Molecular Characterization of a Novel Peroxidase from the Cyanobacterium Anabaena sp. Strain PCC 7120

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The open reading frame alr1585 of Anabaena sp. strain PCC 7120 encodes a heme-dependent peroxidase (Anabaena peroxidase [AnaPX]) belonging to the novel DyP-type peroxidase family (EC 1.11.1.X). We cloned and heterologously expressed the active form of the enzyme in Escherichia coli. The purified enzyme was a 53-kDa tetrameric protein with a pI of 3.68, a low pH optima (pH 4.0), and an optimum reaction temperature of 35°C. Biochemical characterization revealed an iron protoporphyrin-containing heme peroxidase with a broad specificity for aromatic substrates such as guaiacol, 4-aminoantipyrine and pyrogallol. The enzyme efficiently catalyzed the decolorization of anilino dyes like Reactive Blue 5, Reactive Blue 4, Reactive Blue 114, Reactive Blue 119, and Acid Blue 45 with decolorization rates of 262, 167, 491, 401, and 256 μM min⁻¹, respectively. The apparent Km and kcat/Km values for Reactive Blue 5 were 3.6 μM and 1.2 × 10⁷ M⁻¹ s⁻¹, respectively, while the apparent Km and kcat/Km values for H₂O₂ were 5.8 μM and 6.6 × 10⁶ M⁻¹ s⁻¹, respectively. In contrast, the decolorization activity of AnaPX toward azo dyes was relatively low but was significantly enhanced 2- to ~50-fold in the presence of the natural redox mediator syringaldhyde. The specificity and catalytic efficiency for hydrogen donors and synthetic dyes show the potential application of AnaPX as a useful alternative of horseradish peroxidase or fungal DyPs. To our knowledge, this study represents the only extensive report in which a bacterial DyP has been tested in the biotransformation of synthetic dyes.

In textile, food, and dyestuff industries, reactive dyes such as azo and anthraquinone (AQ) and phthalocyanine-based dyes constitute one of the extensively used classes of synthetic dyes. However, it has been estimated that approximately 50% of the applied reactive dye is wasted because of hydrolysis during the dyeing process (26, 35). This results in a great effluent problem for the industries because of the recalcitrant nature of these dyes. With increased public concern and ecological awareness, in addition to stricter legislative control of wastewater discharge in recent years, there is an increased interest in various methods of dye decolorization. Dye decolorization using physical-chemical processes such as coagulation, adsorption, and oxidation with ozone has proved to be effective. However, these processes are usually expensive, generate large volumes of sludge, and require the addition of environmentally hazardous chemical additives (26). There are several reports of microorganisms capable of decolorizing synthetic dyes. This has been attributed to their growth and production of enzymes such as laccase (1, 9, 40), azoreductases (3), and peroxidases, for example, lignin peroxidase (12, 25, 36), manganese peroxidase (10, 38), and versatile peroxidase (16). However, most of the synthetic dyes are xenobiotic compounds that are poorly degraded using the typical biological aerobic treatments. Furthermore, microbial anaerobic reductions of synthetic dyes are known to generate compounds such as aromatic amines that are generally more toxic than the dyes themselves (3). Therefore, for environmental safety, the use of enzymes instead of enzyme-producing microorganisms presents several advantages such as increased enzyme production, enhanced stability and/or activity, and lower costs by using recombinant DNA technology.

Peroxidases are heme-containing enzymes that use hydrogen peroxide (H₂O₂) as the electron acceptor to catalyze numerous oxidative reactions. They are found widely in nature, both in prokaryotes and eukaryotes, and are largely grouped into plant and animal superfamilies. They are one of the most studied enzymes because of their inherent spectroscopic properties and potential use in both diagnostic and bioindustrial applications. In particular, their ability to degrade a wide range of substrates has recently stimulated interest in their potential application in environmental bioremediation of recalcitrant and xenobiotic wastes (10, 25, 26).

Recently, a novel family of heme peroxidases characterized by broad dye decolorization activity has been identified in various fungal species such as Thanatephorus cucumeris Dec1 (18), Termitomyces albuminosus (15), Polyporaceae sp. (15), Pleurotus ostreatus (13), and Marasmius scorodonius (27). Because of their broad substrate specificity, low pH optima, lack of a conserved active site distal histidine, and structural divergence from classical plant and animal peroxidases (32), these proteins have been proposed to belong to the novel DyP per-
oxidase family. Over 400 proteins of prokaryotic and eukaryotic origins have been grouped in the DyP peroxidase family, Pfam 04261 (http://pfam.sanger.ac.uk/), and it is apparent from genome databases that many species possess DyP. The ability of these proteins to effectively degrade hydroxyl-free AQ and azo dyes as well as the specificity for typical peroxidase substrates illustrates their potential use in the bioremediation of wastewater contaminated with synthetic dyes. However, with the exception of a DyP from the plant pathogenic fungus T. cucumeris Dec1 (an anamorph of Rhizoctonia solani, a very common fungal plant pathogen), which has been characterized extensively (18, 28, 30–32, 34), little information is available on other members of the DyP family. In particular, studies on bacterial DyPs have been limited to only the automatically translated sequence or structural data (41, 42). Within the context of further understanding the structure-function and potential applicability of these novel types of enzymes in general, we have taken an interest in DyP-type enzymes, particularly, the less known bacterial groups.

