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The Mechanism of Oxidative Halophenol Dehalogenation by *Amphitrite ornata* Dehaloperoxidase Is Initiated by H_2O_2 Binding and Involves Two Consecutive One-Electron Steps: Role of Ferryl Intermediates[†,‡]

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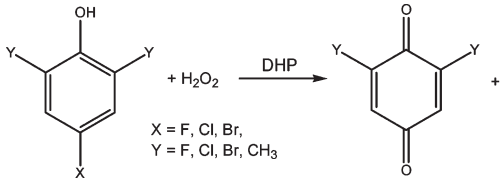
ABSTRACT: The enzymatic globin, dehaloperoxidase (DHP), from the terebellid polychaete *Amphitrite ornata* is designed to catalyze the oxidative dehalogenation of halophenol substrates. In this study, the ability of DHP to catalyze this reaction by a mechanism involving two consecutive one-electron steps via the normal order of addition of the oxidant cosubstrate (H_2O_2) before organic substrate [2,4,6-trichlorophenol (TCP)] is demonstrated. Specifically, 1 equiv of H_2O_2 will fully convert 1 equiv of TCP to 2,6-dichloro-1,4-benzoquinone, implicating the role of multiple ferryl [Fe(IV)=O] species. A significant amount of heterolytic cleavage of the O−O bond of cumene hydroperoxide, consistent with transient formation of a Compound I [Fe(IV)=O/porphyrin π-cation radical] species, is observed upon its reaction with ferric DHP. In addition, a more stable high-valent Fe(IV)=O-containing DHP intermediate [Compound II (Cpd II) or Compound ES] is characterized by UV−visible absorption and magnetic circular dichroism spectroscopy. Spectral similarities are seen between this intermediate and horse heart myoglobin Cpd II. It is also shown in single-turnover experiments that the DHP Fe(IV)=O intermediate is an active oxidant in halophenol oxidative dehalogenation. Furthermore, reaction of DHP with 4-chlorophenol leads to a dimeric product. The results presented herein are consistent with a normal peroxidase order of addition of the oxidant cosubstrate (H_2O_2) followed by organic substrate (TCP) and indicate that the enzymatic mechanism of DHP-catalyzed oxidative halophenol dehalogenation involves two consecutive one-electron steps with a dissociable radical intermediate.

The enzyme dehaloperoxidase (DHP),[1] isolated from the terebellid polychaete *Amphitrite ornata*, metabolizes noxious haloaromatics, including chlorophenols. DHP is composed of two identical subunits with a total molecular mass of ~31 kDa and has been shown to function as a peroxidase converting halogenated phenols to quinones in the presence of H_2O_2 (eq 1) (*1*, *2*).



(1)

The X-ray crystal structure of native DHP revealed that it has the same protein fold as myoglobin (Mb) (*3*, *4*). A halophenol substrate analogue, 4-iodophenol, was shown to bind to DHP in a well-defined binding site above the heme rather than at the heme edge, the site of substrate interaction for horseradish peroxidase (HRP) (*5*). In HRP, the prototypical heme-containing peroxidase, the distal side of the heme iron active site is considered to be too narrow for organic substrates to enter and directly interact with the heme iron center (*6*). The presence of a substrate-binding site and modeling studies led to a proposed mechanism whereby the substrate of DHP is oxidized by a direct two-electron oxygen atom insertion mechanism (Figure 1) (*3*, *4*).

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[1]Abbreviations: DHP, dehaloperoxidase; Mb, horse heart myoglobin; HRP, horseradish peroxidase; CCPO, *Caldariomyces fumago* chloroperoxidase; LiP, lignin peroxidase; Cpd I, Compound I; Cpd II, Compound II; Cpd ES, Compound ES; TCP, 2,4,6-trichlorophenol; DBQ, 2,6-dichloro-1,4-benzoquinone; UV−vis, ultraviolet−visible; MCD, magnetic circular dichroism; GC−MS, gas chromatography−mass spectroscopy.

