Characterization of Dye-decolorizing Peroxidase (DyP) from Thermomonospora curvata Reveals Unique Catalytic Properties of A-type DyPs

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Dye-decolorizing peroxidases (DyPs) are a new family of heme peroxidases, which has received much attention due to their potential applications in lignin degradation. A new DyP from Thermomonospora curvata (TcDyP) was identified and characterized. Unlike other A-type enzymes, TcDyP is highly active toward a wide range of substrates including model lignin compounds, in which the catalytic efficiency with ABTS ($k_{cat} / K_{m}^{app} = (1.7 \times 10^{6}) \, m^{-1} \, s^{-1}$) is close to that of fungal DyPs. Stopped-flow spectroscopy was employed to elucidate the transient intermediates as well as the catalytic cycle involving wild-type (wt) and mutant TcDyPs. Although residues Asp$^{220}$ and Arg$^{327}$ are found necessary for compound I formation, His$^{312}$ is proposed to play roles in compound II reduction. Transient kinetics of hydroquinone (HQ) oxidation by wt-TcDyP showed that conversion of the compound II to resting state is a rate-limiting step, which will explain the contradictory observation made with the aspartate mutants of A-type DyPs. Moreover, replacement of His$^{312}$ and Arg$^{327}$ has significant effects on the oligomerization and redox potential ($E^0$) of the enzyme. Both mutants were found to promote the formation of dimeric state and to shift $E^0$ to a more negative potential. Not only do these results reveal the unique catalytic property of the A-type DyPs, but they will also facilitate the development of these enzymes as lignin degraders.

Continuing interest in utilization of renewable biomass for generation of biofuels and fine chemicals has brought enormous attention to lignin that is the most abundant aromatic polymer on earth (1, 2). However, due to its extreme recalcitrance, lignin degradation has to be performed under harsh conditions, such as strong acids and bases and high temperature, which will produce toxics to inhibit downstream treatment of the biomass (3). Thus, methods of mild lignin degradation are being actively sought. A variety of microorganisms have been known to react with and depolymerize lignin. Among them, the fungal system has the highest efficiency using multiple heme- and copper-containing oxidative enzymes (4). Yet no fungal-based biocatalysts have been developed so far due to difficulties in genetic manipulation and protein expression of fungi. Therefore, there is a growing interest in identifying lignin-degrading enzymes from bacterial sources (5). Thermomonospora curvata is a thermophilic actinomycete isolated from composted stable manure (6). It is used for breaking down plant materials for biomass conversion at high temperatures (7, 8). Its genome has recently been sequenced (9).

Dye-decolorizing peroxidases (DyPs)$^2$ are a new family of heme peroxidases that catalyze $H_2O_2$-dependent oxidation of various molecules (10). Although their physiological substrates are unknown, they have been demonstrated to carry out lignin degradation, albeit with a low activity (11–13). Analysis of the genome sequence has revealed that DyPs are mostly present in bacteria, whereas only a limited number exists in fungi and higher eukaryotes (14). DyPs can be subdivided into four types, A, B, C, and D, based on their primary sequences (15). Identities between different types are at most 15%, each having a unique characteristic sequence. For example, the A-type DyPs contain a Tat-dependent signal sequence. However, tertiary structures of all DyPs are surprisingly similar and adopt a ferredoxin-like fold (14). Among the four classes of DyPs, the A-type is studied the least due to its low activity. However, increasing evidence (13, 16), including the present report, suggests that A-type DyPs may have potentials in lignin depolymerization.

The mechanism of DyPs is proposed to be similar to that of plant peroxidases (17, 18). Generally, the catalytic cycle of a

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$^2$ The abbreviations used are: DyP, dye-decolorizing peroxidase; ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); HQ, hydroquinone; MMA, 4-methoxymandelic acid; ICP-OES, inductively coupled plasma optical emission spectrometry; SEC, size exclusion chromatography; TcDyP, DyP from T. curvata; TcDyP-0/TcDyP-I/TcDyP-II, resting state/compound I/compound II of TcDyP; $E^0$, redox potential.

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plant peroxidase includes the resting state and transient intermediates that consist of compound I and compound II. The resting ferric enzyme $[\text{Fe}^{3+}/\text{H}_2\text{O}_2]$ is oxidized by $\text{H}_2\text{O}_2$ to produce a high-valence compound I that is designated as $[\text{Fe}^{4+/\text{H}_2\text{O}_2}/\text{O}]$. Reaction of compound I with 1 eq electrons from a reducing substrate generates a $[\text{Fe}^{4+/\text{H}_2\text{O}_2}/\text{O}]$ intermediate named compound II. Reduction of compound II by a second substrate molecule regenerates the resting state. Although kinetics of these intermediates has been studied with B-type DypB from Rhodococcus jostii (11), it is still unclear which intermediate is involved in the rate-limiting step of the catalytic cycle. Moreover, the catalytic mechanism is yet to be established with A-type DyPs, although detection of intermediates was briefly mentioned recently (19).

Sequence analysis of four types of DyPs (Fig. 1A) has revealed that three residues surrounding the heme region, histidine, aspartate, and arginine, may play important roles in catalysis. Moreover, a homology model (Fig. 1B) was generated for DyP from T. curvata (TcDyP) complexed with heme (less of iron) using an online COACH server (20, 21). Compared with the structures of classical plant peroxidases that primarily contain $\alpha$-helices (e.g. horseradish peroxidase, HRP, Fig. 1C) (22), DyP enzymes are rich in both $\alpha$-helices and $\beta$-sheets and adopt ferrodoxin-like folds. Similar to the proximal histidine in plant peroxidases (His$^{170}$ in HRP), the histidine in DyPs (His$^{312}$ in TcDyP) binds noncovalently with the heme and acts as the 5th ligand to the iron. The distal histidine essential for the plant peroxidases (His$^{42}$ in HRP) is absent from the DyPs, in which it has been replaced with a conserved aspartate (Asp$^{220}$ in TcDyP). This aspartate also forms the so-called GXXDG motif (boxed in Fig. 1A) that has been assigned as the fingerprint of all DyPs (18, 23). In plant peroxidases, the arginine within the heme distal area (Arg$^{38}$ in HRP) is necessary for peroxidase activity. However, the role of arginine in DyPs (Arg$^{327}$ in TcDyP) is still debatable. Although the arginine residue is indispensable for the activity of DypB (24), its substitution with lysine in DyP from Thanatephorus cucumeris Dec 1 retains partial peroxidase activity (23). Nevertheless, the effects of these three residues on formation of the catalytic intermediates, redox potentials, and oligomeric states of DyPs have yet to be determined.

Here we report identification and biochemical characterization of TcDyP (UniProt accession number D1A807). The matured protein was expressed and purified from Escherichia coli. The catalytic cycle of the wild-type (wt) and mutant TcDyPs were studied by steady-state and transient-state kinetics. Roles of Asp$^{220}$, His$^{312}$, and Arg$^{327}$ were identified using a combination of approaches including stopped-flow spectroscopy, spectrophotometric titration, and size-exclusion chromatography (SEC). Moreover, degradation of model lignin compounds by wt-TcDyP was investigated using HPLC and mass spectroscopy (MS). The results are discussed to reveal the unique catalytic property of A-type DyPs.