Cyanobacteria (blue-green algae) represent the most primitive, oxygenic, plant-type photosynthetic organisms and are thought to have been involved in greater than 20 to 30% of the global photosynthetic primary production of biomass, accompanied by the cycling of oxygen. Anabaena sp. strain PCC 7120 is a filamentous, heterocyst-forming cyanobacterium capable of nitrogen fixation and has long been used as a model organism to study the prokaryotic genetics and physiology of cellular differentiation, pattern formation, and nitrogen fixation (14). This strain’s genome sequence is complete and annotated (17). From bioinformatics analysis of the Anabaena sp. strain PCC 7210 genome, we identified an open reading frame (ORF), alr1585, encoding a putative heme-dependent peroxidase exhibiting homology to T. cucumeris Dec1, DyP. Here, we report on the characterization of this novel bacterial DyP, designated AnaPX (for Anabaena peroxidase), from the cyanobacterium Anabaena sp. strain PCC 7120, with broad specificity for both aromatic compounds and synthetic dyes such as AQ dyes.

MATERIALS AND METHODS

Bacterial strains and materials. The Anabaena sp. strain PCC 7120 (ATCC 27893) was obtained from the ATCC (Virginia). Cell cultures were grown in BG11 medium containing 30°C in air under continuous illumination (40 μmol m−2 s−1). E. coli strains MV1184 [araΔ (lac pro AB) rpsL thi (δ80 lacZΔ M15) Δ (rl recA)606: Tn10 (Tet*) F[traD36 proAB lacI (lacZΔ M15)] and DHT5a [λ δ80ΔlacZΔ M15 Δ (lacZΔ Y argF-196 recA1 endA1 hsdR7 (tk Δmc ) sup64 thi-1 gyrA relA1] were used as host bacteria for standard recombinant constructions while katGE-disrupted E. coli BL21(DE3) [F ompT hsdSB (rB mB ) thi-1 huf β katE12::Tn10 sup64 hsdR endA1 pro thi katG: Tn5 gal dcm (DE3)] (kat mutant E. coli) was used for overexpression of AnaPX. Reactive Red 33, Reactive Yellow 2, and Direct Yellow 12 were kind gifts from Nippon Kayaku Ltd. (Tokyo, Japan). Other reactive dyes were obtained from Sigma-Aldrich Japan (Tokyo, Japan), ICN Biomedicals (Ohio), DyStar Japan (Tokyo, Japan), and Waldeck-Gmbh & Co. (Munster, Germany) were used without further purification. Horseradish peroxidase ([HRP] Reinhart Zahl value of 3.0; >99% purity) in hophorized form, purified from horseradish roots, was purchased from Oriental Yeast Co. Ltd. (Tokyo, Japan), and used without further purification. All other reagents and chemicals used were commercially available and of reagent grade.

Cloning and overexpression of recombinant AnaPX. Genomic DNA was prepared from Anabaena sp. strain PCC 7210 cells according to Ausubel et al. (2). The ORF alr1585 (http://genome.kazusa.or.jp/cyanobase/Anabaena/genes/ALR1585) was amplified by the following two oligonucleotide primers: a sense primer (5'-GGATTTCGATAGAGATTAATTTGCACC-3') containing an EcoRI recognition site (in boldface), and a stop codon (lowercase letters), and a Shine-Dalgarno sequence (underlined) located 7 nucleotides upstream of the start codon ATG (in boldface and underlined) of AnaPX; and an antisense primer (5'-CACGGCGACGCGATAAAATGCTTC-3') containing a Psil recognition site (in boldface). PCR consisted of initial denaturation at 95°C for 1 min followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min, with a final extension step at 72°C for 5 min. The amplified DNA was cloned into pPl7 Blue T-vector (Novagen, WI), and DNA sequences of both strands were checked by an ABI Prism 3100 Genetic Analyser (Applied Biosystems, CA) using a BigDye Terminator, version 3.1, Cycle Sequencing Ready Reaction Kit (Applied Biosystems). The inserted DNA was digested with EcoRI and Psil and then ligated into the respective sites of pUC18 vector (Takara Bio Ltd., Otsu, Japan). The resultant plasmid was designated pUC-AnaPX; in this construction, AnaPX was under the control of the lac promoter. A kat mutant E. coli BL21(DE3) strain (with disrupted catase genes [ΔkatE ΔkatG]) was transformed with pUC-AnaPX, and the recombinant cells were used for the overproduction and purification of Anabaena sp. strain PCC 7120 peroxidase.

