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Oxidation of Pyrrole by Dehaloperoxidase-Hemoglobin: Chemoenzymatic Synthesis of Pyrrolin-2-Ones

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Abstract

The use of oxidoreductases as biocatalysts in the syntheses of functionalized, monomeric pyrroles has been a challenge owing to, among a number of factors, undesired polypyrrole formation. Here, we have investigated the ability of dehaloperoxidase (DHP), the coelomic hemoglobin from the terebellid polychaete *Amphitrite ornata*, to catalyze the H_2O_2 -dependent oxidation of pyrroles as a new class of substrate for this enzyme. Substrate oxidation was observed for all compounds employed (pyrrole, N-methylpyrrole, 2-methylpyrrole, 3-methylpyrrole and 2,5-dimethylpyrrole) under both aerobic and anaerobic conditions. Using pyrrole as a representative substrate, only a single oxidation product, 4-pyrrolin-2-one, was observed, and notably without formation of polypyrrole. Reactivity could be initiated from all three biologically relevant oxidation states for this catalytic globin: ferric, ferrous and oxyferrous. Isotope labeling studies determined that the O-atom incorporated into the 4-pyrrolin-2-one product was derived exclusively from H_2O_2 , indicative of a peroxygenase mechanism. Consistent with this observation, single- and double-mixing stopped-flow UV-visible spectroscopic studies supported Compound I, but not Compounds ES or II, as the catalytically-relevant ferryl intermediate involved in pyrrole oxidation. Electrophilic addition of the ferryl oxygen to pyrrole is proposed as the mechanism of O-atom transfer. The results demonstrate the breadth of chemical reactivity afforded by dehaloperoxidase, and provide further evidence for establishing DHP as a multifunctional globin with practical applications as a biocatalyst.

TOC image

A biocatalytic approach using the enzyme dehaloperoxidase catalyzes the H_2O_2 -dependent oxidation of pyrrole to 4-pyrrolin-2-one and notably without polypyrrole formation.

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[†]Electronic Supplementary Information (ESI) available: Isotope labelling studies, HPLC chromatograms, UV-vis spectra of the oxidation products and ESI-MS total ion chromatograms for the reaction of DHP with N-methylpyrrole and 3-methylpyrrole in the presence of hydrogen peroxide. RFQ-EPR data for the reaction of DHP and pyrrole with H_2O_2 . See DOI: 10.1039/x0xx00000x

1 Introduction

Pyrroles are an important class of heterocyclic compounds, both for being found in a broad range of natural products as well as being a critical building block for organic synthesis and drug design. Notable examples include: i) as the key structural fragment in tetrapyrrole macrocycles¹ (e.g., heme, chlorophyll *a*, vitamin B₁₂), the pyrrole nucleus is fundamental to such biologically-related processes as respiration, photosynthesis, and biocatalysis; ii) pyrrole-containing bioactive natural products comprise a number of marine alkaloids isolated from bacteria, worms, sponges, and other invertebrates,² such as the antihistamine dispacamides from Caribbean *Agelas* sponges,³ the antimicrobial oroidin from *Agelas* sp.,⁴ the anticancer hanishin from the Red Sea sponge *Acanthella carteri* and related longamide B of the Caribbean sponge *Agelas dispar*,⁵ and the tambjamines from bryozoans, nudibranchs and ascidians that exhibit antitumor, antimicrobial and immunosuppressive activities;⁶ iii) pyrrole-based pharmaceuticals⁷ include the non-steroidal anti-inflammatory agent tolmetin,⁸ the antipsychotic elopiprazole,⁹ the antibiotic macrolide nargenicin,¹⁰ and the cholesterol-lowering agent atorvastatin;¹¹ iv) semiconducting materials, including polypyrrole¹² and related conducting polymers,¹³ are of particular interest due to their stability and redox properties. Accordingly, extensive research has been devoted to the synthesis and/or functionalization of pyrrole and its derivatives, with an emphasis on traditional organic methods.^{14–17}

By comparison, relatively little effort has focused on the use of enzymes for pyrrole derivatization. This is not surprising given the significant challenges often associated with biocatalyst development,¹⁸ particularly for oxidoreductases that represent the leading candidates for pyrrole functionalization. For example, the cytochrome P450 enzymes are able to selectively oxidize C-H bonds and alkenes in both a stereo- and regiospecific manner,¹⁹ yet the substrate specificity of P450s often needs to be broadened, either through traditional mutagenesis or directed evolution,²⁰ in order to successfully apply them to the biocatalytic conversion of new substrates.²¹ Moreover, P450s require a reductase partner for electron transfer from NADPH,²² adding both complexity and cost. Peroxidases, on the other hand, are able to resolve some of these issues, and the enzyme-catalyzed oxidative polymerization of pyrrole to polypyrrole has been reported in a number of systems, including horseradish peroxidase (HRP)²³ and laccase.²⁴ However, polypyrrole formation highlights yet another barrier to employing oxidoreductases in the synthesis of pyrrole derivatives: oxidative polymerization via radical chemistry appears to be the dominant reaction for enzymes that function via electron transfer (i.e., peroxidases) for substrate oxidation,^{24–26} rather than O-atom insertion (i.e., P450s). Despite the many advantages of a

biocatalytic approach, the use of a non-native substrate, multicomponent enzyme systems, costly reducing cofactors, and undesired polypyrrole formation are significant. As such, the use of enzymes for the chemoenzymatic synthesis of functionalized, monomeric pyrroles remains elusive.

To facilitate biocatalyst development, identifying an enzyme whose native substrate is pyrrole at the outset is a key first step. *Amphitrite ornata*, a sediment-dwelling marine polychaete, inhabits coastal mudflats that are rich in biogenically produced halometabolites: excreted from other organisms as a repellent or defense mechanism against predation, these include brominated indoles, phenols, and – of particular relevance to the present study – pyrroles (Figure 1).^{27–31} To overcome the challenges posed from this diverse array of toxic haloaromatic compounds, *Amphitrite ornata* employs its hemoglobin to function both as a detoxification enzyme and as an O₂-transport protein.³² Named dehaloperoxidase (DHP), this coelomic hemoglobin^{33–35} possesses a broad substrate specificity for the oxidation of the aforementioned halophenols^{36,37} and haloindoles,³⁸ and is therefore the first known multifunctional catalytic globin to possess biologically-relevant peroxidase³⁶ and peroxygenase activities³⁸, as well as an oxidase activity that leads to the formation of indigo.³⁸ Against compounds of anthropogenic origin, it has recently been shown that DHP catalyzes the H₂O₂-dependent oxidation of nitrophenols,³⁹ and that DHP is also capable of binding azoles, including imidazole, benzotriazole, benzimidazole, and indazole.⁴⁰ The key feature that enables such a broad substrate scope and sets DHP apart from traditionally studied globins (e.g. myoglobin and hemoglobin), or even many heme peroxidases, is a small molecule binding site located in the distal heme pocket that can accommodate a variety of different substrates.^{32,38–45} When paired with the highly reactive H₂O₂-activated forms of DHP, namely the Compound I⁴⁶ or ES⁴⁷ intermediates (the redox potential of the DHP ferryl/Fe(III) couple is estimated to be ~1.58 V³⁹), this hydrophobic cavity enables DHP to function as a versatile biocatalyst against a wide variety of substrates, which may include potential applications in chemoenzymatic synthesis.

To that end, here we have investigated the ability for DHP to catalyze the oxidation of pyrroles in the presence of H₂O₂. The rationale for doing so is as follows: i) (halo)pyrroles are native to the marine environments in which *A. ornata* resides,^{2,48,49} and likely represent physiologically-relevant substrates of the enzyme, ii) DHP has been shown to oxidize indoles,³⁸ substrates that contain a pyrrole nucleus, via a peroxygenase mechanism, and iii) the O-atom insertion chemistry of DHP^{38,39} may circumvent the undesired formation of polypyrrole seen in traditional (monofunctional) peroxidases. In this context, we have employed biochemical assays to monitor the H₂O₂-dependent/DHP-catalyzed oxidation of pyrrole, N-methylpyrrole, 2-methylpyrrole, 3-methylpyrrole, and 2,5-dimethylpyrrole. Using a combination of spectroscopic and spectrometric methods, we have identified the reaction product(s) to be primarily monomeric in nature, in stark contrast to polypyrrole formation when employing other enzymes. Stopped-flow UV-visible and rapid-freeze-quench electron paramagnetic resonance spectroscopies were used to elucidate a peroxygenase mechanism for pyrrole oxidation, and isotope labeling studies provided further support of the proposed catalytic cycle. As will be discussed, the results demonstrate that pyrrole may be considered the third class of biologically-relevant compounds, along with haloindoles and halophenols, upon which dehaloperoxidase is capable of acting. More importantly, the results further

establish DHP as a multifunctional hemoglobin capable of peroxxygenase activity, an adaptation that not only enables *A. ornata* to persist in environments that contain a diverse array of toxic haloaromatic compounds, but one that may possibly be exploited via protein engineering methods for practical applications.

2 Experimental

2.1 Materials and Methods

Reagent-grade chemicals and HPLC grade acetonitrile were purchased from commercial sources (VWR, Sigma-Aldrich or Fisher Scientific) and used without further purification. Isotopically-labeled H_2^{18}O (98% ^{18}O -enriched) and $\text{H}_2^{18}\text{O}_2$ (90% ^{18}O -enriched) were purchased from Icon Isotopes. Stock solutions of pyrrole, N-methylpyrrole, 2-methylpyrrole and 3-methylpyrrole were dissolved in 100 mM KPi buffer at the desired pH and stored at -80°C until needed. UV-visible spectroscopy was performed aerobically on a Varian Cary Bio50 spectrophotometer, or anaerobically on an Agilent 8454 diode-array spectrophotometer in a glovebox. DHP variants were expressed and purified as previously reported.^{46,47,50} Oxyferrous DHP was obtained by the aerobic addition of excess ascorbic acid to a solution of ferric DHP, followed by desalting (PD-10 column). Enzyme concentration was determined spectrophotometrically using $\epsilon_{\text{Soret}} = 116,400 \text{ M}^{-1}\text{cm}^{-1}$ for all isoenzymes.⁴⁷ Lyophilized horseradish peroxidase and horse heart myoglobin were purchased from Sigma-Aldrich and stored at -20°C until utilized. Solutions of H_2O_2 were prepared fresh daily and kept on ice until needed. H_2O_2 concentration was determined by UV-vis ($\epsilon_{240} = 46 \text{ M}^{-1}\text{cm}^{-1}$).⁵¹ Anaerobic studies were performed as described above in an MBraun LABmaster 130 nitrogen-filled glove box using argon degassed or deoxygenated solutions of buffer, peroxide, substrate and enzyme.