### Experimental Procedures

**Instruments, Biochemicals, and Chemicals**—All activity assays and steady-state kinetics were performed in a 96-well format on a SpectraMax Plus 384 microplate reader manufactured by Molecular Devices. Transient-state kinetics was performed using an SX20 stopped-flow spectrometer (Applied
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PhotoPhysics Ltd., UK) equipped with a sequential mixing, PDA detector, and monochromator. Spectroelectrochemical titrations were carried out on a Cary 100 Bio UV-visible spectrometer (Agilent) equipped with a temperature controller and magnetic stirring. The redox potentials were measured by a potentiostat (model 650B, CH Instrument). High-pressure liquid chromatography (HPLC) and SEC were performed with a Waters Breeze 2 system equipped with a PDA detector. The electrospRAY ionization mass spectroscopy (ESI-MS) was performed with Waters Acquity TQD. The inductively coupled plasma-optical emission spectrometry (ICP-OES) was carried out on a Varian 720 series system. All chemical and biochemical reagents were of the highest grade commercially available and were used without further purification. Stocks of H$_2$O$_2$ were prepared fresh before the experiments and the concentrations were determined at 240 nm ($\epsilon_{240} = 43.6$ M$^{-1}$ cm$^{-1}$).

Cloning, Expression, and Purification of Matured TcDyP and Its Mutants—The gene fragments of TcDyP were synthesized by IDT using a gBlocks method. They were amplified and assembled together by PCR following standard protocols (25). After the PCR products were purified by gel extraction, they were inserted into the SspI site of pTBSG (26) to generate pPL2014L01, which contains a His$_6$ tag and tobacco etch virus cleavage site at the N terminus. Mutants of TcDyP were generated using the QuikChange from Agilent Technologies according to the manufacturer’s instructions.

The wt-TcDyP were overexpressed in E. coli BL21(DE3) cells (Lucigen). A 160-ml starter culture was grown overnight from a single colony in LB media in the presence of 100 µg/ml of ampicillin at 37 °C with shaking at 225 rpm and then used to inoculate 8 liters of LB medium. When $A_{600}$ reached 0.6, isopropyl 1-thio-β-d-galactopyranoside and hemin chloride were added to a final concentration of 0.2 mM and 30 µg/ml, respectively. The cells were grown at 30 °C for an additional 14 h and then harvested by centrifugation at 5,000 × g for 30 min at 4 °C. The cell pellets were collected and stored at −80 °C.

All of the following steps were carried out at 4 °C. Purification buffers consisted of buffer A (400 mM NaCl and 50 mM potassium phosphate, pH 7.8) and increasing concentrations of imidazole. Buffers B (lysis), C (wash), and D (elution) contained 10, 30, and 250 mM imidazole, respectively. The cell pellets were re-suspended in buffer B and lysed by sonication (25 × 30-s pulsed cycle). The cell debris was removed by centrifugation at 30,000 × g for 45 min and the supernatant was incubated with 30 ml of nickel-nitritoliatric acid resin that had been pre-equilibrated with buffer B. The resin was washed with 5 column volumes of buffer C and then eluted with buffer D. The fractions containing TcDyP were collected, concentrated, and exchanged into buffer E (100 mM NaCl and 50 mM potassium phosphate, pH 7.8) using an Amicon Ultra-15 (10K, EMD Millipore). The purified protein was stored in aliquots at −80 °C until further use. Protein purity was assessed by 12% acrylamide SDS-PAGE. The protein concentration was determined by the bicinchoninic acid assay using a BSA calibration curve (27).

Expression and purification of the mutants were carried out in the same way as described above for the wt enzyme except that the His$_{312}$ mutants were reconstituted before further analysis. Briefly, 2-fold molar excess of hemin chloride was incubated with the His$_{312}$ mutants at 4 °C for 4 h. The unbound hemin was removed by passing the mixture through DEAE-Sephacel resin eluted with 30–500 mM NaCl in 20 mM potassium phosphate (pH 8.0). Additionally, the excess heme bound with R327A was removed in the same manner as the His$_{312}$ mutants.

Determination of Heme Contents—The heme contents of the wt and mutants were determined by following a protocol described previously (28). The amount was calculated using the absorbance difference between the peak at 557 nm and the trough at 541 nm from the subtraction spectrum using $\epsilon = 20.7$ M$^{-1}$ cm$^{-1}$.

Furthermore, the heme contents were confirmed with ICP-OES. A standard curve of Fe(NO$_3$)$_3$ with concentrations between 100 and 1,000 µg/liter was generated. The protein samples were dialyzed for 12 h against buffer E supplemented with 1 mM EDTA to chelate excess metals and then diluted to a concentration ~500 µg of Fe$^{3+}$/liter (assuming 100% heme occupancy) before analysis.

Enzyme Assay and Steady-state Kinetics—The peroxidase activity of wt-TcDyP against various substrates was determined using a continuous assay by monitoring the absorbance change at a certain wavelength at 22 °C. Briefly, in a 160-µl solution consisting of 50 mM sodium citrate (pH 3.0), 0.5 mM H$_2$O$_2$, 0.5 mM substrate, and 0.2 mg/ml of bovine serum albumin (BSA), the purified wt-TcDyP was added to initiate the reaction. Controls were performed in the absence of enzyme, H$_2$O$_2$, or both. The measurements were performed in triplicates. The tested substrates, wavelengths (nm), and corresponding extinction coefficients ($\epsilon$, cm$^{-1}$ M$^{-1}$), and the enzyme concentrations (µM) used were: 2,2’-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS): 420, 36, 0.006; catechol: 392, 1,456, 2; 2,4-dichlorophenol: 510, 18,5, 2; 2,6-dimethoxyphenol: 470, 49,6, 2; veratryl alcohol: 340, 93, 2; guaiacol: 465, 26,6, 2; hydroquinone (HQ): 247, 21, 0.2; reactive black 5: 598, 30, 2; reactive blue 4 (RB4): 610, 4,2, 0.3; reactive blue 5 (RB5): 600, 8, 0.1; and RB19: 595, 10, 0.05.

To determine steady-state kinetic parameters of the wt-TcDyP, the reactions were performed in the same way as described above except that the concentration of one substrate was varied in the presence of the other substrate at saturation. The rates were determined by the slopes of the initial reactions. The obtained data were fitted either to Michaelis-Menten Equation 1 or Hill Equation 2 using OriginPro 2015. All measurements were done in triplicates.

$$ v = \frac{v_{\text{max}}[S]}{K_M + [S]} \quad \text{(Eq. 1)} $$

$$ v = \frac{v_{\text{max}}[S]^n}{K_M^n + [S]^n} \quad \text{(Eq. 2)} $$

Specific activities of mutants were measured with 0.5 mM ABTS, 5 mM HQ, or 5 mM guaiacol in the presence of 0.5 mM H$_2$O$_2$ following the same protocol as described above. The concentrations of enzyme were: wt, 6 nM; D220H, D220N, H312A, and H312C, 0.2 µM; D220A, D220F, D220G, D220K, and
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R327A, 2 μM. Additionally, steady-state kinetic parameters of D220A and H312C with guaiacol were also determined in the same way as described above for the wt-TcDyP.

Influence of pH on Enzyme Activity—The pH optimum of wt-TcDyP was determined using ABTS in McIlvaine buffers covering pH 2.5–8.0 (29). The assay conditions were the same as described above.

Influence of Temperature on Enzyme Activity—The optimal temperature of wt-TcDyP was determined by measuring ABTS oxidation as described above at temperatures ranging from 15 to 60 °C.

The thermostability of wt-TcDyP was carried out by incubating 12 μM enzyme in buffer E for 1 h in a water bath set to a temperature ranging from 22 to 80 °C. The samples were withdrawn, cooled to 22 °C for 15 min, and immediately analyzed for the residual activity with ABTS as described above.

