by another lactone hydrolase. Thus, two types of intracellular enzymes (i.e., oxygenases and hydrolases) were required to hydrolyze anthraquinones. Therefore, oxidative reactions may not be capable of cleaving the anthraquinone frame, indicating that water molecules are required for ring-cleaving reactions. It may be that generation of phthalic acid by DyP suggests that DyP acts as a hydrolase rather than a peroxidase, although H_2O_2 is required for this reaction.

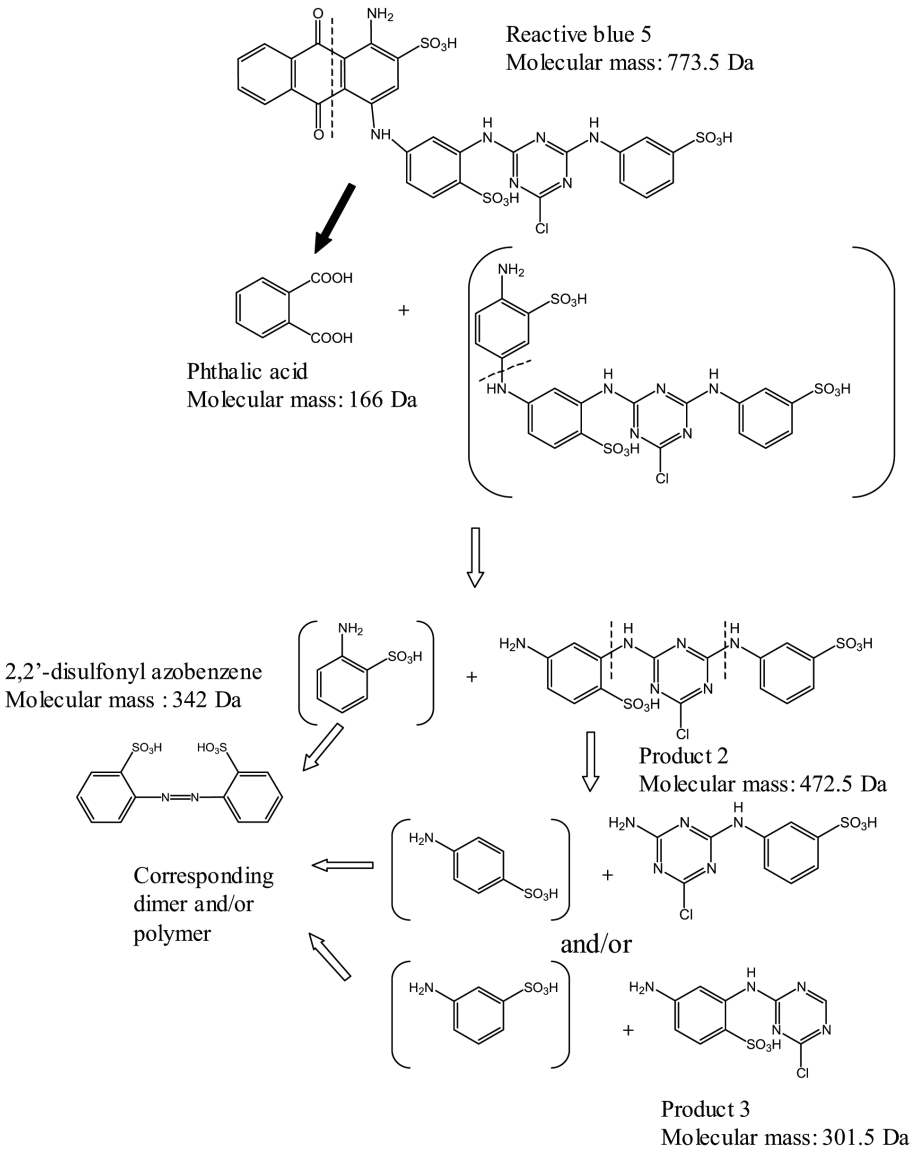

Figure 6. Degradation pathway of RB5 by DyP. Dotted lines indicate putative cleavage sites. Molecules shown in parenthesis were not detected by ESI-MS. Peroxidase and hydrolase reactions are shown in white and black arrows, respectively. Dimerization and polymerization of aminobenzenesulfonate occur during general peroxidase reactions [107] and one of them is displayed as 2,2'-disulfonyl azobenzene.