2.2 Enzyme Assay Protocol

Reactions were performed in triplicate at pH 7 unless otherwise indicated, in 100 mM KPi at 25°C . Buffered solutions of DHP (10 μM final concentration) and pyrrole (500 μM final concentration) were pre-mixed, and the reaction was initiated upon addition of H_2O_2 (500 μM final concentration, $\sim 15 \text{ mM}$ stock concentration). Experiments were performed in the presence of D-mannitol (500 μM), superoxide dismutase (SOD) ($\sim 2 \text{ U}/\mu\text{L}$), and DMSO (10% v/v). For the studies with sodium formate, a buffered solution (100 mM KPi; 500 mM sodium formate) at pH 7 was used. Reactions were quenched at 5 minutes with excess catalase and diluted 1:2 for pyrrole and 1:5 for all other substituted pyrrole substrates. Diluted samples were analyzed using a Waters 2796 Bioseparations Module coupled with a Waters 2996 Photodiode Array Detector, and equipped with a Thermo-Scientific ODS Hypersil (150 mm $\times$ 4.6 mm) 5 μm particle size C_{18} column. Separation of observed analytes was performed using a linear gradient of binary solvents (solvent A - H_2O containing 0.1% trifluoroacetic acid, solvent B - MeCN). Elution was performed using the following conditions: (1.5 mL/min A:B) 99:1 to 0:100 linearly over 18 minutes; 0:100 isocratic for 2 minutes; 0:100 to 99:1 linearly over 1 minute, then isocratic for 4 minutes. Data analysis was performed using the Empower software package (Waters Corp.). Calibration curves for all pyrroles were performed using serial dilutions of commercially available analytes to determine the amount of substrate conversion.

For the ^{18}O -labeling studies, assays were performed using the same final concentrations as above. However, the stock enzyme (220 μM) and substrate (25 mM) concentrations were higher to allow maximum addition of the label in each sample. In the H_2^{18}O studies, 226.9 μL H_2^{18}O (out of 250 μL total reaction volume) was achieved using a stock unlabeled hydrogen peroxide concentration of 18.3 mM in 100 mM KPi . In the $\text{H}_2^{18}\text{O}_2$ experiments, the stock concentration of 90% enriched $\text{H}_2^{18}\text{O}_2$ was 20.1 mM. After 5 minutes, the reactions were quenched with excess catalase, and 20 μL of the undiluted solution was injected immediately for LC-MS analysis.

2.3 LC-MS Studies

Analysis was carried out on a Thermo Hypersil Gold (50×2.1 mm) 1.9 μm particle size C_{18} column with a Thermo Fisher Scientific Exactive Plus Orbitrap mass spectrometer and a heated-electrospray ionization (HESI) probe (Thermo Scientific, San Jose, CA). Analyte separation was achieved by using a linear gradient of binary solvents (solvent A - H_2O containing 0.1% formic acid; solvent B - MeCN), with an elution profile (0.5 mL/min, A:B) of 99:1 to 0:100 over 20 min. Samples were analyzed using electrospray ionization in positive ion mode to provide observation of the $[\text{M} + \text{H}]^+$ species. Spectra were collected each second while scanning in the range from 55 – 1000 m/z. Data analysis was performed using Thermo Xcalibur software.

2.4 Stopped-flow UV-Visible Studies

Optical spectra were recorded using a Bio-Logic SFM-400 triple-mixing stopped flow instrument coupled to a rapid scanning diode array UV-visible spectrophotometer. The temperature was maintained at 20 $^{\circ}\text{C}$ with a circulating water bath, and all solutions were prepared in 100 mM KPi (variable pH). Data were collected (900 scans total) over a three-time domain regime (2.5, 25, and 250 ms; 300 scans each) using the Bio Kinet32 software package (Bio-Logic). Kinetic data of sufficient quality were evaluated using the Specfit Global Analysis System software package (Spectrum Software Associates) and fit to exponential functions as one-step/two-species, two-step/three species, or three-step/four species irreversible mechanisms where applicable. Data were baseline corrected using the Specfit autozero function.

Experiments were performed as follows: i) single-mixing experiments reacted either ferric or oxyferrous WT DHP (10 μM final concentration) that was pre-incubated with pyrrole (10–50 equiv.) with 10 equivalents of H_2O_2 ; ii) double-mixing experiments were performed using an aging line prior to the second mixing step to observe Compound I/ES/II reactivity with pyrrole substrate (2.5–50 eq.): Compound I was pre-formed by reaction of ferric DHP B (Y28F/Y38F) with 10 equiv. of H_2O_2 in an aging line for 150 ms prior to mixing with the substrate, Compound ES was pre-formed by reaction of ferric WT DHP B with 10 equiv. of H_2O_2 in an aging line for 450 ms, and Compound II was formed from oxyferrous DHP B pre-incubated with 1 equiv. of TCP and reacted with 10 equiv. of H_2O_2 in an aging line for 82 s.

2.5 Preparation of Reaction Intermediates by Freeze-Quench Methods

Rapid-freeze-quench experiments were performed with a BioLogic SFM 400 Freeze-Quench apparatus by mixing a 75 μM ferric WT DHP B solution (final concentration) pre-incubated with 50 mol equiv. pyrrole with a 10-fold excess of H_2O_2 in 100 mM KP_i (pH 7) at 25 $^\circ\text{C}$. A standard 4 mm O.D. quartz EPR tube was connected to a Teflon funnel, and both the tube and the funnel were completely immersed in an isopentane bath at -110°C . The reaction mixtures were quenched by spraying them into the cold isopentane, and the frozen material so obtained was packed at the bottom of the quartz tube using a packing rod equipped with a Teflon plunger. In this manner, a packing factor of $60 \pm 2\%$ was consistently achieved. Quench times were 100 ms and 1 s. Samples were then transferred to a liquid nitrogen storage dewar until analyzed. After analysis, the 100 ms sample was allowed to thaw on the bench top and subsequently re-frozen in liquid nitrogen to generate a 10 min sample.

2.6 X-band EPR Spectroscopy

EPR spectra were recorded with an X-band (9 GHz) Varian E-9 EPR spectrometer (Varian, El Palo, CA). A standard 4 mm o.d. quartz EPR tube containing the sample was placed into a quartz finger dewar insert filled with liquid nitrogen. The temperature of the samples was maintained at 77 K for the duration of the data acquisition, which required periodic refilling of the dewar due to the evaporation of the liquid nitrogen during longer acquisition runs. The typical spectrometer settings were as follows: field sweep 200 G, scan rate 3.33 Gauss/s, modulation frequency 100 KHz, modulation amplitude 4.0 G, and microwave power 2 mW. The exact resonant frequency of each EPR experiment was measured by an EIP-578 (PhaseMatrix, San Jose, CA) in-line microwave frequency counter.

2.7 Substrate-Binding Studies

Fluoride-binding studies were performed per literature protocol⁵² to determine the extent of substrate binding. DHP B (50 μM) was incubated with 5 mM pyrrole, and 10 – 400 equiv. of fluoride (using a 180 mM NaF stock solution) were titrated in 100 mM KP_i buffer at pH 7. The absorbance at 605 nm was monitored, and the analysis was performed as published.

2.8 Non-enzymatic Pyrrole Oxidation Studies

Adapted from literature protocols,^{53–56} 100 mM pyrrole dissolved in water was brought to reflux in the presence of 100 mM H_2O_2 . After reflux for 4 hours and subsequent cooling to room temperature, diluted samples (1:25–1:100 in water) were then analyzed by HPLC as described above. Reaction conditions were identical for the non-enzymatic oxidation of N-methylpyrrole and 3-methylpyrrole.

3 Results

3.1 Pyrrole Oxidation by DHP

Substrate Variation—The hydrogen peroxide-dependent oxidation of pyrrole substrates as catalyzed by DHP was monitored by HPLC, and the results are summarized in Table 1. Reactions were initiated upon addition of 500 μM H_2O_2 to a solution containing 10 μM

enzyme and 500 μM pyrrole, incubated at 25 $^{\circ}\text{C}$ for 5 min, and then quenched with catalase. For reactions employing ferric WT DHP B under aerobic conditions, substrate turnover was as follows: 2,5-dimethylpyrrole > 2-methylpyrrole > 3-methylpyrrole > N-methylpyrrole > pyrrole, a trend that mirrors the basicity of pyrrole and its mono-methyl substituted analogs as revealed by their $\text{p}K_{\text{a}}$ values for α -protonation⁵⁷ ($\text{p}K_{\text{a}}$: 2-methyl-, -0.21; 3-methyl-, -1.00; N-methyl-, -2.9; pyrrole, -3.8). When the pyrrole oxidation reaction was repeated under anaerobic conditions, pyrrole and N-methylpyrrole yielded virtually identical amounts of substrate loss regardless of the presence of O_2 , suggesting that the oxidation of these substrates was independent of molecular oxygen and wholly dependent upon H_2O_2 . By contrast, 2-methyl-, 3-methyl-, and 2,5-dimethylpyrrole all exhibited slightly attenuated substrate turnover under anaerobic conditions, results that possibly indicated a secondary dioxygen-dependent pathway in addition to the H_2O_2 -dependent one. It is important to note, however, that the same reactivity trend as a function of substrate $\text{p}K_{\text{a}}$ was noted in the anaerobic reactions as in the aerobic ones, and that no substrate turnover occurred when either DHP (non-enzymatic control) or peroxide (non-oxidant control) were excluded from the reaction for pyrrole, N-methylpyrrole, and 3-methylpyrrole. However, as reactivity was observed for 2-methyl and 2,5-dimethylpyrrole in the absence of hydrogen peroxide, yielding polypyrrole, these substrates were excluded from further investigations here and will be discussed in a later study.