Reactions with Model Lignin Compounds and Mass Analysis—The model lignin dimers, guaiacylglycerol-β-guaiacol ether (1) and veratrylglycerol-β-guaiacol ether (2), were prepared as previously reported (30). Twenty μM wt-TcDyP was incubated with 5.0 mM 1 or 2 and 5.0 mM H2O2 in 50 mM sodium citrate (pH 3.0) at 22 °C for 16 h. The mixture was heated at 95 °C for 5 min and the enzyme precipitates were removed by centrifugation. The supernatant was loaded onto an HPLC column (Luna C18–2, 5 μm, 4.6 mm × 250 mm) that was eluted at 1.00 ml/min using a linear gradient from 30% to 90% methanol in ddH2O over 30 min. Each HPLC peak was collected, lyophilized, and analyzed by ESI-MS. Reactions and analyses with 4-methoxymandelic acid (MMA) were performed in the same way as described above except that 2.5 mM MMA was used in the presence and absence of 5.0 mM MnCl2.

Stopped-flow Kinetics—Transient-state kinetics of wt-TcDyP were performed at 22 °C at pH 3.0 and 7.8. All reactions were investigated both at defined (monochromator) and multiple (PDA) wavelengths. The PDA data were analyzed using singular value decomposition with the Pro-KIV Global Analysis program provided by Applied PhotoPhysics to obtain the number of reaction intermediates and their corresponding spectra. Reactions were then monitored at the selected single wavelengths to follow the formation and decay of intermediates, and single exponential equations were fitted to the data to obtain pseudo-first order rate constants (kobs). Second-order rate constants were calculated from plots of kobs versus substrate concentrations. All experiments were performed in triplicate.

All concentrations mentioned here were concentrations before mixing. Formation of wt-TcDyP-I was carried out in a conventional mixing mode with 5 μM enzyme and equal volume of H2O2 at various concentrations and monitored at 406 nm. Formation of wt-TcDyP-II was performed in a sequential mixing mode, in which 5 μM wt-TcDyP was premixed with equal volume of 5 μM H2O2. After maximum formation of TcDyP-I was reached in 100 ms, HQ at various concentrations were mixed with TcDyP-I and the reaction was followed at 416 nm. Reduction of wt-TcDyP-II was performed in a sequential mixing mode, in which 5 μM wt-TcDyP and 5 μM ferrocyanide were premixed with an equal volume of 5 μM H2O2. The mixture was incubated in the delay line for 5 s and regeneration of wt-TcDyP-0 was monitored at 406 nm after mixing with HQ at various concentrations. Stopped-flow experiments of mutants were carried out in the same way as described above for the wt-TcDyP.

Spectroelectrochemical Determination of Redox Potentials—In a 3.5-ml cuvette, a 3.0-ml solution consisting of 50 mM potassium Pi (pH 7), 100 mM NaCl, and a mixture of redox mediators (10 μM each, methyl viologen, antraquinoine-2,6-disulfonic acid, 2-hydroxy-1,4-naphthoquinone, 2,5-dihydroxy-1,4-benzoquinone, duroquinone, 1,2-naphthoquinone, and ferricyanide) was purged with water-saturated argon for 1 h. The electrodes (Ag/AgCl, 012167; Pt gauze, 011498; ALS Co, Japan) were connected to a potentiostat. The wt- or mutant TcDyP was added to a final concentration of 10 μM. The entire mixture was completely reduced to ferrous state with ~5 μl of 100 mM freshly prepared sodium dithionite stock solution. This was then oxidized by stepwise addition of 2–5-μl aliquots of argon-purged 2.5 mM K3Fe(CN)6. The reaction mixture was kept under water-saturated argon protection during the whole experiment. After each addition, the reaction was allowed to equilibrate with stirring until the difference in potential readings was less than 1 mV/min. Once the equilibrium was established, the UV-visible spectrum was recorded. The fraction of oxidized TcDyP was calculated by the ΔA433 nm and the mid-point reduction potential (E′) was determined by fitting the data into the Nernst Equation 3 using OriginPro 2015,

$$f = \frac{1}{1 + \exp \left( \frac{n \times (E - E^\prime)}{25.6} \right)}$$

(Eq 3)

where f is the reduced fraction, n is the number of electrons, E is the potential at each point in mV, and E′ is the midpoint reduction potential in mV.

The E′′ of wt-TcDyP was also determined under pH 6 and 8. All measurements were done in duplicates.

Size Exclusion Chromatography—The protein sample (120 μM, 20 μl) was injected onto a BioSep SEC-S2000 column (Phenomenex, 3 μm, 290 Å, 7.8 × 300 mm) attached to a HPLC system at room temperature. The eluent consisted of 50 mM potassium Pi (pH 7.8) and 150 mM NaCl. The flow rate was 0.5 ml/min and the sample was monitored at 280 nm. The retention times of molecular mass standards: aprotinin (6.5 kDa, 9.75 ml), horse cytochrome c (12.4 kDa, 9.05 ml), chicken albumin (44.3 kDa, 7.70 ml), bovine albumin (66 kDa, 7.20 ml), yeast alcohol dehydrogenase (150 kDa, 6.85 ml), and horse apoferritin (443 kDa, 6.00 ml).

Results

Protein Purification and Basic Biochemical Properties

The gene encoding a DyP-type peroxidase is predicted from the genome sequence of T. curvata. Because the efforts to clone it were unsuccessful, the gene (Tcur_2987) corresponding to the predicted matured TcDyP, in which the signal sequence was removed, was synthesized and inserted into the vector pTBSG (26) to construct a new plasmid pPL2014L01.

The N-terminal His-tagged wt or mutant TcDyP was purified to homogeneity by nickel-nitrilotriacetic acid affinity chromatography and migrated as a single band at the predicted size of
42.2 kDa on SDS-PAGE (Fig. 2A). The purified TcDyPs exhibited an absorbance maximum at 406–412 nm in its UV-visible spectrum (Fig. 2B), which was consistent with the prediction that TcDyP is a hemoprotein. Although the Reinheitszahl values \(R_z\) of wt and Asp\(^{220}\) mutants were at \(\sim 1.8\), His\(^{312}\) and Arg\(^{327}\) mutants were determined to be at \(\sim 0.9\) (due to loss of proximal histidine) and 2.5 (due to nonspecific heme binding), respectively. Thus, the histidine mutants were reconstituted with hemin chloride before further analysis. The excess heme bound with R327A was removed by DEAE ion-exchange chromatography. Heme quantitation by pyridine hemochrome assay (Fig. 2C) and ICP-OES revealed that one molecule of wt-TcDyP binds one molecule of heme cofactor. The enzyme is most active at pH 3 (Fig. 2D), which is slightly more acidic than the other reported DyPs (12, 31). Although the enzyme could retain \(\sim 70\%\) maximal activity after incubation at 60 °C for 1 h, the optimal enzyme assay temperature was determined to be 30 °C (Fig. 2E). Because the His tag did not interfere with enzyme activity, future experiments were performed with its presence.

**Peroxidase Activity and Steady-state Kinetics of TcDyP**

To explore its substrate scopes, wt-TcDyP was tested against a panel of well known peroxidase substrates including ABTS, aromatics, azo, and anthraquinone dyes. It was found that the peroxidase is inactive toward 2,6-dimethoxyphenol, 2,4-dichlorophenol, veratryl alcohol, and reactive black 5. As shown in Fig. 3A, the wt-TcDyP displayed low activities toward phenolic compounds such as guaiacol, catechol, and HQ. However, unlike other A-type enzymes, the wt-TcDyP exhibits extremely high activity toward reactive blue 4, 5, and 19 (anthraquinone dyes) and ABTS. No catalase activity was detected when the enzyme was incubated with \(H_2O_2\) (data not shown), suggesting that it does not function as a peroxidase-catalase.