Catalytic cycle

As DyP exhibits a unique type of reaction in addition to peroxidase activity, in this section, a probable model of the catalytic cycle of DyP is proposed. The general heme peroxidase catalytic cycle should be briefly reviewed prior to discussing the catalytic model of DyP. The catalytic cycle of general peroxidases has been well studied. Although several variations have been reported, resting, compound I and compound II states are believed to show around the cycle [1]. Compound I represents the intermediate that holds the Fe^{4+} oxoferryl center [86] and a porphyrin-based cation radical [87, 88], whereas compound II is obtained when one electron has been removed from compound I. In general, the overall heme peroxidase catalytic cycle proceeds as follows:

$$\text{Resting peroxidase} + H_2O_2 \rightarrow \text{Compound I} + H_2O$$

$$\text{Compound I} + AH_2 \rightarrow \text{Compound II} + AH\bullet$$

$$\text{Compound II} + AH_2 \rightarrow \text{Resting peroxidase} + AH\bullet + H_2O$$

Finally, peroxidase catalyzes the following reaction:

$$2AH_2 + H_2O_2 \rightarrow 2H_2O + 2\,AH\bullet$$

Enzymes that catalyze the above reaction are defined as peroxidases, even if the catalytic cycle or intermediates obtained differ from those seen in the standard cycle of general heme peroxidases. The resulting AH● is further converted to various products by non-enzymatic reactions (e.g., radical coupling reactions). The absorption spectra of DyP and other representative peroxidases are summarized in Table 1.

Table 1. Absorption maxima of resting state and oxidized intermediates of several peroxidases

Name	resting	compound I	compound II[1)]	ref.
DyP	**406**[2)], 506, 636	**401**, 530, 556, 615, 644(sh)[3)]	**399**, 529, 555, 615, 644(sh)	[17]
LiP	**408**, 496, 630	**408**, 550, 608, 650	**420**, 525, 556	[64]
MnP	**406**, 502, 632	**407**, 558, 617, 650	**420**, 528, 555	[62]
HRP	**403**, 498, 640	**400**, 525(sh), 577, 622(sh), 651	**420**, 527, 555	[1]
YcdB[4)]	**406**, unassigned	**414**, 530, 555, 603	no description	[42]

[1)] In the case of DyP, these absorption maxima correspond to compound I, not compound II.
[2)] Bold typefaces indicate wavelengths that correspond with Soret bands.
[3)] sh, shoulder peak.
[4)] An undetermined amount of H_2O_2 was added to YcdB to form compound I.

The characteristics of resting DyP and DyP in the presence of one equivalent of H_2O_2 resemble those of the resting enzyme and compound I, respectively, for other peroxidases, suggesting that the primary reaction state of DyP is similar to that of compound I for other peroxidases. Previous studies have shown that DyP forms a resting enzyme and compound I, but compound II has not been detected [17]. Although DyP does not form a typical compound II state, this enzyme still satisfies the definition of a peroxidase (i.e., $2AH_2 + H_2O_2 \rightarrow 2H_2O + 2\ AH\bullet$). Moreover, this characteristic may explain the unique function of DyP. The process of generating compound I differs in DyP, as compared with other general peroxidases, particularly in relation to the non-conserved amino acid residues of general peroxidases. The heme-surrounding region of DyP is unique, as shown in Figures 4B and 4C. A proximal histidine is observed, but the distal histidine is absent, indicating that heme binding occurs normally, whereas the H_2O_2 reaction proceeds by a unique mechanism. Recently, a part of the DyP catalytic cycle was proposed [17], in which the D171 replaces the distal histidine seen in general peroxidases. This is important to note, because the optimum pH of DyP (i.e., pH 3) is lower than that of general peroxidases such as HRP (i.e., pH 6). In addition, the pKa values for histidine and aspartic acid are 6.0 and 3.8, respectively. These residues are key components of acid-base catalysts. Similar results have been reported for peroxidase-type reactions of YcdB [42]. It is important to note that the essential role of the R329 has not been defined. In general peroxidases, the arginine residue within the heme-distal area is essential and plays an important role in peroxidase reactions. According to the model catalytic cycle for DyP [17], the R329 seems to be necessary for charge stabilization; however, the R329K mutant partially conserved peroxidase activity (unpublished data). Moreover, the R329 has a different angle to the heme plane as compared with an essential arginine of the plant peroxidases superfamily (e.g., compare Figs. 1 and 4). Thus, R329 may not always be necessary for the reaction. Heme propionate also plays an important role in the catalytic cycle [89]. In the case of TyrA, it has been proposed that the propionate on pyrrole IV rotates into a less common rotamer to accommodate a hydrogen bonding network between the propionic carboxylate, R206, R242, a water molecule , and the propionate of pyrrole III [41].