Enzyme Variation—Nearly the same amount of pyrrole conversion was observed when initiating the pyrrole oxidation reaction under anaerobic conditions with ferrous WT DHP B ($27.9 \pm 3.8\%$) as with the ferric form ($30.6 \pm 2.1\%$). When starting with oxyferrous DHP, however, attenuated substrate conversion (isoenzyme A, $12.7 \pm 3.4\%$; isoenzyme B, $17.8 \pm 1.0\%$) was noted at pH 7. While these results demonstrated that pyrrole oxidation could be initiated directly from the globin active ($\text{Fe}^{\text{II}}\text{-O}_2$) state, they differ from the previously studied halophenol⁵⁸ (peroxidase) and haloindole³⁸/nitrophenol³⁹ (peroxygenase) substrates that showed that reactivity was independent of the starting DHP oxidation state. As one possible explanation for this difference, we surmise that the relatively weak binding of the pyrrole substrate (Section 3.4), when compared with the halophenol, haloindole, and nitrophenol substrates, is unable to promote the known^{32,58–60} substrate-dependent activation of oxyferrous DHP (see Discussion).

Three additional studies were also performed: i) the mutant DHP B(Y28F/Y38F), which forms Compound I rather than the Compound ES species observed in WT DHP B,⁴⁶ exhibited nearly the same reactivity as the native enzyme; ii) studies performed with horse heart myoglobin (Mb), an archetype of the globin superfamily, showed no reactivity with pyrrole; iii) horseradish peroxidase (HRP) was found to react with pyrrole, but differed from the reaction with DHP in that multiple products were observed, consistent with previous studies that showed oligomeric and polypyrrole formation.^{23,25}

pH Dependence—A comparison of pyrrole reactivity between the two DHP isoenzymes, A and B, revealed marked differences between the two in their pH dependences. Isoenzyme A exhibited only a 1.6-fold difference in substrate conversion from pH 5–8: maximal pyrrole oxidation was observed at pH 7 ($42.8 \pm 3.1\%$), with a slight loss in activity at pH 6 (36.1

$\pm 3.4\%$) and pH 5 ($35.2 \pm 2.9\%$), and a more pronounced loss at pH 8 ($26.8 \pm 1.2\%$). By contrast, pyrrole oxidation for isoenzyme B exhibited a ~5-fold difference over the same pH range, and was maximally active at pH 5 ($50.8 \pm 2.0\%$), with activity decreasing progressively as the pH was increased: pH 6 ($38.8 \pm 3.2\%$) > pH 7 ($31.7 \pm 2.5\%$) > pH 8 ($10.8 \pm 2.2\%$). While pH effects have been observed for other DHP substrates,⁶¹ most notably for haloindoles³⁸ and nitrophenols³⁹ that were studied with the identical HPLC assay employed here, their conversion was always higher at lower pH, which differs from the DHP A results reported here for pyrrole. Moreover, DHP B consistently yielded greater substrate turnover when compared to DHP A for those peroxygenase substrates regardless of pH, which again differs from the results with pyrrole for pH 7 and 8. Given its pK_a of -3.8 for α -protonation, as well as a pK_a of 17.5 for the N-H proton,⁵⁷ it is unlikely that the pH effects observed here over the pH range of 5–8 are substrate related. While we surmise that structural differences between DHP A and B may lead to subtle differences in the availability of protons at the active site through alterations of the H-bonding networks that are specific to the pyrrole oxidation pathway, the origins of the differences in the pH-dependences for the two isoenzymes was not further explored.

Mechanistic Studies—The pyrrole oxidation reaction was performed in the presence of radical scavengers to probe if solvent accessible radicals played a role in substrate turnover. While the presence of superoxide dismutase ($29.5 \pm 2.2\%$) did not significantly alter the amount of substrate conversion, the inclusion of both sodium formate ($25.6 \pm 0.2\%$) and D-mannitol ($25.9 \pm 1.6\%$) showed a slight loss in activity when compared to the reaction run in their absence ($31.7 \pm 2.5\%$). The addition of DMSO led to a much greater loss in activity ($8.1 \pm 0.5\%$). No changes in product distribution were observed by HPLC in the presence of these radical scavengers (data not shown). When 4-bromophenol ($K_d = 1.15 \text{ mM}^{62}$), the known inhibitor of DHP peroxidase and peroxygenase activities,^{38,47} was included in the reaction at 500 μM , conversion of pyrrole was significantly reduced ($5.1 \pm 0.6\%$). Taken together, the results suggest that the lower activity of DHP in the presence of DMSO and the other radical scavengers is likely related to the weak binding of pyrrole (Section 3.4) rather than as suggestive of a radical-based mechanism.

3.2 Identification of Reaction Products by HPLC

Pyrrole (1)—A representative HPLC chromatogram of the DHP-catalyzed oxidation of pyrrole as monitored at 248 nm is shown in Figure 2A, and a summary of the reaction products is shown in Chart 1. Under the conditions employed, only one reaction product was observed (Figure 2B, $t_R = 3.5 \text{ min}$). This product exhibited an identical retention time and UV-visible spectrum (Figure 2B), as well as mass (m/z 154, $[M+H]^+$; Figure 2A), as an authentic sample of 4-pyrrolin-2-one (**1a**), providing unequivocal evidence for the identity of this species as being formed from O-atom incorporation into the pyrrole substrate. The authentic sample of 4-pyrrolin-2-one was generated following a literature protocol⁵³ where equimolar pyrrole and hydrogen peroxide were reacted via reflux in water. Per that literature precedent, it was noted that two major products were formed, 4-pyrrolin-2-one (**1a**; $t_R = 3.5 \text{ min}$) and 3-pyrrolin-2-one (**1b**; $t_R = 3.2 \text{ min}$), in a 1:9 ratio, respectively (Figure S1). The non-enzymatic reaction was reported to be unable to generate **1a** in good purity due to its isomerization into the more thermodynamically stable product, 3-pyrrolin-2-one (**1b**), that

occurs rapidly at the elevated reaction temperature.⁵³ Under the room temperature conditions employed for the DHP-catalyzed reaction, only **1a** was formed (Chart 1). However, when the reaction mixture was left at room temperature for 24 hours, isomerization to 3-pyrrolin-2-one with a ~1:9 (**1a**:**1b**) ratio was observed (Figure S2), consistent with the non-enzymatic reaction. Moreover, when the DHP-catalyzed and non-enzymatic reactions were mixed in a 1:1 ratio, only an increase in the **1a** peak was detected (Figure S3). With the above information, it was concluded that the product in the DHP catalyzed oxidation of pyrrole is 4-pyrrolin-2-one (**1a**).

The oxidation of pyrrole was monitored as a function of H₂O₂ concentration at pH 5 and 7 (Figure S4). At pH 7, 4-pyrrolin-2-one (**1a**) was formed linearly across the range studied up until ~150 μ M H₂O₂, at which point product formation plateaued. At pH 5, a similar observation was noted with the plateau occurring at ~200 μ M H₂O₂. The lack of further product formation under conditions of excess peroxide is in line with previous observations that DHP enters a peroxidase-attenuated state,³⁷ Compound RH,^{46,47} under conditions of low [substrate].

N-Methylpyrrole (2)—The DHP-catalyzed oxidation of N-methylpyrrole (**2**) yielded three singly oxygenated products (t_R = 4.1, 4.4 and 4.7 min; Figure S5), all which exhibited a m/z of 98 ($[M+H]^+$: C₅H₈NO). On the basis of their retention times, mass, and UV-visible spectral features (Figure S6) when compared with authentic samples that were synthesized per literature protocol,^{53,56} we conclude that N-methyl-4-pyrrolin-2-one (**2a**) to be the major enzymatic product at 4.4 minutes and N-methyl-3-pyrrolin-2-one (**2b**) is the minor enzymatic product at 4.1 minutes (Chart 1). As with the parent pyrrole reaction, a slow isomerization of **2a** to **2b** was observed after > 24 hours. Similarly, the product at t_R = 4.7 minutes may represent O-atom insertion at the 3 or 4 position, but was not further explored here.

3-Methylpyrrole (3)—The reaction of 3-methylpyrrole (**3**) with DHP and hydrogen peroxide produced four products (t_R = 4.3, 4.4, 4.7 and 4.8 min; Figure S7A), all which exhibited a m/z of 98 ($[M+H]^+$: C₅H₈NO). Consistent with the data reported in Table 1, the reactions ran under anaerobic (data not shown) and aerobic conditions yielded identical products, but with a slightly different distribution. Following literature protocols,^{53,56} an authentic sample of 3-methyl-3-pyrrolin-2-one (**3a**) was synthesized and subjected to HPLC analysis (Figure S7B). The reported product of the non-enzymatic reaction matched the UV-visible spectrum, mass, and retention time of the 3-methylpyrrole/DHP-catalyzed oxidation product at t_R = 4.4 min (Figure S7C). The other three singly oxygenated products observed in the DHP-catalyzed reaction could be the O-atom insertion at the 4- or 5- positions, and/or possibly their corresponding pyrroline isomers, however these were not structurally characterized.

3.3 Isotopically-labeled Oxygen Studies

The identified product in the pyrrole oxidation reaction, 4-pyrrolin-2-one (**1a**), incorporated an O-atom via an O₂-independent reaction (Table 1). Therefore studies employing labeled H₂¹⁸O₂ and H₂¹⁸O (90% and 98% ¹⁸O-atom enriched, respectively) were performed with

pyrrole, and subsequently analyzed by LC-MS (Figure 3) to determine the origin of the O-atom incorporation. The isotopic distributions of the $[m/z]^+$ values are shown in Table S1. In the presence of $H_2^{18}O$ and unlabeled H_2O_2 , no significant increase in mass was observed (m/z : 84, 100%; 86, n.d.; Figure 3A) when compared with the absence of an ^{18}O source (data not shown), thus ruling out solvent water as the source of the O atom incorporated. The same experiment employing $H_2^{18}O_2$ and unlabeled H_2O showed a clear increase to higher mass by 2 Da for 4-pyrroline-2-one (**1a**): m/z : 84, 21%; 86, 100% (Figure 3B). Similarly, the experiment performed with both labels, $H_2^{18}O$ and $H_2^{18}O_2$, showed virtually the same ^{18}O -atom incorporation into the **1a** product as the $H_2^{18}O_2$ only study (m/z : 84, 20%; 86, 100%; Table S1). The results provide unambiguous evidence that the oxygen atom was derived from H_2O_2 , consistent with a peroxygenase mechanism for pyrrole oxidation.