It is worth noting that reactions catalyzed by peroxidases generally do not involve Michaelis-Menten intermediates because the catalytic cycle is considered irreversible (32). However, there is no doubt that adsorption complexes between the enzyme and its substrates exist physically (33). The microscopic constants governing the equilibrium between reducing substrates and peroxidase have been estimated (34). Even though the cosubstrates (reducing donor or \(H_2O_2\)) modulate the affinity of each other to the enzyme, it is possible for the mechanism to proceed via random binding. This observation, together with some special kinetic features (35), supports the notion that there is no need for the peroxide to bind to the enzyme prior to donor adsorption (36). Because the oxidations of the compounds shown in Fig. 3A exhibited classical steady-state kinetics with respect to both substrates, the system was analyzed using Michaelis-Menten equation. The resulting apparent Michaelis constant \(K_m^{app}\) and turnover number \(k_{cat}^{app}\) are summarized in Table 1. It has to be pointed out that oxidation of ABTS and anthraquinone dyes (reactive blue series) displayed sigmoidal rate curves (not shown). This “apparent cooperative phenomenon” suggests that TcDyP may have multiple oxidation
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sites like the fungal DyP from Auricularia auricular-judae (37–39).

To characterize the importance of the proposed catalytic residues, they were mutated and the mutants were tested for their activity against ABTS, HQ, and guaiacol. As shown in Fig. 3B, all mutants lose activity toward ABTS, indicating that these residues are important for ABTS oxidation. In contrast, oxidation of phenolic compounds showed a different profile. Although substitution of His312 and Arg327 resulted in marginal enzyme activities, mutants of Asp220 displayed up to 83% (cross-patterned bars in Fig. 3B). A similar phenomenon was also observed with EfeB from E. coli O157 (16). Thus, questions were raised about the catalytic importance of the aspartate in A-type DyPs (14). The present study of the catalytic cycle has resolved the contradiction, which will be described under “Discussion.” It has to be pointed out that the residual activity of H312C with phenolic compounds may result from partial ligand compensation by cysteine.

To determine kinetic parameters of mutants, D220A and H312C were selected because they exhibited relatively high residual peroxidase activities toward guaiacol and would be used for study of catalytic intermediates. As summarized in Table 1, whereas both mutants display similar catalytic efficiency toward guaiacol as the wt enzyme, their catalytic efficiency toward H2O2 drop by 22- and 88-fold for D220A and H312C, respectively. The decrease is caused by the fact that the mutants have a much higher $K_m$ for H2O2 than the wt.

**Oxidation of Model Lignin Compounds by wt-TcDyP**

Due to the high catalytic efficiency found with wt-TcDyP, we decided to test its ability to degrade lignin model dimers containing the β-O-4 linkage with (1) and without (2) a phenolic site. Their structures and HPLC profiles of the reactions are shown in Fig. 4A. The results indicate that only the phenolic compound 1 could be depolymerized in the absence of redox mediators, which suggests that the low potential phenolic site ($\Delta E^{\text{red}} = 0.6 – 0.8$ V) (40) is required for enzyme activity as no degradation of 2 was observed under the same condition. Similar results have also been obtained with B- and C-type DyPs (12, 13). The incomplete degradation of 1 (retention time at 13.8 min) was due to enzyme instability at pH 3.0 for prolonged time.

To characterize the degradation products of 1, peaks at 6.5, 16.6, 17.5, and 20.8 min were collected and analyzed by ESI-MS. Only the latter two peaks were identified and their mass spectra are shown in Fig. 4B. The peak at 17.5 min had an $m/z$ of 661.43, indicating that the radical recombination was taking place to give a high molecular species that is likely the hydroxylated guaiacol pentamer (MH$^+$ 661.19). The peak at 20.8 min was assigned as a creosol dimer (MH$^+$ 275.13) that corresponds to the observed $m/z$ of 274.47. The pathway for the formation of these products is proposed in Fig. 4C, indicating that C$\alpha$–C$\beta$ cleavage occurred upon incubation with wt-TcDyP.

Because no activity was observed with nonphenolic lignin dimer 2, we decided to test another nonphenolic lignin peroxidase substrate, MMA, 3 (Fig. 5A), which has been used to detect high potential oxidative events ($\Delta E^{\text{red}} > 1.4$ V) occurring in lignin breakdown by fungi (41, 42). MMA has also been reported to undergo decarboxylation to form anisaldehyde 4 through Mn$^{2+}$-dependent oxidase activity of C-type DyP2 (12). In our case, when wt-TcDyP was incubated with MMA, it was found that only H$_2$O$_2$ was required for MMA oxidative decarboxylation (Fig. 5B). Because

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**TABLE 1**

Kinetic parameters of wt and mutant TcDyps

| Enzyme | Substrate | \(K_m\) (μM) | \(k_{cat}/K_m\) (s$^{-1}$) | \(k_{cat}/K_m\) (M$^{-1}$ s$^{-1}$) |
|--------|-----------|-------------|-----------------|------------------|
| wt     | ABTS      | 15 ± 6      | 260 ± 7         | 1.7 × 10$^6$     |
|        | RB4       | 170 ± 80    | 110 ± 9         | 6.5 × 10$^5$     |
|        | RB19      | 52.8 ± 0.5  | 41 ± 1          | 7.8 × 10$^4$     |
|        | RB5       | 43 ± 4      | 29 ± 1          | 6.6 × 10$^4$     |
|        | HQ        | 6300 ± 880  | 31 ± 2          | 4.8 × 10$^3$     |
|        | Catechol  | 19000 ± 3500| 24 ± 3          | 1.5 × 10$^2$     |
|        | Guaiacol  | 370 ± 90    | 0.33 ± 0.03     | 8.8 × 10$^2$     |
|        | ABTS      | 84 ± 6      | 280 ± 6         | 3.4 × 10$^6$     |
|        | HQ        | 33 ± 4      | 27 ± 1          | 8.2 × 10$^5$     |
|        | Guaiacol  | 77 ± 20     | 0.56 ± 0.03     | 4.7 × 10$^4$     |
| D220A  | Guaiacol  | 280 ± 6     | 0.58 ± 0.03     | 2.1 × 10$^4$     |
|        | H$_2$O$_2$| 2000 ± 160  | 0.59 ± 0.02     | 2.1 × 10$^4$     |
| H312C  | Guaiacol  | 660 ± 7     | 0.52 ± 0.02     | 7.9 × 10$^3$     |
|        | H$_2$O$_2$| 11000 ± 1000| 0.58 ± 0.01     | 5.3 × 10$^3$     |

*a* Assays were performed in 50 mM sodium citrate pH 3.0 at 22 °C.

*b* Kinetics of ABTS, RB4, RB19, and RB5 were fitted to the Hill equation.

*c* No activity was detected for H312A and R327A mutants.
wt-TcDyP could not oxidize MMA in the presence of Mn$^{2+}$ and O$_2$, it suggested that the enzyme did not contain oxidase activity like the DyP2 (12).

**Detection of Catalytic Intermediates Using Stopped-flow Spectroscopy**

Because the wt-TcDyP displayed high activities toward various substrates and could depolymerize model lignin compound 1 and MMA, we decided to investigate the intermediates involving the heme center to better understand the catalytic cycle of DyPs.

**Compound I (TcDyP-I) Formation**—The left panel in Fig. 6A shows the spectral changes upon conventional mixing of wt-TcDyP and H$_2$O$_2$ at pH 7.8. When it was analyzed by Pro-KIV, a two-step reaction of A → B → C was given as the best fit. Since the resting state, compound I, and compound II have been reported with many heme peroxidases including their spectral characters (43), A and B were assigned as the resting state (TcDyP-0, blue line) and compound I (TcDyP-I, green line), respectively. As summarized in Table 2, when TcDyP-0 was converted into TcDyP-I, the Soret band shifted slightly to a longer wavelength (406 to 407 nm) along with a new Q-band at 512 nm. To calculate the rate of compound I formation, wt-TcDyP was rapidly mixed with H$_2$O$_2$ and the reaction was monitored at 406 nm for the decay of TcDyP-0 (left panel in Fig. 6B). The results were fitted into a single-exponential equation to obtain the pseudo-first order rate constant $k_{obs}$ (406 nm). By plotting $k_{obs}$ (406 nm) versus H$_2$O$_2$ concentrations, the second-order rate constant was determined to be $(5.92 \pm 0.31) \times 10^6$ M$^{-1}$ s$^{-1}$ at pH 7.8 (right panel in Fig. 6B).