At this point, it is appropriate to describe the role of water molecules generated from H_2O_2. During each catalytic cycle of a general peroxidase reaction, two water molecules are released and one is generated from H_2O_2. Previously, it was thought that the released water molecules played no further role in the catalytic cycle, and that the resulting oxoferryl intermediate was reduced by the substrate and the enzyme returned to the resting state. In our present study, an oxoferryl intermediate of DyP following the addition of H_2O_2 was observed, indicating that the initial stage of the DyP-mediated enzyme reaction resembles that of other studied peroxidases. In the case of DyP, it appears that compound I and H_2O form a complex (i.e., Wet-I). During DyP-mediated degradation of the anthraquinone frame, the H_2O molecule appears to attack the carbonyl on the anthraquinone frame before being released from the Wet-I intermediate. Jones reported that this water molecule provided the structural basis for a redox pathway-switching mechanism [90]. Although his report referred to redox activity in the presence or absence of the water molecule, it is likely that the water molecule is involved in the hydrolysis process, given that DyP performs a unique reaction in addition to the peroxidase-type reaction. Consequently, the potential catalytic cycle of DyP is shown in Figure 7. Wet-I is similar to the wet-compound I described by Jones. However, one H_2O molecule attacks one of two carbonyl sites of the anthraquinone ring; thus, this process should be repeated to generate phthalic acid. Phthalic acid would then be generated for each molecule B that fits the active site, although several molecules (i.e., A, B, C or C-C) may fit the active site after the second catalytic cycle. The yield of phthalic acid was not very