The above isotopically labeled experiments were repeated for the N-methylpyrrole (**2**) and 3-methylpyrrole (**3**) substrates. For N-methylpyrrole (Figure S8 and Table S1), the results provide further evidence for a peroxygenase mechanism: all three products showed no ^{18}O -atom incorporation with $H_2^{18}O/H_2^{16}O_2$, and virtually full label incorporation with $H_2^{16}O/H_2^{18}O_2$ and $H_2^{18}O/H_2^{18}O_2$. In the case of 3-methylpyrrole, as this substrate exhibited a secondary aerobic reaction (Table 1), the isotopically labeled studies were performed anaerobically. Of the four reaction products (Figure S9, Table S1), three (t_R = 4.3, 4.7 and 4.8 min) exhibited a minor amount of label incorporation with $H_2^{18}O/H_2^{16}O_2$, likely indicative of scrambling with the solvent, whereas the product at t_R = 4.4 min showed no ^{18}O addition. In the $H_2^{16}O/H_2^{18}O_2$ reaction, the most abundant peak for each of the four products corresponded to ^{18}O -incorporation, but a significant amount of unlabeled products were still present. However, when both $H_2^{18}O/H_2^{18}O_2$ were present, all four products exhibited virtually full label incorporation. Although it is evident that scrambling of the label with the reaction solvent occurs for the 3-methylpyrrole oxidation products, taken together the results are consistent with a peroxygenase mechanism.

3.4 Fluoride Binding Studies

The optical absorbance spectrum of ferric DHP B [407 (Soret), 508, 540 (sh), 633 nm] was unperturbed even in the presence of >100 equiv. of pyrrole (data not shown), thereby precluding the use of the Soret or Q bands in substrate binding studies for direct measurement of the K_d values. Thus, a competition assay with the heme-fluoride binding equilibrium was employed to indirectly measure substrate binding (Figure S10).⁵² In this method, substrates that bind within the DHP active site lead to a decreased binding affinity of fluoride to the heme ($K_d^{F^-}$ = 2.5 ± 0.2 mM, pH 5). In the presence of 5 mM pyrrole, a ~2-fold increase in the $K_d^{F^-}$ to 5.2 ± 0.4 mM at pH 5 was observed (Table 2), consistent with pyrrole binding to the active site of DHP. Similar $K_d^{F^-}$ values were also obtained for N-methylpyrrole (5.4 ± 0.2 mM) and 3-methylpyrrole (5.1 ± 0.2 mM). When compared with the $K_d^{F^-}$ values reported for other peroxygenase (5-bromoindole, 13.2 ± 0.5 mM; 4-nitrophenol, 18 ± 1 mM) and peroxidase (2,4,6-tribromophenol, 23.8 ± 1.0 mM) substrates, it is evident that the pyrrole substrates bind with a significantly weaker affinity to DHP.

3.5 Stopped-flow UV-visible Spectroscopic Studies with Pyrrole

Single and double-mixing stopped-flow UV-visible spectroscopic methods were used to investigate the reaction of pyrrole with DHP and hydrogen peroxide. Studies were performed with H₂O₂-activated DHP (i.e., pre-formed as Compound I [DHP B(Y28F/Y38F)],⁴⁶ Compound ES,⁴⁷ or Compound II⁵⁸), or by pre-incubating DHP with pyrrole followed by H₂O₂ addition.

Compound I Reactivity—Preformed Compound I, itself generated in an initial mixing step of ferric DHP B(Y28F/Y38F) with 10 equiv. H₂O₂ in an ageing line for 150 ms,⁴⁶ was reacted with 50 equiv. pyrrole at pH 7. No activated enzyme (i.e., Compound I [406 (Soret), 528, and 645 nm]) was initially observed (Figure 4A). Rather, ferric DHP B(Y28F/Y38F) [406 (Soret), 507, 540 (sh) nm] was the first spectrum recorded, suggestive that Compound I was reduced by the substrate within the mixing time of the stopped-flow apparatus. The rapid reaction of Compound I with pyrrole is reminiscent of its reactivity with 5-Br-indole³⁸ and 4-nitrophenol³⁹ in that both were also found to reduce Compound I to the ferric enzyme within the mixing time of the stopped-flow apparatus via a peroxxygenase mechanism. Due to the experimental conditions of excess H₂O₂ employed here, the resultant ferric enzyme subsequently converted within 2 s to a ferryl containing species [416 (Soret), 545, 585 (sh), blue] assigned as Compound II (see Section 3.6), which further formed oxyferrous DHP [416 (Soret), 542, 578 nm, red] after 20 seconds. For both of these, a small amount of ferric enzyme was also observed to be present. At longer observation times (data not shown), the reaction underwent bleaching. We surmise that the known reactivity of Compound II with H₂O₂ to yield the oxyferrous state in other hemoproteins^{63–65} as well as in the DHP A(Y38F) mutant⁴⁶ is likely the origin of oxyferrous DHP B (Y28F/Y38F) here.

Single-mixing studies were also performed: ferric DHP B(Y28F/Y38F) preincubated with 50 eq. of pyrrole was reacted with 10 eq. of H₂O₂ at pH 7 (Figure S11A). No formation of Compound I was seen under these conditions, likely due to its immediate reduction in the presence of pyrrole substrate. Rather, the ferric enzyme was initially observed [406 (Soret), 507, 540 (sh) nm, black; t = 2.5 ms], followed by conversion to a ferryl species [419 (Soret), 545, 585 nm, blue; t = 2.6 s] that reformed the resting state ferric enzyme [406 (Soret), 507, 540 (sh) nm, red] after 83 s. No formation of oxyferrous DHP B(Y28F/Y38F) was noted under these conditions.

Compound ES Reactivity—Ferric WT DHP B (10 μM) was reacted with 10 molar equivalents of H₂O₂ at pH 7, incubated for 450 ms to allow for the maximum accumulation of Compound ES,⁴⁷ and subsequently mixed with 10–50 equiv. pyrrole. As a representative reaction, the preformed Compound ES [419 (Soret), 545, 584 (sh) nm, black; t = 0 s] was converted in the presence of 50 eq. pyrrole to a second ferryl-containing species [419 (Soret), 545, 577 (sh) nm, blue; t = 22 s] that underwent further reduction to the resting ferric state after 830 s (Figure S11B). Under these conditions, Compound RH,^{47,50} the stable yet inactive form of DHP that arises from H₂O₂-activation in the absence of substrate,³⁷ was not observed. Moreover, no product-driven reduction of the enzyme to the oxyferrous form was observed, a departure from similar assays that employed other physiological substrates (i.e., trihalophenols⁴⁷ and haloindoles³⁸).

To further investigate this reaction, single-mixing studies were also conducted: ferric WT DHP B (10 μ M) preincubated with 50 eq. of pyrrole was reacted with 10 eq. of H₂O₂ at pH 5 (Figure 4B). The ferric enzyme [UV-visible: 407 (Soret), 510, 635 nm, black] was converted to a ferryl species [417 (Soret), 544, 584 nm, blue; $k_{\text{obs}} = 5.35 \pm 0.09 \text{ s}^{-1}$] that subsequently underwent slow reduction back to the ferric state [407 (Soret), 510, 540 (sh) nm, red; $k_{\text{obs}} = 0.11 \pm 0.01 \text{ s}^{-1}$]. Similar results were seen at pH 7 (data not shown). Neither Compound RH nor oxyferrous DHP were observed.

For both of the above reaction conditions, several lines of evidence suggest the identity of the second ferryl species as DHP Compound II rather than Compound ES: i) the spectral features are subtly different from Compound ES (i.e., ~5% hypochromicity of the Soret band and a slight blue shift in the broad Q band shoulder from >583 nm to 578 nm), and match the substrate-bound forms of Compound II observed previously with 5-bromoindole³⁸ and 4-nitrophenol³⁹; ii) Compound II has been previously shown to exhibit a slower substrate-dependent reduction to the ferric enzyme than Compound ES for 2,4,6-trichlorophenol,^{47,58} 5-bromoindole,³⁸ and 4-nitrophenol.³⁹ As such, in the above experiments employing preformed Compound ES, the persistence of the second ferryl species for over 800 seconds is consistent with Compound II reactivity; iii) were the Compound ES species not to react with pyrrole to generate Compound II, Compound RH would be observed in the above experiments,^{47,50} however this was not the case; iv) the lack of an EPR spectroscopic signature consistent with Compound ES^{46,47,50} by rapid-freeze-quench EPR studies (Section 3.6).

Compound II Reactivity—Previous studies have shown that oxyferrous DHP can only be activated by H₂O₂ in the presence of substrate (TCP,^{59,60} 5-bromophenol,³⁸ or 4-nitrophenol³⁹), leading to Compound II formation, whereas its activation in the absence of a substrate leads to the bleaching of the Soret band and/or slow conversion to Compound RH without the formation of any observable fast timescale reactive intermediates.⁵⁸ Here, we investigated the reactivity of preformed DHP Compound II with pyrrole using sequential double-mixing stopped-flow methods: oxyferrous DHP B containing 1 equivalent TCP was reacted with 10 molar equivalents of H₂O₂ at pH 6, incubated in an aging line for 83 s to allow for the maximum accumulation of Compound II [419 (Soret), 545, 585 (sh) nm, black],⁵⁸ and subsequently mixed with an additional 50 equiv of pyrrole (Figure S11C). The Compound II spectrum was found to convert to ferric DHP [407 (Soret), 505, 540 (sh) nm, blue] within 152 s, followed by formation of a species whose spectral features [411 (Soret), 540, 576 (sh) nm, blue] matched those of a mixture of ferric DHP B and the oxyferrous enzyme, the latter likely arising from the known secondary reaction between Compound II with excess H₂O₂ that generates the oxyferrous state in hemoproteins.^{63–65} Little to no optical changes were observed when pyrrole was reacted with Compound II at pH 7 or 8 (data not shown).

Oxyferrous Reactivity—Single-mixing stopped-flow methods were used to investigate if pyrrole was able to initiate the substrate-dependent activation of oxyferrous DHP B. Upon rapid mixing of a solution of oxyferrous DHP B pre-incubated with 50 eq. of pyrrole with 10 equivalents of H₂O₂ at pH 8, no significant optical changes were observed (data not shown),

consistent with the low reactivity of oxyferrous DHP with pyrrole at this pH (Table 1). However, when repeated at pH 7, a slow conversion of oxyferrous DHP B [418 (Soret), 543, 578 nm, black] to a transiently observable Compound II intermediate [419 (Soret), 545, 578 (sh) nm, blue; $k_{\text{obs}} = (1.87 \pm 0.05) \times 10^{-2} \text{ s}^{-1}$] was seen (Figure 4C), although without full formation owing to its decay with nearly the same rate [$k_{\text{obs}} = (6.98 \pm 1.02) \times 10^{-3} \text{ s}^{-1}$] to a mixture of Compound RH and oxyferrous DHP [410 (Soret), 536, 575 (sh) nm, red] after 830 s.