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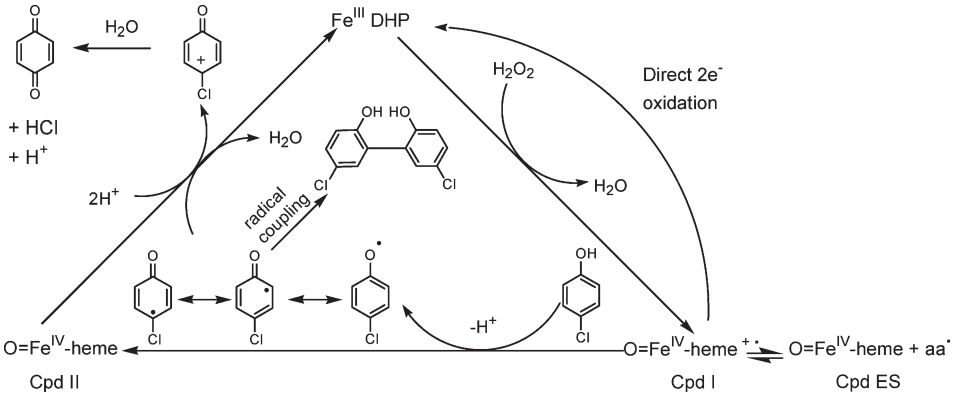

FIGURE 1: Possible reaction mechanisms for the DHP-catalyzed oxidative dehalogenation of 4-chlorophenol proposed to occur via consecutive one-electron transfers facilitated by both Compound I (or Compound ES) and Compound II and nucleophilic attack on a phenoxy radical intermediate by a water molecule or a single two-electron oxidation whereby an oxygen atom is inserted specifically from Compound I.

Numerous spectroscopic studies have demonstrated the globin-like nature of DHP. Vibrational data for DHP are similar to those previously reported for neutral histidine/imidazole-ligated globins (*7*). Resonance Raman data collected on a range of ferric [Fe(III)] DHP complexes is consistent with a modified charge relay in which a strongly hydrogen bonded backbone carbonyl polarizes the proximal histidine (*8*). Magnetic circular dichroism (MCD) studies on a series of Fe(III) and ferrous complexes revealed that the spectra observed for parallel forms of DHP and Mb are virtually identical to each other and less similar to the spectra of corresponding HRP derivatives (*9*, *10*). In particular, exogenous ligand-free Fe(III) DHP is six-coordinate with water and neutral histidine as ligands, a structure distinct from the five-coordinate Fe(III) resting-state structure of histidine-ligated peroxidases (*10*). Recently, results from continuous wave electron paramagnetic resonance spectroscopy and hyperfine sublevel correlation spectroscopic analysis of Fe(III) DHP indicate the water molecule bound to the heme iron of DHP is displaced upon halophenol substrate binding (*11*). Kinetic assays have revealed that the rate of DHP-catalyzed reactions is slower than that of HRP but faster than that of Mb (*12*, *13*). Additionally, DHP forms a trapped state when a large excess of H_2O_2 is present in the absence of organic substrates (*12*). Alternatively, HRP and Mb form Compound III [Fe(II)−O_2] and the ferryl [Fe(IV)═O] Compound II (Cpd II) upon reaction with excess peroxide, respectively (*14*). The compilation of spectroscopic and kinetic data indicates that DHP does not activate bound peroxide to form Compound I (Cpd I) [Fe(IV)═O/porphyrin π-cation radical] through a mechanism dependent on a push effect imparted by a partially ionized proximal histidine as proposed for typical heme peroxidases (*15*). Although the DHP mechanism does not involve an imidazolate push, the DHP crystal structure reveals that the distal histidine is located in the peroxidase position to facilitate O−O bond cleavage rather than the globin position to stabilize dioxygen binding (*3*, *4*). The crystal structure also shows that a halophenol substrate analogue-bound form has the distal histidine moved out of the active site (*3*), presumably to avoid inadvertent heme bleaching.