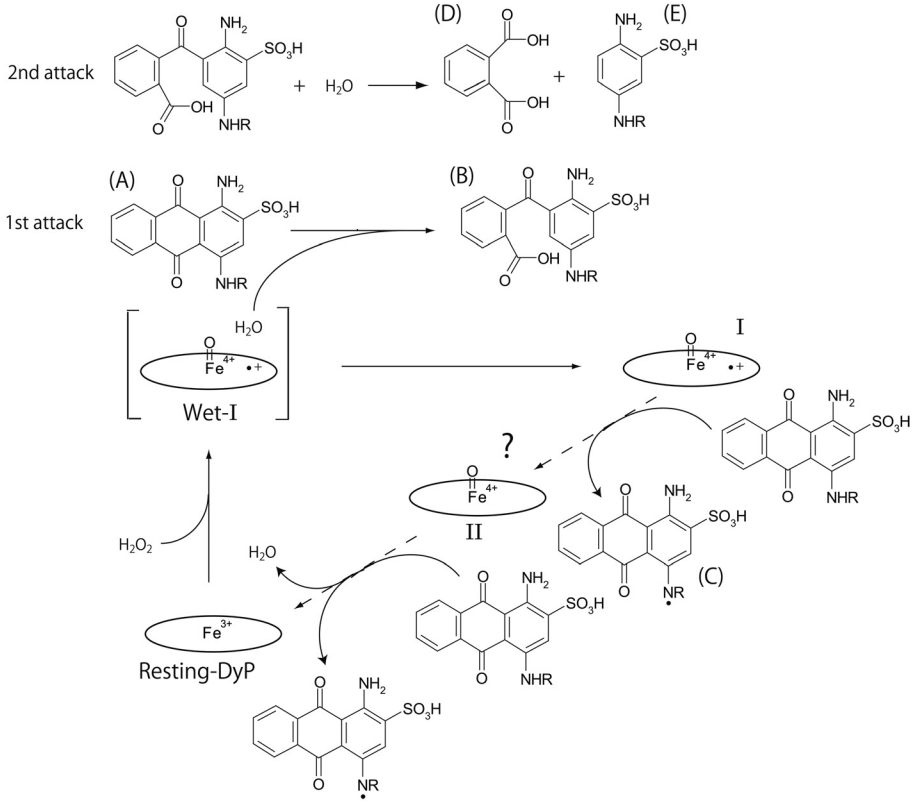

Figure 7. Putative catalytic cycle of DyP with an anthraquinone model compound. The enzyme intermediate shown in bracket (Wet-I) is the deduced state on the basis of the hypothetical concept proposed by Jones [90]. Compounds I, II and the resting state are indicated as I, question mark, and Resting-DyP, respectively. As compound II has not yet been detected, pathways from compound I to the resting state via hypothetical compound II are shown in dotted arrows. Although molecule A is the initial substrate of the first catalytic cycle, several other molecules (e.g., A, B, C or C-C) are possible in the following cycle. Phthalic acid is generated when molecule B is the substrate. The reaction of DyP shown here is one of many possible patterns, involving several potential molecules. Please also note that radical coupling reactions are chemical, rather than enzymatic.

high (7% by HPLC), even though RB5 was completely degraded. Thus, DyP may simultaneously catalyze peroxidase and hydrolase activities.

Functional relationship between peroxygenase P450, chloroperoxidase, and DyP

Recently, two unique P450 s ($P450_{SP\alpha}$ and $P450_{BS\beta}$) were described [91]. These enzymes have been categorized as members of the monooxygenase P450 superfamily on the basis of their primary sequences, tertiary homologies, and spectroscopic properties [92–94]; however, their catalytic reactions differ from those of general P450 s. Reactions by these enzymes are advanced in the presence of H_2O_2, suggesting that they function as peroxidases rather than as typical P450 monooxygenases. Therefore, $P450_{SP\alpha}$ and $P450_{BS\beta}$ are now called peroxygenase P450 s. Compound I formation by peroxygenase P450 has not been confirmed, although this activity is thought to be important for the formation of highly reactive intermediates such as those formed by DyP. Interestingly, P450 s are thought to form an intermediate with a fatty acid, R242, and H_2O_2. In this case, fatty acids function as acid-base catalysts (e.g., as does the D171 residue of DyP) at low pH. Similarly, the E183 of chloroperoxidase functions as an acid-base catalyst at low pH. Moreover, chloroperoxidase is considered to be a functional hybrid of heme peroxidase and cytochrome P450 [12]. However, DyP-type peroxidases and peroxygenases and chloroperoxidase differ in that the heme ligand of DyP-type peroxidases is histidine, whereas the heme ligand of the others is cysteine. With regard to the acid-base catalyst of compound I, DyP-type peroxidases more closely resemble chloroperoxidases and peroxygenases, rather than general heme peroxidases. On the other hand, DyP-type peroxidases resemble neither haloperoxidases nor members of the P450 family. It is noteworthy that the intermediate formed by fatty acids and peroxygenases may also be formed by DyP-type peroxidases, suggesting that these enzymes possesses oxygenase activity in addition to peroxidase activity.