The main observations from these stopped-flow studies with pyrrole were i) Compound I was rapidly reduced by pyrrole within the mixing time of the instrument to the ferric enzyme, ii) Compounds ES was initially reduced to Compound II, followed by further reduction to the ferric enzyme, iii) Compound II reacted with pyrrole to reform ferric DHP, and iv) pyrrole was found to activate oxyferrous DHP towards reactivity with H_2O_2 via a ferryl (likely Compound II) intermediate.

3.6 Confirmation of Compound II by EPR Spectroscopy

In an effort to unambiguously establish the ferryl intermediate observed in the stopped-flow UV-visible studies as Compound II, rapid-freeze-quench methods were employed to stabilize this species for subsequent characterization by continuous wave (CW) EPR spectroscopy. Specifically, X-band EPR spectra at quench times of 100 ms and 1 s were obtained for the reaction of 75 μM ferric DHP B premixed with 50 equiv of pyrrole with 10 equiv of H_2O_2 at pH 7. After spectral acquisition, the 100 ms sample was gently warmed to room temperature, and then refrozen in liquid nitrogen to obtain the 10 minute quench sample. Very weak EPR signals at $g = 2.0059$ that lacked hyperfine structure were observed for all three quench times (Figure S12), none of which were reminiscent of the strong tyrosyl radical signal(s) attributable to fully formed Compound ES^{46,47,50} in WT DHP B. As the free radical concentration in Compound ES is nearly quantitative with respect to the enzyme concentration (~ 0.93 spin/heme) in DHP,⁶⁶ the very weak signals could be attributed to a minor amount of protein that remained unreacted with pyrrole, leading to a trace amount of Compound ES and its corresponding tyrosyl radical EPR signal. We rule out the signals as being attributable to the presence of the monomeric substrate pyrrole radical cation (singlet, $g = 2.0025$ ⁶⁷) as this has been reported to have a lifetime of 30 μs ,⁶⁸ which is shorter than the freeze-quench times employed here; however, oligomeric pyrrole radical cations may still be a possibility. In all, the RFQ-EPR spectroscopic data unequivocally rule out the intermediate observed above in the stopped-flow studies as Compound ES.

4. Discussion

The activity studies reported here demonstrate that dehaloperoxidase was able to catalyze the conversion of all pyrroles studied under physiological conditions, representing a new class of substrate for this enzyme. Although brominated analogues were not investigated due to lack of commercial availability, we suggest that they are likely a biologically-relevant substrate: bromopyrroles have been isolated in the benthic ecosystems within which *A. ornata* resides,^{2,48,49} and the oxidation chemistry observed with the other known naturally-occurring haloaromatic substrates of DHP, i.e. halophenols and haloindoles, also correlates

well with their non-halogenated analogs.^{38,69} The isotopic labeling studies unequivocally show that the oxygen atom incorporated into the products for pyrrole and N-methylpyrrole originate exclusively from H₂O₂, analogous to the “peroxide-shunt” pathway of the cytochrome P450s, and consistent with previous reports of DHP peroxxygenase reactivity with the physiological haloindole³⁸ and non-physiological nitrophenol³⁹ substrates. Labeling studies also confirmed the presence of a peroxxygenase oxidation mechanism acting upon 3-methylpyrrole under anaerobic conditions, although significant solvent scrambling was noted for this substrate.

Enzyme-catalyzed oxidations of pyrrole have been well-investigated in the literature^{70–72} owing to their formation of polypyrrole, a conductive polymer that has applications in electronic devices and chemical sensors. Notable examples include i) the oxidation of pyrrole by horseradish peroxidase^{23,73,74} in the presence of an oxidant generates water-soluble polypyrrole at low pH (< 4), and with only minor amounts of smaller molecular weight species observed; ii) production of polypyrrole with glucose oxidase⁷⁵ and H₂O₂ (or glutaraldehyde) has also been reported, and is optimal at pH 6.5 with no evidence of lower molecular weight compounds being formed; iii) laccase^{24,76} has also been shown to catalyze the polymerization of pyrrole into a conducting polymer using dioxygen as the terminal oxidant; iv) soybean peroxidase^{77,78}/H₂O₂ has also been shown to form polypyrrole, with increased yields in the presence of redox mediators. The consensus first step for the mechanism of polypyrrole formation as catalyzed by these enzyme/oxidant systems is that the oxidative polymerization proceeds via a one-electron oxidation of pyrrole monomer ($E_0^o = 1.31$ V vs SCE⁷⁹) yielding a pyrrole radical cation.^{26,80} Coupling of two radical cations followed by deprotonation yields 2,2'-bipyrrole, with chain elongation following from either the successive coupling of the pyrrole oligomer with the monomeric radical cation, or from the coupling of the oligomer radical cation with a neutral monomer.^{24,81} By contrast, the H₂O₂-dependent peroxxygenase reactivity exhibited by DHP for pyrrole yielded a single oxygenated product, 4-pyrroline-2-one. Moreover, the DHP-catalyzed reaction showed no polypyrrole formation by UV-visible spectroscopy (absence of the characteristic polypyrrole absorbance at ~450 nm⁸²), and only trace oligomeric products detected by LC/MS. These observations suggest that pyrrole oxidation to 4-pyrroline-2-one likely does not proceed via a radical mechanism (or at least one that involves diffusible radical species), and is consistent with a single two-electron oxidation step for O-atom transfer via a peroxxygenase mechanism.

Peroxygenase activity necessitates a close interaction between the pyrrole substrate and the catalytically-relevant H₂O₂-activated form(s) of DHP in order to effect O-atom transfer. Thus, the Compound I, ES and II intermediates were explored here for their role(s) in the DHP-catalyzed oxidation of pyrrole. While all three activated forms were found to react with pyrrole, an analysis of their relative rates reveals mechanistic insight: Compound I, the two-electron oxidized form of DHP that contains an Fe(IV)-oxo and a porphyrin π -cation radical,⁴⁶ itself pre-formed from the reaction of the ferric DHP B (Y28F/Y38F) mutant with hydrogen peroxide, was found to react within the mixing time of the stopped-flow apparatus (<2.5 ms), yielding the ferric enzyme as the first observable species. By way of comparison, Compound ES, the two-electron oxidized form of DHP comprised of an Fe(IV)-oxo and a

tyrosyl radical,^{46,50} reforms the ferric enzyme from its reaction with pyrrole $\sim 10^4$ times slower than Compound I. Compound II, pre-formed here from the reaction of oxyferrous DHP with 1 equiv H_2O_2 in the presence of trihalophenol substrate, possesses an Fe(IV)-oxo species but no additional oxidizing equivalent residing on either the protein or the heme cofactor,⁵⁸ and was found to react with pyrrole (yielding a mixture of the ferric and oxyferrous forms) $\sim 10^4$ – 10^5 times slower than Compound I. Taken together, the results strongly implicate Compound I as the species responsible for pyrrole oxidation via O-atom transfer.

Based on the available evidence, and paralleling well-established mechanisms for O-atom insertion into an arene by hemoproteins,^{83–87} the following mechanism is proposed for the H_2O_2 -dependent oxidation of pyrrole (**1**) to 4-pyrrolin-2-one (**1a**) as catalyzed by DHP (Scheme 1): the ferric enzyme reacts with 1 equiv of H_2O_2 to yield Compound I (step *i*). Electrophilic addition of the ferryl oxygen to the pyrrole yields a tetrahedral-like intermediate (step *ii*) that undergoes further rearrangement to form the 4-pyrrolin-2-one product and regenerate the resting enzyme (steps *iii* and *iv*). The distal histidine likely plays the role of the general base/acid for the deprotonation/protonation events in steps *iii* and *iv*, respectively, although we cannot rule out that a solvent water molecule or other amino acid is involved. Displacement of the product upon binding a second pyrrole molecule (step *v*) readies the system for reactivation with H_2O_2 and subsequent turnover via an additional peroxygenase cycle, allowing for further production of 4-pyrrolin-2-one.

Although Scheme 1 shows the catalytic cycle initiating from the ferric oxidation state, substrate conversion was also observed when starting with ferrous or oxyferrous DHP. It is known that DHP from both of these oxidation states forms Compound II⁵⁸ when activated with H_2O_2 in the presence of either trihalophenol,^{59,60} nitrophenol,³⁹ or haloindole substrates,³⁸ and is therefore able to initiate a peroxygenase (or peroxidase) catalytic cycle. In the present work, Compound II formation was also observed when employing pyrrole and starting from oxyferrous DHP, yet is excluded from Scheme 1 on the basis of the slow reactivity observed for this intermediate. Rather, we suggest that Compound II is simply reduced via pyrrole to the ferric state, which is then available for turnover as described by Scheme 1. A similar argument can also be made for the exclusion of Compound ES from Scheme 1 owing to its slow reactivity.

Interestingly, the substrate conversion percentage was lower for oxyferrous DHP by ~ 1.6 -fold when compared to ferrous DHP, and lower by ~ 1.8 -fold when compared to ferric DHP. As no differences in peroxygenase activity have been noted previously between the oxyferrous and ferric oxidation states for haloindole³⁸ and nitrophenol³⁹ substrates, the differential reactivity noted here is unique to the pyrrole substrate. Previous studies^{58,60} have shown that oxyferrous DHP activation in the absence of a substrate leads to the bleaching of the Soret band and/or slow conversion to Compound RH without the formation of any observable fast timescale reactive intermediates. In the presence of a substrate, we have proposed that substrate binding to DHP destabilizes the oxyferrous state in the presence of hydrogen peroxide, leading to a reaction between ferrous DHP and H_2O_2 that activates the enzyme via a Compound II intermediate that is subsequently quickly reduced to the ferric enzyme.⁵⁸ In line with this hypothesis, the haloindole and nitrophenol substrates possess a

high binding affinity and thus show no differences in reactivity between oxyferrous and ferric DHP (Table 2), whereas we surmise that the relatively weak binding of pyrrole is therefore less able to destabilize the bound molecular oxygen in oxyferrous DHP that is ultimately necessary for enzyme activation. Alternatively, it has also been proposed that substrate radicals, themselves formed from trace amounts of the ferric enzyme reacting with substrate and hydrogen peroxide, oxidize oxyferrous DHP to the catalytically-active ferric form.^{59,60} Here, the slow reactivity of Compounds ES/II with pyrrole, leading to the pyrrole radical cation, would also be consistent with the substrate radical activation of oxyferrous DHP in a manner that would lead to the observed differential reactivity when compared to the ferric enzyme.