The spectrum corresponding to intermediate C (red line) has a Soret band at 416 nm and two Q-bands at 524 and 555 nm, which is consistent with features of compound II identified in DyPs and plant peroxidases (18, 19, 44). Thus, it was initially assigned as a compound II species and was expected to have a relationship with H$_2$O$_2$. To define the relationship, formation of C was monitored at 416 nm (left panel in Fig. 6C). It was found that the $k_{obs}$ (416 nm) was independent of H$_2$O$_2$ concen-
tration (right panel, Fig. 6C), which indicated that C was not a compound II species. Due to its spectral resemblance to the reported compound II-type species (43), it was reassigned as a compound II-like decay product of \( \text{TcDyP-I} \).

To facilitate comparison with the steady-state results, the transient kinetics was repeated at pH 3.0. The second-order rate constant of \( \text{TcDyP-I} \) formation was determined to be \((4.06 \pm 0.02) \times 10^6 \text{M}^{-1} \text{s}^{-1}\) (right panel in Fig. 6D), which is comparable with the rate constant at pH 7.8.

**Compound II (TcDyP-II) Formation**—Because TcDyP-II was not found with \( \text{H}_2\text{O}_2 \), we decided to determine whether it would form in a reaction in the presence of a substrate. HQ was selected because its oxidation does not interfere with absorbance of the enzyme at the 380–450 nm range (48). The reaction was performed in a sequential mixing mode and the delay time was set at 100 ms, during which maximal TcDyP-I had been reached but the decay had not occurred yet. As shown in Fig. 7A, the initial spectrum (green lines) corresponds to TcDyP-I. When the reaction was carried out under neutral conditions (top panel in Fig. 7A), a Soret band at 416 nm and two Q-bands at 524 and 555 nm appeared and their intensities were increased over time. In contrast with the experiments where only \( \text{H}_2\text{O}_2 \) was present, the pseudo-first order rate constants monitored at 416 nm displayed a linear relationship with the HQ concentrations (right panel in Fig. 7B) giving a second-order rate constant of \((2.24 \pm 0.09) \times 10^4 \text{M}^{-1} \text{s}^{-1}\). Thus, this new species was assigned as the TcDyP-II. Because the spectral change at pH 3 (bottom panel in Fig. 7A) was small, the second-order rate constant was not determined due to inability to identify an appropriate wavelength for monitoring TcDyP-II.

**Reduction of TcDyP-II**—Finally, the reduction of compound II was investigated using HQ as the substrate in a sequential
mixing mode. The compound II was produced by premixing solution of \(5 \text{ M} \) TcDyP and \(5 \text{ M} \) ferrocyanide with 1 eq of \( \text{H}_2\text{O}_2 \). Reduction of TcDyP-II was not observed at pH 7.8, which agrees with our steady-state results that the enzyme displayed marginal activity under neutral conditions. In contrast, TcDyP-II was reduced at pH 3.0 and the intensity of the Soret band at 406 nm that corresponded to the resting state \( \text{TcDyP-0} \) was increased as depicted in Fig. 8A. Plot of the pseudo-first order rate constants monitored at 406 nm with the HQ concentrations exhibited a saturation kinetics (right panel in Fig. 8B), which suggested that binding between HQ and TcDyP-II prior to the reduction was required as described by Equation 4. The observed \( k_{\text{obs}} \) was represented by Equation 5 and the second-order rate constant, \( k/K_D \) was calculated by nonlinear fit of the data to Equation 6, giving a value of \((6.36 \pm 0.52) \times 10^{-2} \text{ M}^{-1} \text{ s}^{-1}\). The corresponding \( K_D \) and \( k \) are 6.7 mM and 42 s\(^{-1}\), which is in excellent agreement with the results of steady-state kinetics summarized in Table 1.

\[ \text{TcDyP-II} + \text{HQ} \xrightarrow{k} \text{TcDyP-It-HQ} \xrightarrow{k} \text{TcDyP-0} + \text{HQ} \]  

(Eq. 4)

\[ k_{\text{obs}} = \frac{k}{K_D} \frac{1}{1 + [\text{HQ}]} \]  

(Eq. 5)

\[ k_{\text{obs}} = \frac{k/K_D [\text{HQ}]}{1 + [\text{HQ}]} \]  

(Eq. 6)

**Formation of TcDyP-I with Mutants**

To understand how the catalytic residues surrounding the heme influence the formation of TcDyP-I, the UV-visual spectra of the reactions between mutants and \( \text{H}_2\text{O}_2 \) were recorded and are shown in Fig. 9, A–D. Normalized spectra of the resting state and compound I of the \( \text{wt} \) and mutant TcDyPs are shown in Fig. 9, E and F. Their spectral characters are summarized in Table 2.

Substitution of Asp\(^{220} \) with the alanine did not prevent formation of TcDyP-I. The second-order rate constant of its formation was determined to be \((1.24 \pm 0.04) \times 10^{-6} \text{ M}^{-1} \text{ s}^{-1}\) at pH 7.8, which is 4 orders of magnitude slower than the \( \text{wt} \). Significant red shift was observed for the Soret bands of TcDyP-0 and TcDyP-I, suggesting that the aspartate plays important roles in heme microenvironment. Moreover, two Q-bands at 542 and 572 nm appeared in the resting state of D220A (red line in Fig. 9E), which is reminiscent of 6-coordinated low spin heme and indicates the potential reactivity difference between the \( \text{wt} \) and mutant.

The most interesting observation was made with the His\(^{312} \) mutants. The proximal histidine is considered as a fifth ligand to the heme iron. A Fe-His-Glu triad has been identified in EfeB, which is thought to play important roles in maintaining the heme center for high peroxidase activity (16). Thus, substitution of histidine was expected to result in a significant change of heme microenvironment as well as the catalytic intermediates due to loss of the ligand. However, in contrast to our prediction, both the H312A and H312C mutants reacted rapidly with \( \text{H}_2\text{O}_2 \) (pH 7.8) to form TcDyP-I at the second-order rate constants of \((2.04 \pm 0.03) \times 10^{-6} \text{ M}^{-1} \text{ s}^{-1}\) and \((3.28 \pm 0.03) \times 10^{-6} \text{ M}^{-1} \text{ s}^{-1}\), respectively, which are close to the rate of the \( \text{wt} \) enzyme. This suggests that histidine is not absolutely necessary in the formation of TcDyP-I. The loss of interaction in the Fe-His-Glu triad must have been compensated by reorganization of local residues and/or other factors such as hydrophobicity, electrostatic interaction, and hydrogen bonding. The observed faster rate of the cysteine mutant than the alanine one may result from partial ligation of the thiolate. Additionally, the TcDyP-0 of H312A loses the charge transfer band at 624 nm that exists in the \( \text{wt} \) enzyme. The H312C has a much broader...
Soret band than the other mutants for both TcDyP-0 and TcDyP-I. These spectral changes indicate that the histidine mutation has altered the heme microenvironment. Moreover, the broadening effect of H312C may also reflect formation of multiple species (thio-ligated and non-ligated species).