Despite extensive investigation, the mechanism of oxidative halophenol dehalogenation catalyzed by DHP remains unresolved. As shown in Figure 1, classical peroxidases typically oxidize organic substrates, including phenols, by first reacting with H_2O_2 to form the transient high-valent intermediate, Cpd I (*14*). In some peroxidases, Cpd I rapidly converts to a state known as Compound ES (Cpd ES) consisting of an Fe(IV)═O heme and a nearby amino acid (Tyr or Trp) radical (*15*). The normal order of substrate addition continues with organic substrate added to Cpd I (or Cpd ES), which then undergoes two consecutive one-electron reductions to regenerate the Fe(III) enzyme with concomitant organic substrate oxidation (*15*, *16*). The first reduction yields a dissociable organic radical and a second Fe(IV)═O enzyme intermediate, Cpd II (*17*). Alternatively, direct insertion of an oxygen atom derived from peroxide into organic substrates involves a single two-electron oxidation mechanism utilizing Cpd I (Figure 1) (*16*, *18*). Peroxidases catalyze such a single two-electron oxidation with alkenes and thioethers to form epoxides and sulfoxides, respectively (*19*−*24*). The differences between mechanisms involving two consecutive one-electron steps and a single two-electron step and between obligatory initial binding of H_2O_2 (normal order) and first binding of organic substrate (reverse order) are distinct. In fact, Belyea et al. proposed a direct two-electron mechanism for the DHP-catalyzed reaction in which Cpd II is not catalytically active and in which there is an obligatory reverse order of addition (substrate binding before H_2O_2) (*25*). If this proposed mechanism is correct, DHP dehalogenates halophenols in a manner never before observed by any peroxidase. A third alternative mechanism was proposed by Franzen et al. in which two consecutive but tightly coupled one-electron oxidations take place with the substrate remaining tightly bound until fully oxidized and where catalysis can be initiated only from the Cpd I state (i.e., not from Cpd ES or Cpd II) (*26*). Whether DHP catalyzes halophenol oxidation by a normal peroxide-initiated mechanism involving two consecutive one-electron steps with a dissociable radical intermediate and a catalytically active Cpd II state has been challenged (*25*−*27*).

The heme enzyme-catalyzed oxidative dehalogenation reaction has been investigated for numerous enzymes. The oxidative dechlorination reaction catalyzed by lignin peroxidase (LiP) was investigated to examine how enzymes degrade environmental pollutants such as 4-chlorophenol (*28*). HRP was shown to break down 2,4,6-trichlorophenol (TCP) at appreciable rates (*12*, *29*). The use of $H_2{}^{18}O$ indicated that the oxygen atom incorporated into the quinone was derived from water and not H_2O_2. Similarly, *Caldariomyces fumago* chloroperoxidase (CCPO), a cysteine thiolate ligated peroxidase, catalyzes the oxidative dechlorination of TCP (*30*). CCPO-catalyzed dehalogenation of mono-*p*-halophenols resulted in dimeric products, consistent with a mechanism involving two consecutive one-electron oxidations via a dissociable phenoxy radical intermediate (*30*). Furthermore, the

mechanism with two consecutive one-electron transfers is supported by rapid scan stopped-flow techniques (*31*). Similar results have recently been obtained with HRP (*32*). Interestingly, Feducia et al. recently characterized DHP Cpd ES and reported that this DHP intermediate can dehalogenate TCP by a direct two-electron mechanism (*33*).[2] The formation and proposed reactivity of Cpd ES are inconsistent with previously proposed mechanisms for DHP-catalyzed reactions (*25*−*27*). Mb, best known for O_2 storage, catalyzes the same reaction under conditions of oxidative stress (*13*). Single-turnover experiments demonstrated that Mb Cpd II is an active oxidant and supported a normal order of addition of H_2O_2 before organic substrate (*13*). The mechanism of oxidative halophenol dehalogenation catalyzed by CCPO, HRP, and Mb has been extensively studied, and a process involving two consecutive one-electron steps and a normal order of substrate binding is consistently observed (*13*, *30*−*32*).

To clarify the controversial mechanism of oxidative halophenol dehalogenation by DHP, we present compelling evidence for a mechanism accomplished by two consecutive one-electron steps via a dissociable phenoxy radical intermediate as previously suggested for the same reaction catalyzed by other heme-containing peroxidases and Mb (*13*, *28*−*32*). Turnover studies reveal that an Fe(IV)═O-containing DHP intermediate can dehalogenate TCP. In addition, the detection of dimeric products upon reaction of DHP with 4-chlorophenol and the isolation of 2,6-dimethyl-1,4-benzoquinone after catalysis by an Fe(IV)═O DHP species is consistent with a mechanism involving two consecutive one-electron transfers (*30*). Previously, an obligatory reverse order of addition of organic substrate before oxidant cosubstrate, H_2O_2, was proposed (*25*). However, the ability of a preformed Fe(IV)═O DHP intermediate (Cpd II or Cpd ES) to catalyze the oxidative dehalogenation reaction reported herein is consistent with the normal order of addition of H_2O_2 before organic halophenol substrate (*15*).