5. Conclusions

In summary, it has now been shown that dehaloperoxidase is capable of peroxygenase activity with pyrrole substrates. While enzyme-catalyzed oxidations of pyrrole have been previously reported, the reactivity observed here with DHP is unique in that a single oxygenated product is produced with virtually no detectable polypyrrole or oligomeric species as seen in other systems. Moreover, evidence strongly suggests that the mechanism for pyrrole oxygenation proceeds via electrophilic addition of Compound I. It is interesting to note for DHP that while peroxidase activity has so far been observed only with phenolic substrates, peroxygenase chemistry is observed across pyrrole, (halo)indole and nitrophenol substrates. When evaluated together, it is likely that the orientation (relative to the activated ferryl heme) and chemical reactivity of the substrate appear to play pivotal roles in determining which enzyme activity DHP performs: sterically accessible substrates are oxidized via a peroxygenase mechanism, whereas sterically hindered substrates proceed via a peroxidase pathway (as O-atom transfer is blocked). This plasticity of chemical reactivity for DHP that is related to the substrate itself leads to a new view (specific to multifunctional catalytic globins) for interrogating protein-structure function relationships: drawing from the language of hemoglobin regulation, the various substrates act as allosteric effectors, not just of O₂ binding, but of function itself. Taken together, the breadth of chemical reactivity demonstrated by DHP not only provides a unique system to explore our understanding of metalloprotein mechanism, activation, and design, but also highlights the flexibility of a single heme active site within a globin-fold to function as an O₂-carrier for aerobic respiration, as a peroxygenase for pyrrolic and indolic substrate oxygenation, as a peroxidase for phenolic substrate oxidation, as well as an oxidase for secondary metabolite formation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Notes and references

1. Warren, MJ., Smith, AG. Tetrapyrroles: Birth, Life and Death. Springer; New York: 2009.
2. Gribble, GW. Naturally Occurring Organohalogen Compounds - A Comprehensive Update. Vol. 91. Springer; Vienna: 2010.
3. Cafieri F, Fattorusso E, Mangoni A, Tagliatela-Scafati O. Dispacamides, anti-histamine alkaloids from Caribbean Agelas sponges. *Tetrahedron Letters*. 1996; 37:3587–3590.
4. Zidar N, Montalvão S, Hodnik Ž, Nawrot D, Žula A, Ilaš J, Kikelj D, Tammela P, Maši L. Antimicrobial Activity of the Marine Alkaloids, Clathrocin and Oroidin, and Their Synthetic Analogues. *Marine Drugs*. 2014; 12:940. [PubMed: 24534840]
5. Zhao D-G, Ma Y-Y, Peng W, Zhou A-Y, Zhang Y, Ding L, Du Z, Zhang K. Total synthesis and cytotoxic activities of longamide B, longamide B methyl ester, hanishin, and their analogues. *Bioorg Med Chem Lett*. 2016; 26:6–8. [PubMed: 26615890]
6. Carbone M, Irace C, Costagliola F, Castelluccio F, Villani G, Calado G, Padula V, Cimino G, Lucas Cervera J, Santamaria R, Gavagnin M. A new cytotoxic tambjamine alkaloid from the Azorean nudibranch *Tambja ceutae*. *Bioorg Med Chem Lett*. 2010; 20:2668–2670. [PubMed: 20227875]
7. Bhardwaj V, Gumber D, Abbot V, Dhiman S, Sharma P. Pyrrole: a resourceful small molecule in key medicinal hetero-aromatics. *RSC Advances*. 2015; 5:15233–15266.
8. Cordrey LJ. Tolmetin Sodium, a New Anti-Arthritis Drug: Double-Blind and Long-Term Studies. *J Am Geriatr Soc*. 1976; 24:440–446. [PubMed: 61224]
9. Van Wijngaarden I, Kruse CG, Van Hes R, Van der Heyden JAM, Tulp MTM. 2-Phenylpyrroles as conformationally restricted benzamide analogs. A new class of potential antipsychotics. 1. *J Med Chem*. 1987; 30:2099–2104. [PubMed: 2889830]
10. Painter, Ronald E., Adam, Gregory C., Arocho, M., DiNunzio, E., Donald, Robert GK., Dorso, K., Genilloud, O., Gill, C., Goetz, M., Hairston, Nichelle N., Murgolo, N., Nare, B., Olsen, David B., Powles, M., Racine, F., Su, J., Vicente, F., Wisniewski, D., Xiao, L., Hammond, M., Young, K. Elucidation of DnaE as the Antibacterial Target of the Natural Product, Nargenicin. *Chem Biol*. 2015; 22:1362–1373. [PubMed: 26456734]
11. Roth, BD. Progress in Medicinal Chemistry. King, FD.A. W. O. A. B. R. Scott, LD., editors. Vol. 40. Elsevier; 2002. p. 1-22.
12. Tat'yana VV, Oleg NE. Polypyrrole: a conducting polymer; its synthesis, properties and applications. *Russ Chem Rev*. 1997; 66:443.
13. MacDiarmid AG. "Synthetic Metals": A Novel Role for Organic Polymers (Nobel Lecture). *Angew Chem Int Ed*. 2001; 40:2581–2590.
14. Schmuck C, Rupprecht D. The Synthesis of Highly Functionalized Pyrroles: A Challenge in Regioselectivity and Chemical Reactivity. *Synthesis*. 2007; 2007:3095–3110.
15. Estevez V, Villacampa M, Menendez JC. Multicomponent reactions for the synthesis of pyrroles. *Chem Soc Rev*. 2010; 39:4402–4421. [PubMed: 20601998]
16. Joule, JA., Mills, K. Heterocyclic chemistry. 5th. Wiley; Hoboken, N.J: 2009.
17. Howard JK, Rihak KJ, Bissember AC, Smith JA. The Oxidation of Pyrrole. *Chem Asian J*. 2016; 11:155–167. [PubMed: 26294175]
18. Mortison JD, Sherman DH. Frontiers and Opportunities in Chemoenzymatic Synthesis. *J Org Chem*. 2010; 75:7041–7051. [PubMed: 20882949]
19. Denisov IG, Makris TM, Sligar SG, Schlichting I. Structure and chemistry of cytochrome P450. *Chem Rev*. 2005; 105:2253–2277. [PubMed: 15941214]
20. Bloom JD, Meyer MM, Meinhold P, Otey CR, MacMillan D, Arnold FH. Evolving strategies for enzyme engineering. *Curr Opin Struct Biol*. 2005; 15:447–52. [PubMed: 16006119]
21. Girhard, M., Bakkes, PJ., Mahmoud, O., Urlacher, VB. Cytochrome P 450: Structure, Mechanism, and Biochemistry. Ortiz de Montellano, PR., editor. Springer International Publishing; Cham: 2015. p. 451-520.

22. Waskell, L., Kim, JJP. Cytochrome P 450: Structure, Mechanism, and Biochemistry. Ortiz de Montellano, PR., editor. Springer International Publishing; Cham: 2015. p. 33-68.
23. Cruz-Silva R, Amaro E, Escamilla A, Nicho ME, Sepulveda-Guzman S, Arizmendi L, Romero-Garcia J, Castillon-Barraza FF, Farias MH. Biocatalytic synthesis of polypyrrole powder, colloids, and films using horseradish peroxidase. *J Colloid Interf Sci*. 2008; 328:263–269.
24. Song H-K, Palmore GTR. Conductive Polypyrrole via Enzyme Catalysis. *J Phys Chem B*. 2005; 109:19278–19287. [PubMed: 16853490]
25. Hollmann F, Arends IWCE. Enzyme Initiated Radical Polymerizations. *Polymers*. 2012; 4:759.
26. Sadki S, Schottland P, Brodie N, Sabouraud G. The mechanisms of pyrrole electropolymerization. *Chem Soc Rev*. 2000; 29:283–293.
27. Lincoln DE, Fielman KT, Marinelli RL, Woodin SA. Bromophenol accumulation and sediment contamination by the marine annelids *Notomastus lobatus* and *Thelepus crispus*. *Biochem Syst Ecol*. 2005; 33:559–570.
28. Fielman KT, Woodin SA, Lincoln DE. Polychaete indicator species as a source of natural halogenated organic compounds in marine sediments. *Environ Toxicol Chem*. 2001; 20:738–747. [PubMed: 11345448]
29. Woodin SA, Lindsay SM, Lincoln DE. Biogenic bromophenols as negative recruitment cues. *Mar Ecol-Prog Ser*. 1997; 157:303–306.
30. Zhang E, Chen YP, Roach MP, Lincoln DE, Lovell CR, Woodin SA, Dawson JH, Lebioda L. Crystallization and initial spectroscopic characterization of the heme-containing dehaloperoxidase from the marine polychaete *Amphitrite ornata*. *Acta Crystallogr D Biol Crystallogr*. 1996; 52:1191–1193. [PubMed: 15299583]
31. Woodin SA, Walla MD, Lincoln DE. Occurrence of brominated compounds in soft-bottom benthic organisms. *J Exp Mar Biol Ecol*. 1987; 107:209–217.
32. Franzen, S., Ghiladi, RA., Lebioda, L., Dawson, J. Heme Peroxidases. *The Royal Society of Chemistry*; 2016. p. 218-244.
33. Weber RE, Mangum C, Steinman H, Bonaventura C, Sullivan B, Bonaventura J. Hemoglobins of two terebellid polychaetes: *Enoplobranchus sanguineus* and *Amphitrite ornata*. *Comp Biochem Physiol A Comp Physiol*. 1977; 56:179–187. [PubMed: 11931]
34. Chiancone E, Ferruzzi G, Bonaventura C, Bonaventura J. *Amphitrite ornata* erythrocrurin. 2. Molecular controls of function. *Biochim Biophys Acta*. 1981; 670:84–92. [PubMed: 7272332]
35. Chiancone E, Brenowitz M, Ascoli F, Bonaventura C, Bonaventura J. *Amphitrite ornata* erythrocrurin. 1. Structural properties and characterization of subunit interactions. *Biochim Biophys Acta*. 1980; 623:146–162. [PubMed: 7378469]
36. Chen YP, Woodin SA, Lincoln DE, Lovell CR. An unusual dehalogenating peroxidase from the marine terebellid polychaete *Amphitrite ornata*. *J Biol Chem*. 1996; 271:4609–4612. [PubMed: 8617721]
37. Osborne RL, Taylor LO, Han KP, Ely B, Dawson JH. *Amphitrite ornata* dehaloperoxidase: enhanced activity for the catalytically active globin using MCPBA. *Biochem Biophys Res Commun*. 2004; 324:1194–1198. [PubMed: 15504340]
38. Barrios DA, D'Antonio J, McCombs NL, Zhao J, Franzen S, Schmidt AC, Sombers LA, Ghiladi RA. Peroxygenase and oxidase activities of dehaloperoxidase-hemoglobin from *Amphitrite ornata*. *J Am Chem Soc*. 2014; 136:7914–7925. 4063182. [PubMed: 24791647]
39. McCombs NL, D'Antonio J, Barrios DA, Carey LM, Ghiladi RA. Nonmicrobial Nitrophenol Degradation via Peroxygenase Activity of Dehaloperoxidase-Hemoglobin from *Amphitrite ornata*. *Biochemistry*. 2016; 55:2465–2478. [PubMed: 27070125]
40. McCombs NL, Moreno Chicano T, Carey LM, Franzen S, Hough MA, Ghiladi RA. Interaction of Azole-based Environmental Pollutants with the Coelomic Hemoglobin from *Amphitrite ornata*: a Molecular Basis for Toxicity. *Biochemistry*. 2017; 56:2294–2303. [PubMed: 28387506]
41. Sun S, Sono M, Wang C, Du J, Lebioda L, Dawson JH. Influence of heme environment structure on dioxygen affinity for the dual function *Amphitrite ornata* hemoglobin/dehaloperoxidase. Insights into the evolutionary structure–function adaptations. *Arch Biochem Biophys*. 2014; 545:108–115. [PubMed: 24440609]