Consistent with the steady-state kinetics, TcDyP-I was not found for the R327A mutant. The decrease of intensity at 406 nm (Fig. 9D) was attributed to the enzyme instability at a high concentration of H2O2, which also occurred in R244L-DypB (24). The R327A mutant and wt enzyme have nearly identical spectra for their resting states, which suggests that the distal arginine does not have direct interaction with the heme.

**Formation and Reduction of TcDyP-II with Mutants**

Because TcDyP-I was observed only with Asp220 and His312 mutants, they were selected for further study of formation and reduction of TcDyP-II. However, the efforts were only successful with H312A.

Due to slow rate of formation of TcDyP-I, a high concentration of more than 20 mM H2O2 was needed for D220A to rapidly produce the precursor to TcDyP-II in a sequential mixing mode. However, such a high concentration of H2O2 was found to be detrimental to the enzyme, which is not uncommon in heme peroxidases (49). For the H312C mutant, a broad peak and small shift of the Soret band prevented us from monitoring TcDyP-II (data not shown). Thus, the second-order rate constants of formation and reduction of TcDyP-II with D220A and H312C were not pursued although their residual activities with HQ were observed in steady-state kinetics.

**Roles of Catalytic Residues in A-type DyPs**

![A-type DyPs](image)

![A-type DyPs](image)

FIGURE 7. Reactions of TcDyP-I with HQ in a sequential mixing mode. A, spectral transition of 3 mM HQ and TcDyP-I at pH 7.8 (top) and 3.0 (bottom) recorded in 1.0 s. The green and red lines represent TcDyP-I and TcDyP-II, respectively. Arrows indicate changes of absorbance over time. B, typical time trace at 416 nm (left panel) and dependence of kobs (416 nm) versus [HQ] (right) for TcDyP-II formation at pH 7.8.

![A-type DyPs](image)

![A-type DyPs](image)

FIGURE 8. Reductions of TcDyP-II with HQ in a sequential mixing mode. A, spectral transition of 5 mM HQ and TcDyP-II at pH 3.0 recorded in 1.0 s. The blue and red lines represent TcDyP-0 and TcDyP-II, respectively. Arrows indicate changes of absorbance over time. B, typical time trace at 406 nm (left panel) and dependence of kobs (406 nm) versus [HQ] (right panel) for TcDyP-II reduction.

FIGURE 9. Reductions of TcDyP-II with HQ in a sequential mixing mode. A, spectral transition of 5 mM HQ and TcDyP-II at pH 3.0 recorded in 1.0 s. The blue and red lines represent TcDyP-0 and TcDyP-II, respectively. Arrows indicate changes of absorbance over time. B, typical time trace at 406 nm (left panel) and dependence of kobs (406 nm) versus [HQ] (right panel) for TcDyP-II reduction.

Roles of Catalytic Residues in A-type DyPs

Because TcDyP-I was observed only with Asp220 and His312 mutants, they were selected for further study of formation and reduction of TcDyP-II. However, the efforts were only successful with H312A.

Due to slow rate of formation of TcDyP-I, a high concentration of more than 20 mM H2O2 was needed for D220A to rapidly produce the precursor to TcDyP-II in a sequential mixing mode. However, such a high concentration of H2O2 was found to be detrimental to the enzyme, which is not uncommon in heme peroxidases (49). For the H312C mutant, a broad peak and small shift of the Soret band prevented us from monitoring TcDyP-II (data not shown). Thus, the second-order rate constants of formation and reduction of TcDyP-II with D220A and H312C were not pursued although their residual activities with HQ were observed in steady-state kinetics.

Reactions of H312A, H2O2, and HQ in a sequential mixing mode generated a TcDyP-II intermediate. The corresponding spectral change from TcDyP-I to TcDyP-II is shown in Fig. 10A. Formation of TcDyP-II was monitored at 412 nm, giving a second-order rate constant of (1.62 ± 0.03) × 104 M⁻¹ s⁻¹, which is close to that of the wt ((2.24 ± 0.09) × 104 M⁻¹ s⁻¹).
FIGURE 9. Reactions of mutants with H$_2$O$_2$ (A–D) and spectral overlay (E and F). The blue, green, and red lines in A–D represent initial, intermediate, and final states of the mutants, respectively. Reactions were performed with 5 μM mutants and different concentrations of H$_2$O$_2$ at pH 7.8 and monitored for 5 s. Arrows indicate changes of absorbance over time. The black, red, green, blue, and cyan lines in E and F represent overlay of normalized spectra of wt, D220A, H312A, H312C, and R327A, respectively. A, D220A with 5 mM H$_2$O$_2$. B, H312A with 5 μM H$_2$O$_2$. C, H312C with 5 μM H$_2$O$_2$. D, R327A with 500 μM H$_2$O$_2$. E, enzyme resting state. F, compound I.
Roles of Catalytic Residues in A-type DyPs

Reduction of TcDyP-II was performed in the same way as the wt enzyme by premixing and incubating equal amounts of H312A, ferrocyanide, and H₂O₂ for 5 s. Addition of HQ produced a new species that has a Soret band at 392 nm, Q-band at 505 nm, and charge transfer band at 638 nm as depicted in Fig. 10B. This indicates that the H312A mutant did not return to the resting state to complete a catalytic cycle, which explains the observed loss of enzyme activity in steady-state kinetics. It has to be noted that the new species was formed extremely fast and then decayed slowly. When the HQ was added at 1 eq to TcDyP-II (final concentration of 2.5 μM), formation of the new species was complete in less than 100 ms. It then underwent decomposition over the next 10 s as the intensity of 392 nm decreased without further spectral shift (Fig. 10B). Attempts to slow down the rate of formation resulted in difficulty to monitor the species. Thus, the second-order rate constant for its formation was not determined.

Spectroelectrochemical Titration and Redox Potentials

The redox behavior of heme iron is an indicator of peroxidase functionality, although it is not directly involved in the catalytic cycle (50). In fact, a stable Fe³⁺ is required for the peroxidases to carry out H₂O₂-mediated oxidation to form compound I, which depends on the redox potential (E°) of the Fe³⁺/Fe²⁺ couple. To date, the E° have only been reported for three DyPs: BsDyP (~40 mV, A-type), PpDyP from Pseudomonas putida MET94 (~260 mV, B-type), and DyP2 (~85 mV, C-type) (12, 31). Moreover, it is unknown how the catalytic residues surrounding the heme affect the E° of DyPs. Thus, we decided to determine the E° of wt and mutant TcDyPs.

The experiments were carried out under argon using a spectroelectrochemical titration method. A typical UV-visible spectrum for the stepwise oxidation of Fe²⁺-TcDyP is shown in Fig. 11A and the titration curves of the wt and mutants are depicted in Fig. 11, B and C, respectively. The data were fitted to Nernst Equation 3 and the results are summarized in Table 3.

The titration of the wt enzyme under neutral conditions revealed an E° of −136 mV and apparent number of transferred electrons (n_app) of 0.61. Similar to BsDyP (31), the transition was broad (Fig. 11B), which was thought to result from multiple 5- and 6-coordinated iron species consisting of low- and high-spin states (31). Thus, the n_app was off from the theoretical number of 1.00 (Fe³⁺ + e → Fe²⁺) due to difficulty to fit multiple species into the Nernst equation. Because the enzyme displayed the highest activity at pH 3, it was predicted that the heme species would become homogeneous under an acidic condition and exist as a reactive high-spin state. A narrow transition, more positive E°, and higher n_app were expected. Indeed, when the titration was performed at pH 6, the observed n_app (0.79) was much closer to the theoretical value. Moreover, the E° of TcDyP was strongly influenced by pH, which is consistent with other peroxidases (52–54). One pH unit corresponded to a ΔE° of ~60 mV for wt-TcDyP.