The transformed cells were incubated with reciprocal shaking at 37°C in LB medium containing 50 μg/ml ampicillin. After overnight cultivation, the culture was inoculated into the same medium, supplemented with 1 mM 5-aminolevulinic acid and 10 μM hemin chloride, followed by incubation with shaking at 37°C until an optical density at 600 nm of 0.8 was reached. Isopropyl-β-D-thiogalactopyranoside was then added to a final concentration of 0.1 mM to induce the lac promoter, and further cultivation was carried out at 25°C for 18 h. Cells were harvested by centrifugation (10,000 × g) and washed twice with 50 mM potassium phosphate (KP) buffer, pH 7.0, and the pellet was stored at −80°C until purification.

Purification of recombinant AnaPX. The harvested cells were suspended in 50 mM KP buffer, pH 7.0 (buffer A), disrupted by a French press, and cell debris was removed by ultracentrifugation. The resulting supernatant was dialyzed overnight against 5 liters of buffer A. The dialyzed lysate was loaded onto a Toyopearl DEAE-650M column (2.5 by 20 cm; Tosoh Corp., Tokyo, Japan), equilibrated with buffer A and eluted with a linear gradient of 0 to 500 mM NaCl. The active fractions were pooled, solid ammonium sulfate was added to a 20% saturation concentration; fractions were subjected to a Toyopearl HTP-650M column (2.5 by 20 cm; Tosoh Corp.) equilibrated with buffer A containing 20% saturated ammonium sulfate and eluted with a linear gradient of 20 to 0% saturated ammonium sulfate. The active fractions were pooled and desalted by a PD 10 column (Amersham Pharmacia Biotech, NJ). The resultant fraction was subjected to a Hypatite C (hydroxylapatite) column (2 by 15 cm; Clarkson Chemical, PA) equilibrated with 10 mM KP buffer, pH 7.0, and washed with 5 volumes of buffer A and the protein was eluted with a linear gradient of 0 to 200 mM KP. The active fractions containing purified enzyme were pooled and desalted by a PD 10 column. The homogeneity of the purified protein was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Enzymatic analysis. AnaPX activity was assayed with a spectrophotometer (UV-1601; Shimadzu, Kyoto, Japan) at the maximum absorption wavelength of each dye and model compound at pH 4.0. Measurement of enzyme activity was initiated by the addition of 0.4 mM H2O2 at 37°C except for the assay of optimal temperature for decolorization. One unit of enzyme activity was defined as the amount of enzyme required for the decolorization of 1 μmol of Reactive Blue 5 (RBS) per min in the reaction mixtures. For dye decolorization experiments, the reaction mixtures contained 1 absorbance unit of dye at the maximal visible absorbance wavelength and 2.1 mM enzyme in 50 mM citrate buffer (pH 4.0 to 4.4), and the reactions were initiated by 0.4 mM hydrogen peroxide addition or in the presence of 40 μM syringaldialdehyde for mediator experiments. Also, each dye was treated under similar conditions for 2 h, and the decrease in absorbance of the dye solutions at their respective maximum wavelength (λmax) was monitored. The percent decolorization was calculated by taking untreated dye solution as a control (100%). The wavelengths and absorption coefficients used for various substrates were as follows: guaiacol, ε470 = 29.3 mM−1 cm−1; pyrogallol, ε430 = 2.47 mM−1 cm−1; ascobate, ε290 = 2.8 mM−1 cm−1; 4-aminoantipyrine, ε510 = 6.58 mM−1 cm−1; d-iso-ascorbate, ε290 = 3.3 mM−1 cm−1; syringaldialdehyde, ε285 = 8.5 mM−1 cm−1; ABTS [2,2’-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)], ε410 = 29.3 mM−1 cm−1; NADH and NADPH, ε440 = 6.2 mM−1 cm−1.

The protein stability of AnaPX was determined by monitoring the change in peroxidase activity at different temperatures (30°C, 40°C, 50°C, and 60°C) in 50 mM KP buffer, pH 7.0. The residual activity was determined at appropriate intervals using a standard assay method. To determine its pH stability, the enzyme was incubated for 30 min at 37°C in various pHs, and the remaining activity was again determined using a standard assay method. Kinetic parameters of
AnaPX activities for H₂O₂ and RB5 were calculated by decolorization of the dye at 600 nm (ε = 11 mM⁻¹ cm⁻¹).