42. Wang C, Lovelace LL, Sun S, Dawson JH, Lebioda L. Complexes of dual-function hemoglobin/dehaloperoxidase with substrate 2,4,6-trichlorophenol are inhibitory and indicate binding of halophenol to compound I. *Biochemistry*. 2013; 52:6203–6210. [PubMed: 23952341]
43. Du J, Huang X, Sun S, Wang C, Lebioda L, Dawson JH. *Amphitrite ornata* Dehaloperoxidase (DHP): Investigations of Structural Factors That Influence the Mechanism of Halophenol Dehalogenation Using “Peroxidase-like” Myoglobin Mutants and “Myoglobin-like” DHP Mutants. *Biochemistry*. 2011; 50:8172–8180. [PubMed: 21800850]
44. LaCount MW, Zhang E, Chen YP, Han K, Whitton MM, Lincoln DE, Woodin SA, Lebioda L. The crystal structure and amino acid sequence of dehaloperoxidase from *Amphitrite ornata* indicate common ancestry with globins. *J Biol Chem*. 2000; 275:18712–18716. [PubMed: 10751397]
45. Lebioda L, LaCount MW, Zhang E, Chen YP, Han K, Whitton MM, Lincoln DE, Woodin SA. An enzymatic globin from a marine worm. *Nature*. 1999; 401:445. [PubMed: 10519547]
46. Dumarieh R, D’Antonio J, Deliz-Liang A, Smirnova T, Svistunenko DA, Ghiladi RA. Tyrosyl Radicals in Dehaloperoxidase: How nature deals with evolving an oxygen-binding globin to a biologically relevant peroxidase. *J Biol Chem*. 2013; 288:33470–33482. 3829192. [PubMed: 24100039]
47. D’Antonio J, D’Antonio EL, Thompson MK, Bowden EF, Franzen S, Smirnova T, Ghiladi RA. Spectroscopic and mechanistic investigations of dehaloperoxidase B from *Amphitrite ornata*. *Biochemistry*. 2010; 49:6600–6616. 2921985. [PubMed: 20545299]
48. Gribble GW. The natural production of organobromine compounds. *Environ Sci Pollut Res Int*. 2000; 7:37–47. [PubMed: 19153837]
49. Gribble GW. The diversity of naturally occurring organobromine compounds. *Chem Soc Rev*. 1999; 28:335–346.
50. Feducia J, Dumarieh R, Gilvey LB, Smirnova T, Franzen S, Ghiladi RA. Characterization of dehaloperoxidase compound ES and its reactivity with trihalophenols. *Biochemistry*. 2009; 48:995–1005. [PubMed: 19187035]
51. Beers RF Jr, Sizer IW. A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. *J Biol Chem*. 1952; 195:133–140. [PubMed: 14938361]
52. Zhao J, Moretto J, Le P, Franzen S. Measurement of internal substrate binding in dehaloperoxidase-hemoglobin by competition with the heme-fluoride binding equilibrium. *J Phys Chem B*. 2015; 119:2827–2838. [PubMed: 25611370]
53. Bocchi V, Chierici L, Gardini GP, Mondelli R. On pyrrole oxidation with hydrogen peroxide. *Tetrahedron*. 1970; 26:4073–4082.
54. Pelkey, ET., Pelkey, SJ., Greger, JG. *Advances in Heterocyclic Chemistry*. Eric, FVS., Christopher, AR., editors. Vol. 115. Academic Press; 2015. p. 151-285.
55. Gardini, GP. *Advances in Heterocyclic Chemistry*. Katritzky, AR., Boulton, AJ., editors. Vol. 15. Academic Press; 1973. p. 67-98.
56. Gardini GP, Bocchi V. The oxidation of mono- and dimethylpyrroles. *Gazz Chim Ital*. 1972; 102:91–101.
57. Jones, RA., Bean, GP. *Organic Chemistry: A Series of Monographs*. Blomquist, AT., Wasserman, HH., editors. Vol. 34. Academic Press; New York: 1977. p. 525
58. D’Antonio J, Ghiladi RA. Reactivity of deoxy- and oxyferrous dehaloperoxidase B from *Amphitrite ornata*: Identification of compound II and its ferrous-hydroperoxide precursor. *Biochemistry*. 2011; 50:5999–6011. 3137918. [PubMed: 21619067]
59. Sun S, Sono M, Du J, Dawson JH. Evidence of the direct involvement of the substrate TCP radical in functional switching from oxyferrous O₂ carrier to ferric peroxidase in the dual-function hemoglobin/dehaloperoxidase from *Amphitrite ornata*. *Biochemistry*. 2014; 53:4956–4969. [PubMed: 24972312]
60. Du J, Sono M, Dawson JH. Functional switching of *Amphitrite ornata* dehaloperoxidase from O₂-binding globin to peroxidase enzyme facilitated by halophenol substrate and H₂O₂. *Biochemistry*. 2010; 49:6064–6069. [PubMed: 20565134]
61. Franzen S, Gilvey LB, Belyea JL. The pH dependence of the activity of dehaloperoxidase from *Amphitrite ornata*. *Biochim Biophys Acta*. 2007; 1774:121–130. [PubMed: 17182294]

62. Thompson MK, Davis MF, de Serrano V, Nicoletti FP, Howes BD, Smulevich G, Franzen S. Internal binding of halogenated phenols in dehaloperoxidase-hemoglobin inhibits peroxidase function. *Biophys J*. 2010; 99:1586–1595. [PubMed: 20816071]
63. Kohler H, Taurog A, Dunford HB. Spectral studies with lactoperoxidase and thyroid peroxidase: interconversions between native enzyme, compound II, and compound III. *Arch Biochem Biophys*. 1988; 264:438–449. [PubMed: 3401009]
64. Jakopitsch C, Wanasinghe A, Jantschko W, Furtmueller PG, Obinger C. Kinetics of interconversion of ferrous enzyme, compound II and compound III of wild-type *synechocystis* catalase-peroxidase and Y249F. Proposal for the catalytic mechanism. *J Biol Chem*. 2005; 280:9037–9042. [PubMed: 15637065]
65. Ghiladi RA, Cabelli DE, Ortiz de Montellano PR. Superoxide reactivity of KatG: insights into isoniazid resistance pathways in TB. *J Am Chem Soc*. 2004; 126:4772–4773. [PubMed: 15080669]
66. Thompson MK, Franzen S, Ghiladi RA, Reeder BJ, Svistunenko DA. Compound ES of dehaloperoxidase decays via two alternative pathways depending on the conformation of the distal histidine. *J Am Chem Soc*. 2010; 132:17501–17510. [PubMed: 21090708]
67. Davies AG, Julia L, Yazdi SN. An electron spin resonance study of the radical cations of pyrroles, furans, and thiophenes in liquid solution. *J Chem Soc, Perkin Trans*. 1989; 2:239–244.
68. Andrieux CP, Audebert P, Hapiot P, Saveant JM. Observation of the cation radicals of pyrrole and of some substituted pyrroles in fast-scan cyclic voltammetry. Standard potentials and lifetimes. *J Am Chem Soc*. 1990; 112:2439–2440.
69. Zhao J, Franzen S. The regulatory implications of hydroquinone for the multifunctional enzyme dehaloperoxidase-hemoglobin from *Amphitrite ornata*. *J Phys Chem B*. 2013; 117:14615–24. [PubMed: 24176025]
70. Otrokhov GV, Morozova OV, Vasil'eva IS, Shumakovich GP, Zaitseva EA, Khlopova ME, Yarpolov AI. Biocatalytic synthesis of conducting polymers and prospects for its application. *Biochemistry (Mosc)*. 2013; 78:1539–1553. [PubMed: 24490739]
71. Cruz-Silva, R., Roman, P., Romero, J. *Biocatalysis in Polymer Chemistry*. Wiley-VCH Verlag; GmbH & Co. KGaA: 2010. p. 187-210.
72. Bouldin, R., Kokil, A., Ravichandran, S., Nagarajan, S., Kumar, J., Samuelson, LA., Bruno, FF., Nagarajan, R. *Green Polymer Chemistry: Biocatalysis and Biomaterials*. Vol. 1043. American Chemical Society; 2010. p. 315-341.
73. Kupriyanovich YN, Sukhov BG, Medvedeva SA, Mikhaleva AI, Vakul'skaya TI, Myachina GF, Trofimov BA. Peroxidase-catalysed synthesis of electroconductive polypyrrole. *Mendeleev Commun*. 2008; 18:56–58.
74. Nabid MR, Entezami AA. A novel method for synthesis of water-soluble polypyrrole with horseradish peroxidase enzyme. *J Appl Polym Sci*. 2004; 94:254–258.
75. Ramanavicius A, Kausaite A, Ramanaviciene A, Acaite J, Malinauskas A. Redox enzyme – glucose oxidase – initiated synthesis of polypyrrole. *Synth Met*. 2006; 156:409–413.
76. Junker K, Zandomenighi G, Schuler LD, Kissner R, Walde P. Enzymatic polymerization of pyrrole with *Trametes versicolor* laccase and dioxygen in the presence of vesicles formed from AOT (sodium bis-(2-ethylhexyl) sulfosuccinate) as templates. *Synth Met*. 2015; 200:123–134.
77. Grijalva-Bustamante GA, Evans-Villegas AG, del Castillo-Castro T, Castillo-Ortega MM, Cruz-Silva R, Huerta F, Morallón E. Enzyme mediated synthesis of polypyrrole in the presence of chondroitin sulfate and redox mediators of natural origin. *Mater Sci Eng, C*. 2016; 63:650–656.
78. Bouldin R, Ravichandran S, Kokil A, Garhwal R, Nagarajan S, Kumar J, Bruno FF, Samuelson LA, Nagarajan R. Synthesis of polypyrrole with fewer structural defects using enzyme catalysis. *Synth Met*. 2011; 161:1611–1617.
79. Audebert P, Hapiot P. Fast electrochemical studies of the polymerization mechanisms of pyrroles and thiophenes. Identification of the first steps. Existence of π -dimers in solution. *Synth Met*. 1995; 75:95–102.
80. Curran D, Grimshaw J, Perera SD. Poly(pyrrole) as a support for electrocatalytic materials. *Chem Soc Rev*. 1991; 20:391–404.