To understand the contribution of catalytic residues to redox potentials of the Fe³⁺/Fe²⁺ couple, the E° of the alanine mutants were determined. It was found that the aspartate mutation had a small effect, with the E° becoming more positive by 7 mV relative to the wt. Both H312A and R327A mutants had more negative E° than the wt-TcDyP, suggesting that the Fe³⁺ state is stabilized in these two substitutions. The observed changes were relatively large, −44 mV for H312A and −74 mV for R327A. The implications of these changes will be discussed in the next section.

Effects of Catalytic Residues on Oligomeric States

No study has been reported on the effects of catalytic residues on the oligomerization of DyPs. X-ray structure of DypB has shown that the arginine located at the distal side of the heme is connected to the surface residues through networks of hydrogen bonding (24), which implies that the conserved residues in the active site may play roles in protein oligomerization. As shown in
Roles of Catalytic Residues in A-type DyPs

Fig. 12, substitution of His$^{312}$ or Arg$^{327}$ with an alanine indeed resulted in change of the oligomeric state. Although the wt enzyme is a mixture of dimer, tetramer, and octamer, both mutants exclusively exist as a dimer. Mutation of Asp$^{220}$ has minimal impact on oligomerization, suggesting that it is not involved in networks interacting with the surface residues.

Discussion

Biochemical Property of wt-TcDyP

It was found that wt-TcDyP was highly active toward a variety of substrates. As summarized in Table 4, bacterial A- and B-type DyPs generally show much lower catalytic efficiency ($k_{cat}/K_m^{app}$) than the C- and D-type DyPs. However, the wt-TcDyP was found to have as high catalytic efficiency with ABTS as the most active D-type fungal enzymes. Furthermore, its activity with anthraquinone dyes is much higher than the other A- and B-type DyPs and comparable with C- and D-type enzymes. In addition, the wt-TcDyP also showed peroxidase activity toward model lignin compounds 1 and 3. Analysis of the degradation products indicated that the C$_6$-C$_7$ bonds in both compounds were cleaved, which represents the first example of such activity with the type-A DyPs. Combined with the recent report on BsDyP (13), our results suggest that type-A DyPs could be potentially useful for lignin degradation.

Catalytic Cycle and Transient Kinetics of wt-TcDyP

The proposed catalytic cycle and obtained second-order rate constants for each step are summarized in Fig. 13. To date,

### TABLE 3

| Enzyme | pH | $E^o$ | $n_{app}$ |
|--------|----|-------|-----------|
| wt     | 6  | $-77 \pm 0.8$ | 0.79 ± 0.02 |
| wt     | 7  | $-136 \pm 1.4$ | 0.61 ± 0.02 |
| wt     | 8  | $-182 \pm 1.7$ | 0.52 ± 0.02 |
| D220A  | 7  | $-129 \pm 0.9$ | 0.68 ± 0.02 |
| H312A  | 7  | $-180 \pm 1.4$ | 0.77 ± 0.04 |
| R327A  | 7  | $-210 \pm 1.0$ | 0.69 ± 0.02 |
Roles of Catalytic Residues in A-type DyPs

Table 4

Comparison of peroxidase activities

Blank spaces represent not reported; ND indicates data not detected.

| Subclass | Protein | ABTS | H₂O₂(ABTS) | RB4 | RB5 | RB19 | Guaiacol | Ref. |
|----------|---------|------|-------------|-----|-----|------|----------|------|
| A        | TcDyP   | 1.7 x 10⁻² | 3.4 x 10⁻⁶ | 6.5 x 10⁻⁵ | 6.6 x 10⁻⁵ | 7.8 x 10⁻⁵ | 8.8 x 10⁻⁵ | This study |
|          | TjDyP   | 2.0 x 10⁻³ | 1.7 x 10⁻⁶ | 1.3 x 10⁻⁶ | 3.5 x 10⁻⁶ | 5.5 x 10⁻⁶ | 19 |
|          | DyPA    | 7.0 x 10⁻⁸ | 2.0 x 10⁻⁶ | 5.0 x 10⁻⁶ | 3.0 x 10⁻⁶ | 31 |
|          | DyPB    | 2.4 x 10⁻² | 2.1 x 10⁻⁷ | 1.0 x 10⁻⁷ | 19 |
| B        | DyPP_a  | 2.2 x 10⁻⁷ | 7.0 x 10⁻⁷ | 5.0 x 10⁻⁷ | 57 |
|          | TfeA    | 8.0 x 10⁻¹ | 1.8 x 10⁻⁷ | 2.0 x 10⁻⁷ | 3.4 x 10⁻⁷ | 31 |
|          | PpDyP   | 6.6 x 10⁻⁶ | 1.8 x 10⁻⁷ | 7.1 x 10⁻⁷ | 12 |
|          | DyP     | 1.2 x 10⁻⁷ | 4.8 x 10⁻⁷ | 1.2 x 10⁻⁷ | 58 |
|          | AjP I   | 1.8 x 10⁻⁷ | 2.7 x 10⁻⁷ | 5.0 x 10⁻⁷ | 60 |
|          | AjP II  | 1.6 x 10⁻⁷ | 4.8 x 10⁻⁷ | 1.7 x 10⁻⁷ | 60 |
|          | TAP     | 2.5 x 10⁻⁷ | 1.2 x 10⁻⁷ | 1.2 x 10⁻⁷ | 61 |
| Other    | LiP     | 5.6 x 10⁻⁷ | ND | 1.7 x 10⁻⁷ | 62 |
|          | MnP     | 10⁻⁷ | ND | 1.7 x 10⁻⁷ | 63, 64 |
|          | VP      | 9.4 x 10⁻⁷ | ND | ND | 65 |

DyPA is the only A-type DyP that has been briefly studied for its reaction with H₂O₂, in which formation of compound II was claimed based on the UV-visible spectra (19). Similar spectral transition was also observed with wt-TcDyP. However, fitting the multiwavelength data revealed that a two-step reaction involving TcDyP-0, TcDyP-I, and the compound II-like decay product of TcDyP-I occurred. Thus, for the first time, compound I was observed in A-type DyPs. Its second-order rate constant was found to be independent of pH. Because the rate of compound I formation is only reported for DypB (∼1.80 x 10⁻⁵ M⁻¹ s⁻¹ at pH 7.5) in all DyPs (19), a comparison reveals that the catalytic efficiency of TcDyP (5.92 x 10⁶ M⁻¹ s⁻¹) is nearly 30-fold higher than that of DypB.

The decay product of TcDyP-I could be a protein radical that has been observed with many heme peroxidases (66, 67). Analysis of residues within 8 Å of the heme center using our homology model reveals that Tyr₃₃₂, which is highly conserved among all DyP enzymes (Fig. 1), is a candidate residue for radical localization. However, mutation of this residue did not affect the peroxidase activity of TcDyP. Identification of the radical residue is being actively pursued.

Formation and reduction of the TcDyP-II were elucidated with HQ in a sequential mixing mode. It was determined that the rate of TcDyP-II formation was ∼2.5-fold slower than that of TcDyP-I formation. Reduction of TcDyP-II was found to be the slowest in the catalytic cycle, suggesting that regeneration of TcDyP-0 from TcDyP-II is a rate-limiting step in HQ oxidation. Thus, the catalytic cycle involving an A-type DyP was fully characterized for the first time. It has to be pointed out that the rate-limiting step may be substrate-dependent. For example, the k₁/kₐᵣratio of ABTS (1.7 x 10⁻⁷ M⁻¹ s⁻¹) is higher than the second-order rate constant of TcDyP-I formation (4.06 x 10⁻⁵ M⁻¹ s⁻¹) at pH 3. Thus, it is reasonable to assume that the formation of TcDyP-I in ABTS oxidation is the rate-limiting step.