Molecular mass, polyacrylamide gel electrophoresis, and protein determination. The purified enzyme sample was applied to a Superdex 200 HR 10/30 column, which was attached to a BioLogic high-performance liquid chromatograph (Bio-Rad, Tokyo, Japan), and then eluted with 50 mM KP buffer containing 0.15 M NaCl at a flow rate of 0.5 ml/min. The absorbance of the effluent was recorded at 280 nm. The molecular mass of the enzyme was calculated from the mobilities of the standard proteins glutamate dehydrogenase (290 kDa), lactate dehydrogenase (142 kDa), enolase (67 kDa), adenylate kinase (32 kDa), and cytochrome c (12.4 kDa). Matrix-assisted laser desorption ionization—time-of-flight mass spectrometry analyses were also used to determine protein molecular weights, and analysis was performed using a Voyager-DE PRO Bio-spectrometry Workstation mass spectrometer (Applied Biosystems, CA). In addition, SDS-PAGE was performed according to Laemmli (19) using a 12% polyacrylamide gel and gel stained with Coomassie brilliant blue R250. Isoelectric focusing was performed by a PhastSystem (GE Japan, Tokyo, Japan), using polyacrylamide gel and gels stained with Coomassie brilliant blue R250. Isoelectric focusing was performed by a PhastSystem (GE Japan, Tokyo, Japan), using polyacrylamide gel and gels stained with Coomassie brilliant blue R250. Isoelectric focusing was performed by a PhastSystem (GE Japan, Tokyo, Japan), using polyacrylamide gel and gels stained with Coomassie brilliant blue R250. Isoelectric focusing was performed by a PhastSystem (GE Japan, Tokyo, Japan), using polyacrylamide gel and gels stained with Coomassie brilliant blue R250. Isoelectric focusing was performed by a PhastSystem (GE Japan, Tokyo, Japan), using polyacrylamide gel and gels stained with Coomassie brilliant blue R250. 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nal sequence exactly matched the amino acid sequence (residues 2 to 8) deduced from the nucleotide sequence. The estimated molecular masses by SDS-PAGE and matrix-assisted laser desorption ionization mass spectrometry analysis were ~54 kDa (Fig. 2, lane 2) and 53,280 Da, respectively. Indeed, these findings agree with the calculated molecular mass (53,985 Da) of both the deduced amino acid sequence (53,368 Da) and the heme moiety (617 Da). Size exclusion chromatography using a Superdex 200HR 10/30 column yielded an experimental native molecular mass of 209 kDa.

**Properties of purified AnaPX.** Fig. 3 depicts the stability of the enzyme to temperature, pH, and H₂O₂. The enzyme was considerably more stable at 30°C and 40°C, where it retained more than 90% of its activity. In addition, the enzyme retained 90% activity when stored in 50 mM KP buffer, pH 7.0, at 4°C for 40 days, illustrating the robustness of the enzyme tetrameric structure under the storage conditions. However, the enzyme lost more than 90% of its activity after incubation at 50°C and 60°C for 3 h (Fig. 3a). Evaluation of the enzyme activity (decolorization of RB5 dye) at different temperatures ranging from 25°C to 45°C revealed an optimum temperature of approximately 35°C and pH optima of 4.0 to 4.4 (Fig. 3b).
When maintained at 40°C for 20 min, the enzyme was stable at pH values between 3.5 and 9.5 (Fig. 3c). AnaPX also exhibited a higher tolerance to peroxide than HRP (Fig. 3d), where it retained >50% of its activity at 2.5 mM H₂O₂ compared to that observed for HRP at 1 mM H₂O₂. The apparent optimum concentration of H₂O₂ required for the decolorization of RB5 by AnaPX was 0.4 mM; thus, this concentration was used for the peroxidase analysis experiments. Isoelectric focusing with the PhastSystem yielded only one band with a pI of 3.68 (data not shown).

Absorption spectra of AnaPX. The purified enzyme was brownish red in the solution, showing the existence of the heme group. The UV-visible spectrum of resting-state AnaPX showed Soret, and charge transfer band maxima at 404, 500, and 630 nm, respectively (Fig. 4a). The \( A_{404} / A_{280} \) ratio, or the Reinheit Zahl value, which reflects the purity and spectral characteristics of hemoproteins, was 1.4, with a molar absorption coefficient of 106 mM\(^{-1}\) cm\(^{-1}\) at 404 nm. As shown in Fig. 4a (inset), the pyridine hemochrome of AnaPX had absorption peaks at 416 (Soret band), 524 (band), and 557 nm (band) that are characteristic of iron protoporphyrin IX. By using a molar absorption coefficient of 34.5 mM\(^{-1}\) cm\(^{-1}\) at 557 nm for pyridine hemochrome, the heme content was estimated to be 0.91 mol per mole of protein. The reduction of the enzyme with dithionite decreased the Soret band and shifted it to 435 nm, and a new peak appeared at 557 nm, while the addition of cyanide resulted in a shift of the Soret band to 423 nm and the formation of a new peak at 537 nm as the peaks at 500 and 600 nm decreased (Fig. 4a). In the presence of equimolar H₂O₂ at
pH 4.4, AnaPX was oxidized to compound I, characterized with a shift in the Soret band to 401 nm with additional peaks at 359 (shoulder), 524, 557 (shoulder), and 651 nm (Fig. 4b).