81. Tan Y, Ghandi K. Kinetics and mechanism of pyrrole chemical polymerization. *Synth Met.* 2013; 175:183–191.
82. Lee JY, Kim DY, Kim CY. Synthesis of soluble polypyrrole of the doped state in organic solvents. *Synth Met.* 1995; 74:103–106.
83. Gillam EM, Notley LM, Cai H, De Voss JJ, Guengerich FP. Oxidation of indole by cytochrome P450 enzymes. *Biochemistry.* 2000; 39:13817–13824. [PubMed: 11076521]
84. Guengerich FP. Common and uncommon cytochrome P450 reactions related to metabolism and chemical toxicity. *Chem Res Toxicol.* 2001; 14:611–650. [PubMed: 11409933]
85. Sono M, Roach MP, Coulter ED, Dawson JH. Heme Containing Oxygenases. *Chem Rev.* 1996; 96:2841–2887. [PubMed: 11848843]
86. Meunier B, de Visser SP, Shaik S. Mechanism of oxidation reactions catalyzed by cytochrome P450 enzymes. *Chem Rev.* 2004; 104:3947–3980. [PubMed: 15352783]
87. Guengerich FP, Munro AW. Unusual Cytochrome P450 Enzymes and Reactions. *J Biol Chem.* 2013; 288:17065–17073. [PubMed: 23632016]

Figure 1.

The known dehaloperoxidase (DHP) substrates 2,4,6-trihalophenol (left) and haloindole (middle), and the substrate investigated in the current study, pyrrole (right).

Figure 2.

(A) HPLC chromatogram of the reaction of 500 μ M pyrrole with 10 μ M DHP B in the presence of 500 μ M H₂O₂ at 25 $^{\circ}$ C in 100 mM KP_i (pH 7). The reaction was quenched after 5 minutes with the addition of excess catalase. (B) UV-visible spectra of pyrrole (**1**, t_R = 4.7 min) and the reaction product 4-pyrroline-2-one (**1a**, t_R = 3.5 min).

Figure 3.

ESI-MS total ion chromatogram for the DHP/pyrrole reaction product: (A: $\text{H}_2^{18}\text{O}/\text{H}_2^{16}\text{O}_2$, B: $\text{H}_2^{16}\text{O}/\text{H}_2^{18}\text{O}_2$). Reaction conditions: $[\text{pyrrole}] = [\text{H}_2\text{O}_2] = 500 \mu\text{M}$, $[\text{DHP}] = 10 \mu\text{M}$, 100 mM KPi (pH 7), 25 °C.

Figure 4.

Kinetic data for the reaction of H_2O_2 -activated DHP with pyrrole. (A) **DHP B (Y28F/Y38F)**: *top panel*, stopped-flow UV–visible spectra of the double-mixing reaction of preformed Compound I (10 μM) with a 50-fold excess of pyrrole at pH 7.0 (650 scans over 20 s); *inset*: the single wavelength (416 nm) dependence on time obtained from the raw data and its fit with a superposition of the calculated spectral components; *middle panel*, calculated spectra of the three reaction components derived from the SVD analysis: ferric DHP B (black); ferryl/ferric DHP mixture (blue), and an oxyferrous/ferric DHP mixture (red); *bottom panel*, time dependences of the relative concentrations for the three components shown in the middle panel as determined from the fitting of the spectra in top panel. (B) **Ferric DHP B**: *top panel*, stopped-flow UV–visible spectra of the single-mixing reaction between ferric DHP B (10 μM) preincubated with 50 equiv pyrrole and reacted with 10 eq. of H_2O_2 at pH 5.0 (900 scans over 83 s); *inset*: the single wavelength (417 nm) dependence on time obtained from the raw data and its fit with a superposition of the calculated spectral components; *middle panel*, calculated spectra of the three reaction components derived from the SVD analysis: ferric DHP B (black), formation of ferryl DHP B (blue), and its reduction back to ferric DHP (red) in the presence of pyrrole; *bottom panel*, time dependences of the relative concentrations for the three reaction components shown in the middle panel as determined from the fitting of the spectra in the top panel. (C) **Oxyferrous DHP B**: *top panel*, stopped-flow UV–visible spectra of the single-mixing

reaction between oxyferrous DHP B (10 μ M) preincubated with 50 equiv pyrrole and reacted with 10 eq. of H_2O_2 at pH 6.0 (900 scans over 830 s); inset: the single wavelength (418 nm) dependence on time obtained from the raw data and its fit with a superposition of the calculated spectral components; *middle panel*, calculated spectra of the three reaction components derived from the SVD analysis: oxyferrous DHP B (black); oxyferrous/ferryl DHP mixture (blue), and the final observed DHP species (red); *bottom panel*, time dependences of the relative concentrations for the three components shown in panel B as determined from the fitting of the spectra in panel A.

Scheme 1.

Proposed Peroxygenase Cycle for Pyrrole Oxidation with Dehaloperoxidase B.

Chart 1.
Peroxygenase products identified by HPLC/LC-MS for the DHP-catalyzed oxidation of pyrrole (**1**), N-methylpyrrole (**2**) and 3-methylpyrrole (**3**).

Table 1Enzyme-Catalyzed Reactivity of Pyrrole Substrates.^a

Enzyme	Conversion (%)
<i>Substrate Variation</i>	
<i>DHP B Ferric</i>	
+ pyrrole	31.7 (± 2.5)
anaerobic	30.6 (± 2.1)
+ N-methylpyrrole	40.8 (± 2.2)
anaerobic	40.1 (± 3.1)
+ 2-methylpyrrole	84.9 (± 1.9)
anaerobic	70.0 (± 3.3)
+ 3-methylpyrrole	58.4 (± 2.4)
anaerobic	42.2 (± 3.7)
+ 2,5-dimethylpyrrole	~ 100%
anaerobic	92.2 (± 1.9)
<i>Enzyme Variation with Pyrrole</i>	
DHP B Ferrous	27.9 (± 3.8)
DHP B Oxyferrous	17.8 (± 1.0)
DHP A Ferric	42.8 (± 3.1)
DHP A Oxyferrous	12.7 (± 3.4)
DHP B (Y28F/Y38F)	25.6 (± 1.7)
Mb	n. d. ^b
HRP	8.6 (± 1.9)
<i>pH Effects with Pyrrole</i>	
<i>DHP B Ferric</i>	
pH 5	50.8 (± 2.0)
pH 6	38.8 (± 3.2)
pH 8	10.8 (± 2.2)
<i>DHP A Ferric</i>	
pH 5	35.2 (± 2.9)
pH 6	36.1 (± 3.4)
pH 8	26.8 (± 1.2)
<i>DHP B Oxyferrous</i>	
pH 5	45.9 (± 2.9)
pH 6	23.9 (± 3.4)
pH 8	10.3 (± 1.2)
<i>Mechanistic Probes with Pyrrole</i>	
<i>DHP B Ferric</i>	
+ 10% DMSO	8.1 (± 0.5)
+ 500 mM formate	25.6 (± 0.2)

Enzyme	Conversion (%)
+ 500 μ M mannitol	25.9 ($\pm$ 1.6)
+ SOD ^c	29.5 ($\pm$ 2.2)
+ 4-bromophenol	5.1 ($\pm$ 0.6)
<i>DHP A Ferric</i>	
+ 4-bromophenol	5.4 ($\pm$ 0.4)

^aReaction conditions: [pyrrole] = [H₂O₂] = 500 μ M, [enzyme] = 10 μ M, 100 mM KP₁ buffer at pH 7 (unless otherwise indicated), 25 °C, 5 min.;

^bn. d. = not detected;

^cSOD = $\sim$ 2 U/ μ L.

Table 2

$K_d F^-$ Values for Substrate Binding to Ferric DHP.

Substrate	$K_d F^-$ (mM)	K_d (μ M), pH 5	K_d (μ M), pH 7	ref.
pyrrole	$5.2 \pm 0.4^{a,d}$	n. d. ^f	n. d.	<i>h</i>
N-methylpyrrole	$5.4 \pm 0.2^{a,d}$	n. d.	n. d.	<i>h</i>
3-methylpyrrole	$5.1 \pm 0.2^{a,d}$	n. d.	n. d.	<i>h</i>
4-bromophenol	$13 \pm 1^{b,d}$	n. r. ^g	1150	52,62
2,4,6-tribromophenol	$23.8 \pm 1.0^{b,e}$	n. r.	n. r.	52
5-bromoindole	$13.2 \pm 0.5^{b,e}$	n. r.	150 ± 10	38,52
4-nitrophenol	$18 \pm 1^{c,d}$	134 ± 7	262 ± 23	39
4-nitrocatechol	$31 \pm 3^{c,d}$	79 ± 4	40 ± 1	39
2,4-dinitrophenol	$90 \pm 9^{c,d}$	48 ± 8	105 ± 21	39
2,4,6-trinitrophenol	$30 \pm 1^{c,d}$	$5.9 \pm 1.0^{c,e}$	n. d.	39

K_d value of fluoride binding in the presence of

^a 5 mM substrate,

^b 1 mM substrate,

^c 500 μ M substrate;

^d pH 5;

^e pH 7;

^f n. d. = not determinable due to lack of spectral changes;

^g n. r. = not reported in reference;

^h This work.