Physiological roles of DyP-type peroxidases

In animals, the relationship between peroxidases and disease has been widely studied [14, 15]. In particular, the roles of peroxisomes in aging, Alzheimer's disease, and Parkinson's disease are thought to involve peroxidases [13, 95–97]. However, in the plant peroxidase superfamily, generation of peroxidases may have promoted the removal of H_2O_2. For instance, ascorbate peroxidase plays an important role in intra-

cellular H_2O_2 scavenging by ascorbate [98]. Most basidiomycetes, especially white-rot fungi, degrade lignin, an important component of wood, by producing LiP, MnP, and VP. This allows for the relatively easy assimilation of nutrients, such as glucose from cellulose.

As mentioned above, DyP decolorizes synthetic dyes. However, these dyes are not essential substrates because they are xenobiotics, rather than natural compounds. The natural role of DyP is unknown, because peroxidase activity has been observed outside the cells. Arylalcohol oxidase, which generates H_2O_2, is secreted by *T. cucumeris* Dec 1 [99], suggesting that DyP is capable of extracellular peroxidase activity. This characteristic is similar to those of LiP, MnP, and VP. Whereas *T. cucumeris* exhibits lignin-degrading activity, the lignin-degrading activity of DyP is relatively low [100]. It is plausible that DyP decomposes extracellular compounds to protect against hazardous materials such as xenobiotics. Similarly, the roles of TyrA and *Bt*DyP are unknown and may differ from that of DyP because they are not extracellular enzymes.

Perspectives and frontiers

DyP-type peroxidases comprise a novel peroxidase family, with DyP being the best characterized. The tertiary structures of most DyP-type peroxidases are unique, compared with those of other general peroxidases. In particular, the H_2O_2 binding site (i.e., the acid-base catalyst) of DyP is aspartic acid, whereas the H_2O_2 binding sites of other general heme peroxidases are found at histidine. Interestingly, the binding sites of DyP-type peroxidases resemble those of chloroperoxidase (i.e., glutamic acid). Chloroperoxidase resembles peroxygenase $P450_{BS\beta}$. Thus, the relationships between members of the DyP-type peroxidase family and chloroperoxidases or peroxygenases should be carefully considered. DyP-type peroxidases are likely distributed among various organisms, as DyP and *Bt*DyP are produced by a fungus and a bacterium, respectively. Moreover, according to a homology search of the InterPro (http://www.ebi.ac.uk/interpro/) database, DyP-type peroxidases are distributed among the kingdom Metazoa. This is surprising because other plant peroxidases are conserved strictly within classes, whereas DyP-type peroxidases appear to be universally conserved in the living world. In general, molecular structures are more reflective of evolutionary relationships than are classical phenotypes. Therefore, the basis for the definition of taxa has progressively shifted from the organismal to the cellular to the molecular level. Woese and colleagues proposed a new taxon called a "domain" above the level of the kingdom, that is, the Bacteria, the Archaea and the Eucarya [101]. According to the proposal, DyP-type peroxidases have been discovered from the both domains of the Bacteria and the Eucarya. This means that the prototype of DyP-type peroxidases has an ancient root before phylogenetic branching between the Bacteria and the Eucarya. No other peroxidases, in particular in the plant peroxidase superfamily, have such a long evolutionary history. Thus, DyP-type peroxidases must be suitable materials to research the molecular evolution of proteins. Tertiary structural and functional analyses are needed to confirm this hypothesis. In any case, the function of DyP-type peroxidases suggests that these proteins possess general peroxidase activities, as well as another type of enzymatic activity, such as hydrolase activity. The versatility of these enzymes may be useful in solving various scientific, environmental, and industrial problems such as recalcitrant waste water contaminated with xenobiotics, although the true physiological roles of these proteins remain unknown. Recent studies have made much progress toward determining the productivity and stability of DyP [31, 102, 103].

Throughout this review, I have described DyP-type peroxidases as members of a novel family of heme peroxidases. I furthermore propose that this family should be divided into various subfamilies. For instance, the secretion system for DyP differs from that for YcdB, although both enzymes exhibit peroxidase activity and possess DyP-type conserved residues. It is our hope that future research will focus on the molecular evolution and the intriguing properties of this novel peroxidase family.

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