**Enzyme activity inhibitors.** AnaPX also showed variable inhibition profiles in the presence of various inhibitor compounds. In particular, AnaPX was highly sensitive to the prototypical catalase inhibitor 3-amino-1,2,4-triazole in the presence of ascorbic acid (1.0 mM). Mn$^{2+}$ inhibition was found to be moderate activity toward two guaiacyl lignin unit (G-type) - derived phenolic compounds characterized by two methoxy substituents in the ortho position to the phenolic hydroxyl, syringaldehyde (54 U mg$^{-1}$) and 2-DMP (71 U mg$^{-1}$) (Table 2). The activity toward syringaldehyde was associated with a decrease in absorption peak at 320 nm and formation of a new peak at 315 nm due to product formation (data not shown).

Due to overlapping of the substrate and product absorption peaks observed in our study, the actual AnaPX activity toward this substrate may be higher than reported.

**Dye decolorization activity of AnaPX.** The ability of the purified AnaPX to decolorize representative synthetic dyes was tested under standard conditions, as described in the Materials and Methods section. The enzyme efficiently decolorized RB5 (262 U mg$^{-1}$), RB4 (167 U mg$^{-1}$), RB114 (491 U mg$^{-1}$), and RB19 (401 U mg$^{-1}$), with >90% decolorization of these dyes obtained within 5 min (Table 3). The enzyme also decolorized azo dyes at rates of 8, 13, 91, and 21 U mg$^{-1}$ for Reactive Yellow 86, Reactive Red 120, Reactive Green 19, and Reactive Black 5, respectively. Except for Direct Sky Blue 6B and Reactive Green 19, the decolorization activity of AnaPX toward azo dyes was <20% relative to activity toward RB5. However, AnaPX could not decolorize two azo dyes, namely,

<table>
<thead>
<tr>
<th>Dye</th>
<th>Type</th>
<th>$\lambda_{\text{max}}$</th>
<th>Optimal pH</th>
<th>Decolorization rate (U mg$^{-1}$)$^b$</th>
<th>Decolorization (%)$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>RB5</td>
<td>AQ</td>
<td>600</td>
<td>4.8</td>
<td>262 ± 13.0 (100)</td>
<td>91 ± 5.2</td>
</tr>
<tr>
<td>RB4</td>
<td>AQ</td>
<td>597</td>
<td>4.4</td>
<td>167 ± 5.2 (64)</td>
<td>94 ± 2.3</td>
</tr>
<tr>
<td>RB114</td>
<td>AQ</td>
<td>620</td>
<td>5</td>
<td>491 ± 23.1 (187)</td>
<td>96 ± 2.8</td>
</tr>
<tr>
<td>RB19</td>
<td>AQ</td>
<td>590</td>
<td>4.8</td>
<td>401 ± 10.5 (153)</td>
<td>94 ± 3.2</td>
</tr>
<tr>
<td>Acid Blue 45</td>
<td>AQ</td>
<td>602</td>
<td>4.6</td>
<td>256 ± 26.5 (98)</td>
<td>91 ± 5.0</td>
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<tr>
<td>Reactive Black 5</td>
<td>Azo</td>
<td>598</td>
<td>4.4</td>
<td>21 ± 3.5 (8)</td>
<td>12 ± 3.4</td>
</tr>
<tr>
<td>Reactive Green 19</td>
<td>Azo</td>
<td>622</td>
<td>4</td>
<td>91 ± 6.0 (35)</td>
<td>27 ± 8.7</td>
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<tr>
<td>Reactive Red 120</td>
<td>Azo</td>
<td>535</td>
<td>4</td>
<td>13 ± 2.6 (5)</td>
<td>12 ± 2.7</td>
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<tr>
<td>Reactive Yellow 86</td>
<td>Azo</td>
<td>417</td>
<td>4.2</td>
<td>8 ± 1.3 (3)</td>
<td>1 ± 0.5</td>
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<tr>
<td>Reactive Red 33</td>
<td>Azo</td>
<td>622</td>
<td>4-5</td>
<td>NRD (0)</td>
<td>0</td>
</tr>
<tr>
<td>Reactive Yellow 2</td>
<td>Azo</td>
<td>390</td>
<td>4-5</td>
<td>NRD (0)</td>
<td>0</td>
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<tr>
<td>Reactive Orange 14</td>
<td>Azo</td>
<td>434</td>
<td>4</td>
<td>26 ± 4.1 (10)</td>
<td>5 ± 0.9</td>
</tr>
<tr>
<td>Direct Sky Blue 6B</td>
<td>Azo</td>
<td>610</td>
<td>4</td>
<td>131 ± 16.7 (50)</td>
<td>44 ± 8.0</td>
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<tr>
<td>Congo Red</td>
<td>Azo</td>
<td>512</td>
<td>4.2</td>
<td>32 ± 5.0 (12)</td>
<td>4 ± 0.5</td>
</tr>
<tr>
<td>Acid Red 151</td>
<td>Azo</td>
<td>484</td>
<td>4.0</td>
<td>32 ± 4.3 (12)</td>
<td>12 ± 4.8</td>
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<tr>
<td>Procion Blue H-ERD</td>
<td>Triazine</td>
<td>618</td>
<td>4.4</td>
<td>236 ± 11.4 (90)</td>
<td>80 ± 10</td>
</tr>
<tr>
<td>Procion Blue H-EXL</td>
<td>Triazine</td>
<td>628</td>
<td>4.4</td>
<td>188 ± 9.0 (72)</td>
<td>72 ± 5.6</td>
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</tbody>
</table>

$^a$ Values in parentheses are relative decolorization rates (%) with the value for RB5 set at 100%. NRD, no reaction detected.