Roles of Catalytic Residues Surrounding the Heme Center

To probe the roles of catalytic residues, steady-state and transient kinetics, spectroelectrochemical titrations, and SEC chromatography were performed.

Aspartate—It is proposed that the catalytic importance of aspartate is dependent on the enzyme types (14). It has also been demonstrated that aspartate is essential for D-type DyPs, but not required for some B-type enzymes (14). Its role in A-type DyPs is still in question (16). In the present study, whereas mutation of Asp₂²₀ in DyP resulted in the loss of enzyme activity toward ABTS, the mutants retained up to 85% wt activity with guaiacol.

The seemingly contradictory observations can be explained as follows. Based on the structural similarity between HQ and guaiacol, it is reasonable to propose that the reduction of TcDyP-II to TcDyP-0 is a rate-limiting step as well for guaiacol oxidation, in which the rate is estimated at 0.33 s⁻¹ according to its steady-state kinetics. This is close to the rate of TcDyP-I formation in D220A at 0.07 s⁻¹. Thus, the aspartate mutation will not have significant impact on guaiacol oxidation because it does not cause the change of rate-limiting step in the catalytic cycle of wt-TcDyP. Second-order rate constants are shown for each step.

FIGURE 13. Catalytic cycle of wt-TcDyP. Second-order rate constants are shown for each step.
cycle. However, TcDyP-I formation is proposed as the rate-limiting step for ABTS oxidation. A decrease of rate by 4 orders of magnitude caused by the aspartate mutation will result in the loss of activity toward ABTS. Thus, our results have shown that aspartate is crucial for the formation of compound I as well as the peroxidase activity of A-type DyPs.

As shown in Fig. 9, E and F, and summarized in Table 2, substitution of Asp220 has the most significant impact on the heme microenvironment among all active site mutants. It causes a red shift in UV-visible spectra of TcDyP-0 and TcDyP-I as well as the appearance of two Q-bands in TcDyP-0, all of which are indicative of generation of a six-coordinated iron species, a disfavored species for peroxidases. However, the aspartate mutation has minimal effects on the oligomeric state of the protein, which may imply that the aspartate is not involved in the networks extended to the protein surface. Finally, a predicted increase (more positive) of $E''$ was observed due to loss of the electrostatic interaction between the Fe$^{3+}$ in heme and carboxylate anion of the aspartate side chain.

Histidine—It has been reported that mutation of the proximal histidine in DyPs prevents formation of holoenzymes (16, 55). Furthermore, this mutation was found to decrease the stability of plant peroxidases (68). However, these deleterious effects were not observed in our histidine mutants. Substitution of His312 with a non-ligating (Ala312) or ligating (Cys312) residue has significant impacts on the enzyme activity. Although H312A showed no peroxidase activity, H312C displayed 0.5, 1.1, and 5.4% wt activity toward ABTS, HO2, and guaiacol, respectively (Fig. 3B). This suggests that His312 is catalytically important. Loss of the proximal ligation (H312A) inactivated the enzyme. Ligation compensation from cysteine (H312C) partially recovered the enzyme activity although the recovery was marginal.

Several striking features were observed with histidine mutants. Replacement of the proximal histidine is expected to result in the change of Fe$^{3+}$ coordination and thus, affect the formation of TcDyP-I and TcDyP-II. Yet, it was discovered that both mutants were able to form compound I as efficiently as the wt-TcDyP. Our results also demonstrated that the rate of formation of TcDyP-II was not retarded by a histidine mutation. The spectral features of histidine mutants and wt enzyme were nearly identical for all intermediates (TcDyP-0, TcDyP-I, and TcDyP-II), which led us to propose that His312 may not be the only residue that can serve as the axial ligand. In fact, His312 is located on a short α-helix between two large loops (Gly276–Ala311 and His317–Pro329) according to our homology model (Fig. 1B). The high structural flexibility in this region may allow other residues to fill the role of proximal histidine when it is mutated. Yet, the mutation to alanine prevents the enzyme from returning to the resting state. A new species with the Soret band at 392 nm was rapidly formed during reduction of TcDyP-II. Its identity still needs to be characterized.

Additionally, the H312A mutant had a more negative $E''$ than the wt-TcDyP by $-44$ mV, suggesting that Fe$^{3+}$ was stabilized by the alanine substitution. Combining the results of catalytic intermediates discussed above, our study indicates that introduction of alanine may reorganize local structure, resulting in a net stabilization of Fe$^{3+}$ to facilitate the formation of compound I and compound II. The reorganization is also amplified in the shift of oligomeric state from a mixture of dimer, tetramer, and octamer for the wt to a dimer for the H312A in solution.

Arginine—Our results have shown that arginine is essential for compound I formation in A-type DyPs, which is similar to B-type DypB (24) yet quite different from HRP and cytochrome c peroxidase (69, 70). For the latter two peroxidases, replacement of this distal arginine does not inhibit the formation of compound I, but lowers the rate by 2–3 orders of magnitude (69, 70). It has been proposed that the arginine is involved in the general acid-base catalysis in DypB (24), which may also be the case for Arg327 in TcDyP. An x-ray crystal structure is needed to identify the residues involved in the acid-base catalysis.

Electrostatic repulsions may exist between the Fe$^{3+}$ and positively charged guanidinium group of the arginine in wt-TcDyP. Thus, replacement of Arg327 with Ala327 is predicted to reduce interaction and stabilize the Fe$^{3+}$, which will shift the $E''$ to a more negative value. Indeed, a large change of redox potential was observed with R327A that has a more negative potential than the wt enzyme by $-74$ mV. Furthermore, the crystal structure of DypB has shown that arginine participates in a network that consists of hydrogen bonds extending to the protein surface (24). The network is thought to be important for peroxidase activity by mediating proton transfer as has been suggested in ascorbate peroxidase and other enzymes (71, 72). This same network may be also present in A-type DyPs because the oligomeric state of the R327A mutant is different from the wt enzyme and exists as a dimer in solution. The observed loss of peroxidase activity in R327A could also be attributed to disruption of this network that has been considered important for compound I formation (24).

Summary

A new DyP-type peroxidase, TcDyP, has been purified and characterized. Unlike other A-type DyPs, the TcDyP is highly active toward a wide range of substrates including model lignin compounds. Transient kinetics was employed to reveal the catalytic cycle involving TcDyP, which is used to resolve the contradiction regarding the catalytic importance of aspartate in A-type DyPs. It is concluded that Asp220 and Arg327 are crucial for compound I formation, whereas His312 is involved in reduction of compound II to resting state. Moreover, His312 and Arg327 also play important roles in the oligomeric state and regulating redox potential of TcDyP. Substitution of each residue with alanine resulted in the formation of dimeric state and shift of $E''$ to a more negative potential. The latter observation suggests that Fe$^{3+}$ in TcDyP appears to be stabilized in a non-polar environment, which is different from plant peroxidases (50). This may explain the difference in substrate specificity and catalytic efficiency between DyPs and plant peroxidases. Thus, fine-tuning the heme microenvironment may allow TcDyP to efficiently oxidize substrates with high redox potentials. The present study offers insights into the unique catalytic property of the A-type DyPs and facilitates them to develop into a bio-catalyst for lignin degradation.
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Author Contributions—P. L. and C. C. designed the study, C. C. performed the experiments of mutagenesis, steady-state and transient kinetics, degradation of model compounds, and SEC. R. S. and K. J. performed protein expression and purification. R. S. carried out the spectrophotometric titrations. P. F. G. constructed the plasmid of wt enzyme. S. H. B., J. S., and B. V. G. critically revised the manuscript. All authors analyzed the results and approved the final manuscript.

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