$^b$ Percent decrease in absorbance of the dye solutions at their respective $\lambda_{\text{max}}$ values after 2 h.
Indeed, 0.635 U/ml of AnaPX in the presence of 40-fold, 15-fold, 2-fold, and 7-fold improvements, respectively, active Red 120, Reactive Green 19, and Acid Red with 50-fold, decolorization of Reactive Black 5, Reactive Orange 14, Reactive Red 120, Reactive Green 19, and Acid Red, respectively (data not shown). However, Reactive Brown 10 and Reactive Orange 86 were recalcitrant to decolorization, even in the presence of syringaldehyde (Fig. 6).

Kinetic parameter analysis. The steady-state kinetics parameters of the AnaPX are summarized in Table 4. The \( K_m \) values (\( H_2O_2 \) concentration that gives 50% of the apparent maximal activity) for \( H_2O_2 \) (5.8 \( \mu M \)) and RB5 (3.6 \( \mu M \)) were smaller than those reported for other peroxidases. The second-order plots of 1/AnaPX versus 1/[\( H_2O_2 \)] at various fixed concentrations of \( H_2O_2 \) yielded a set of parallel lines, indicating a ping-pong mechanism for the oxidation of RB5 by AnaPX (data not shown). Additionally, the secondary plot of the primary y-intercepts versus 1/[\( H_2O_2 \)] revealed a linear relationship, indicating that the AnaPX activity on RB5 involves classical ping-pong bi-bi kinetics.


discussion

Compared to class I, II, and III peroxidases, DyPs represent a large group of hemoproteins that are yet to be understood although they are found in almost all bacterial genomes deposited in gene databases. The peroxidases encoded by \( alr1585 \) and its three cyanobacterial homologues do not show any significant homologies to other bacterial DyPs (Fig. 1b). However, AnaPX and its cyanobacterial homologues do not show any significant homologies to other bacterial DyPs.

The recent resolution of the X-ray crystal structures of three DyPs—\( T. \) cucumeris Dec1 DyP (32), \( Bacteroides thetaiotamicron \) VP (4), and \( S. \) oneidensis TyrA DyP (41)—revealed a unique tertiary structure and distal heme region that differ from those of most other peroxidases. The catalytically important distal histidine and distal heme region that differ from those of most other peroxidases. The catalytically important distal histidine residue conserved in the typical peroxidases is absent in DyPs.

## Table 4. Steady-state kinetic parameters of AnaPX and other peroxidases for RB5 and \( H_2O_2 \)

<table>
<thead>
<tr>
<th>Peroxidase</th>
<th>( K_m ) (( \mu M ))</th>
<th>( k_{cat} ) (s(^{-1} ))</th>
<th>( k_{cat}/K_m ) (M(^{-1} ) s(^{-1} ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>AnaPX</td>
<td>3.6</td>
<td>5.8</td>
<td>384</td>
</tr>
<tr>
<td>HRP</td>
<td>58</td>
<td>36</td>
<td>140</td>
</tr>
<tr>
<td>DyP</td>
<td>54</td>
<td>26</td>
<td>260</td>
</tr>
<tr>
<td>VP</td>
<td>4.0</td>
<td>5–10</td>
<td>4.0</td>
</tr>
<tr>
<td>TyrA</td>
<td>84</td>
<td>ND</td>
<td>5.9</td>
</tr>
</tbody>
</table>

\( ^a \)Kinetic constants were obtained from the literature in the case of HRP and \( T. \) cucumeris Dec1 DyP (18), \( P. \) eryngii versatile peroxidase (VP) (4), and \( S. \) oneidensis TyrA DyP (41). ND, not determined.

For Reactive Black 5, Reactive Orange 14, Reactive Red 120, Reactive Green 19, and Acid Red, respectively, (data not shown). However, Reactive Brown 10 and Reactive Orange 86 were recalcitrant to decolorization, even in the presence of syringaldehyde (Fig. 6).
AnaPX, like most members of the DyP family, exhibited an acidic isoelectric point (pI) of 3.68, which does not differ from that reported for T. cucumeris Dec1 DyP (3.8) (18) or the MsP1 (3.1) and MsP2 (3.7) isozymes of M. scorodonius peroxidase (27). However, AnaPX exhibited slightly higher pH optima (pH 4.0 to 4.4) (Fig. 3b) than T. cucumeris Dec1 DyP (pH 3.0 to 3.2).

The absorption maxima of AnaPX were comparable to those of high-spin heme peroxidases (24, 39). In the presence of cyanide, conversion of the ferric high-spin state to the low-spin state was observed in AnaPX. The absorption patterns were in agreement with those of low-spin, hexacoordinate species of peroxidases with histidine as the fifth ligand (24, 39), suggesting that the fifth ligand to the heme iron of AnaPX is a histidine residue. The oxidized species of AnaPX formed in the presence of H₂O₂ also showed absorption spectra typical of a compound I product.

Although less stable at 50°C and 60°C, AnaPX showed high stability at 30°C and 40°C (Fig. 3a). The enzyme maintained 83% and 78% residual activities when kept at 30°C and 40°C, respectively, for 4 days. The stabilities of AnaPX and HRP were also compared by exposing them to different H₂O₂ concentrations (Fig. 3d). Both peroxidases exhibited similar decay curves as the H₂O₂ concentration increased, with a >90% loss in activity at 15 mM. This indicates low stability at high peroxide concentrations. This is consistent with other peroxidases; it appears that an excess of H₂O₂ causes the irreversible conversion from compound II to inactive compound III, which in turn decreases activity. However, AnaPX exhibited a relatively higher tolerance than HRP at similar H₂O₂ concentrations (Fig. 3d). AnaPX also showed different inhibition profiles in the presence of various inhibitor compounds. Unlike cytochrome c peroxidases (23) and HRP (6), which are strongly inhibited by DEPC at neutral pH because of the modification of the essential distal histidine, AnaPX was only slightly inhibited (<50% inhibition at 50 mM DEPC). Therefore, the differences in the inhibition profiles of AnaPX and other plant-type peroxidases in the presence of KCN, NaN₃, and DEPC may be attributable to the differences in the accessibility of the heme active-site and catalytic residues.

Peroxidases are generally specific for H₂O₂ as the substrate but can use a number of hydrogen donors. AnaPX can oxidize both phenolic compounds such as guaiacol, 4-aminooantipyrine, and 2,6-dimethoxyphenol with activities of 230, 1,478, and 71 U mg⁻¹, respectively, more effectively than HRP. Remarkably, the enzyme could also oxidize t-ascorbate and 6-isoascorbate, which are typical ascorbate peroxidase substrates. Compared to the T. cucumeris Dec1 DyP1 isozyme, which shows >1% oxidative activities of both guaiacol and 2,6-DMP relative to RB5 activity (33), AnaPX showed high activity for the two substrates. Since the natural substrate of AnaPX remains unknown, the specific peroxidase activity may be significantly higher in vivo. AnaPX could also effectively degrade various AQ dyes compared to HRP (Fig. 5). In particular, AnaPX showed more effective decolorization activities (>90%) toward RB19 (Remazol brilliant blue R) and RB114 (Drimarene brilliant blue K-BL) with a vinyl sulfonic reactive moiety in their structures (Table 3); these dyes are generally resistant to chemical oxidation because their aromatic anthracene-9,10-dione structure is highly stabilized by resonance. In addition, the enzyme showed higher decolorization activity toward RB5, RB4, Acid Blue 45, and the triazine dyes Procion Blue H-ERD and Procion Blue H-EXL, with >70% decolorization within 2 h. Decolorization of RB5 and Acid Blue 45 by AnaPX resulted in a decrease in absorbance at 600 nm and an increase in absorbances at 400 to 500 nm accompanied with formation of a reddish-brown product with an azo link (2,2-disulfonil azobenzene). These findings are consistent with those reported for the DyP1 isozyme (31). Interestingly, the formation of reddish-brown product was not observed in the vinyl sulfonate AQ dyes (RB19, RB114, and RB4), indicating that AnaPX uses different degradative pathways for these dyes and for RB5 and Acid Blue 45. The apparent AnaPX affinity for H₂O₂ (Kₘ = 5.8 μM) and RB5 (Kₘ = 3.6 μM) was higher than that reported for the partially purified native T. cucumeris Dec1 DyP and HRP (18) but was in the same range as the affinity of the Pleurotus eryngii versatile peroxidase (4). The kinetic parameters determined for AnaPX clearly revealed that it has a higher affinity and greater redox potential than HRP and other peroxidases for H₂O₂ and RB5. Indeed, this may explain AnaPX's higher decolorization activity toward RB5. Thus, this enzyme appears to be unique because it has broader substrate specificity in addition to dye decolorization activity than T. cucumeris Dec1 DyP, which shows higher activity only toward AQ dyes rather than toward a typical substrate like guaiacol.

In contrast to AQ dyes, AnaPX weakly decolorized azo dyes, a property also observed in other DyP enzymes (13, 18). The majority of azo dyes, due to presence of azo linkages (R-N=N-R) in their structure, are generally recalcitrant to the action of oxidoreductive enzymes including peroxidases and laccases; however, the presence of redox mediators can significantly improve their decolorization (8, 11, 22). In our study, the decolorization range and oxidation rates of AnaPX compared to HRP for azo dyes were also drastically enhanced in the presence of a phenolic redox mediator, syringaldehyde. The role of redox mediators in the laccase oxidation reaction is now well characterized, and redox mechanisms have been proposed. The cationic radical is involved in either one-electron oxidation of the substrate to a radical cation or abstraction of a proton from the substrate, converting it into a radical (7, 37). It is very likely the phenoxy radicals formed during oxidation of syringaldehyde by AnaPX have similar properties to those of the laccase-mediator system; however, the actual fate and possible reactions of the mediator itself need further clarification. Compared to synthetic mediators that have the drawbacks of cost and potential toxicity, however, syringaldehyde is a potentially promising natural and cheap mediator for industrial application of AnaPX and other DyP-type enzymes, particularly in the bioremediation of azo dyes.

The AnaPX structural model showed a conserved tertiary structure of a two-domain, α+β protein, with each domain consisting of a four-stranded, antiparallel β-sheet sandwiched by α-helices in a ferredoxin-like fold (32, 42). In addition, the heme is sandwiched between the proximal and distal domains, with the heme edge relatively inaccessible. However, AnaPX and other DyPs possess a “funnel” or V-shaped channel extending ~20 Å from the surface opening directly on the distal side of the heme pocket between catalytic residues Asp204 and Arg365 (Fig. 7). This channel presumably permits the transport of smaller aromatic substrates and peroxides into and out
of the active site but may also form the substrate-binding site for large substrate molecules in DyPs. The size and hydrophobicity of this substrate channel in AnaPX model were relatively similar to the size and hydrophobicity of the T.  
cucumeris  Dec1 DyP structure, with the AnaPX channel having 468 and 65 total contact and hydrophobic contact atoms, respectively, while the T.  
cucumeris  Dec1 DyP channel consists of 424 and 62 total contact and hydrophobic contact atoms, respectively. We performed docking simulation of guaiacol (a small aromatic substrate) and RB5 (a bulkier substrate) using the ASEDock function of the MOE software to better understand the substrate binding and access modes in these two proteins. As shown in Fig. 7b, both proteins showed similar binding levels of RB5, with the anthraquinone moiety oriented ~5 Å toward the heme pocket entrance; that may explain the similar reactivities of AnaPX and the fungal DyP toward RB5. However, these proteins showed different accessibilities of guaiacol to the active site (Fig. 7b). In the AnaPX model, guaiacol easily accesses the active site and binds ~2.5 Å from the iron center within the heme pocket. In contrast, the fungal DyP has a constricted heme pocket entrance that provides steric hindrance, limiting accessibility of substrates, and thus the guaiacol molecule binds within a hydrogen bond distance to catalytic residues Asp171 and Arg329 outside the heme pocket. AnaPX shows relatively higher activities toward both guaiacol and RB5 than T.  
cucumeris  Dec1 DyP, which shows a higher specificity toward AQ than guaiacol that may be attributable to different accessibilities of the guaiacol molecule to the heme active site, as deduced by the above docking experiments. The substrate access channel of both proteins was also inaccessible to Reactive Black 5, which is bulkier than RB5, which may explain the low reactivity of DyPs toward azo dyes. It is notable that DyPs utilize different substrate binding sites from typical peroxidases, and their broad activities characterized thus far may suggest the involvement of unique radicals or an active oxygen species. In addition, due to the multiple oligomerization states exhibited by AnaPX, it was unclear whether oligomerization also affects the substrate channel and substrate specificity in contrast to the monomeric fungal DyPs. However, these hypotheses based on in silico analysis require further study.

In conclusion, we have shown that the alr1585 in the  
Anabaena  sp. strain PCC 7120 genome encodes a novel heme-dependent peroxidase. The enzyme shares certain unique molecular characteristics with fungal DyP proteins. Its low pH optima, enzymatic properties, and broad specificity for aromatic compounds and recalcitrant synthetic dyes make AnaPX a versatile DyP. The enlarged substrate spectrum of AnaPX may open new possibilities for biotechnological applications of DyP-type peroxidases, including bioremediation of wastewater contaminated by xenobiotic compounds. Furthermore, its high level of activity toward peroxidase substrates in addition to its high overproduction in recombinant hosts indicates that AnaPX could be a useful alternative to HRP or fungal DyP in biotechnological or bioindustrial applications. It may be that the same peculiarity in structure, including the nature of the axial ligands and the environment of the substrate-binding site in AnaPX, is also responsible for the other observed differences between the DyPs and the plant superfamily peroxidases. Further physicochemical and kinetic studies of DyPs are presently being carried out in our laboratory to better understand how the protein environment modulates the activity of the heme.

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