EXPERIMENTAL PROCEDURES

Materials and Instrumentation. Reagent-grade chemicals (Aldrich, ACROS, or Fisher) were used without further purification except for potassium ferricyanide, which was recrystallized from water. H_2O_2 was taken from a 30% stock solution. Dehaloperoxidase (DHP) was expressed and purified as previously reported (*12*). We note, however, that a more efficient expression system has been reported by Belyea et al. (*25*). UV−visible (UV−vis) absorption spectra were measured using a Cary 400 spectrophotometer interfaced with a Dell personal computer. Transient kinetics and the detection of enzyme intermediates were monitored with a stopped-flow spectrophotometer (model SF-61 DX2, Hi-Tech Scientific, Salisbury, U.K.). Magnetic circular dichroism (MCD) spectra were recorded at −40 °C with a magnetic field strength of 1.41 T using a JASCO J600 spectropolarimeter equipped with a JASCO MCD 1B electromagnet and interfaced with a Silicon Solutions personal computer through a JASCO IF-600-2 interface unit. Data acquisition and manipulation were accomplished as described previously (*10*). UV−vis spectra were re-recorded following the MCD measurements to confirm the integrity of the samples.

Preparation of Samples. DHP was completely oxidized to the Fe(III) state by treatment with a slight excess of potassium ferricyanide, followed by desalting with a P-6 DG (Bio-Rad) column in 100 mM potassium phosphate buffer (pH 5.5) at 4 °C. DHP concentrations were determined using published molar absorptivities (ε_{406} = 116.4 mM^{-1} cm^{-1}) (*10*). Fresh 10 mM H_2O_2 stocks in deionized water were made daily, and H_2O_2 concentrations were confirmed spectrophotometrically using an ε_{240} of 39.4 M^{-1} cm^{-1} (*34*). Halophenol stocks (10−50 mM) were made in a 50/50 ethanol/deionized water mixture. 4-Chloro-, 2,4,6-trichloro-, and 4-chloro-3,5-dimethylphenol were the substrates used for this study.

Stoichiometric Dehaloperoxidase Activity Assay. UV−vis absorption spectroscopy was used to assay for DHP activity using H_2O_2 to initiate the reaction. The ability of DHP to dehalogenate TCP was followed by addition of four equal aliquots (one total equivalent) of H_2O_2 (250 μM) to a cuvette containing protein (15 μM) and TCP (250 μM). UV−vis absorption spectra were recorded before the addition of H_2O_2 and 2 min after the addition of each H_2O_2 aliquot. The reactions were conducted in a 0.5 cm cuvette in 100 mM potassium phosphate buffer (pH 5.5 or 7.0) at 4 °C.

Ferryl DHP Activity Assay. Rapid scan stopped-flow spectrophotometry was used in the double-mixing mode. In the first mix, DHP (13−20 μM) was mixed with H_2O_2 (200−500 μM) in 100 mM potassium phosphate buffer (pH 5.5 or 7.0) for 750 ms to generate an Fe(IV)═O DHP intermediate. This species was reacted with deionized water or TCP (250−1000 μM) in the second mix and allowed to react for 7.5−3000 s.

Cumene Hydroperoxide Assays. The products obtained by reaction of DHP with cumene hydroperoxide were characterized by GC−MS. The 2 mL reaction mixtures at 20 °C consisted of 25 μM Fe(III) recombinant DHP and 240 μM cumene hydroperoxide in 100 mM potassium phosphate buffer (pH 5.5), and the samples were incubated for ~1 h. The organic products were extracted from the reaction mixture with ethyl acetate. The cumyl alcohol and acetophenone products were identified by GC−MS analysis and their retention times compared to retention times of authentic samples. Four individual reactions were conducted and averaged to estimate the ratio of heterolytic to homolytic cleavage.

GC−MS Analyses. An HP5890 gas chromatograph (Agilent Technologies, Palo Alto, CA) equipped with a 30 m × 0.25 mm RTX-5 capillary column (Restek Corp., Bellefonte, PA) was used for quinone and cumene hydroperoxide reaction product identification. The initial temperature of the column was set at 50 °C for 2 min and then increased at a rate of 10 °C/min to a final temperature of 300 °C and held for 10 min. The GC effluent went directly into a VG70S mass spectrometer (Waters Corp., Milford, MA), which was operated in electron impact (EI) ionization mode, and scanned from 50 to 450 amu. Authentic samples of 2,6-dimethyl-1,4-benzoquinone gave essentially identical GC retention times and MS fragmentation patterns. Conditions for the detection of the 4-chlorophenol dimer product have been described previously (*30*).

Ferryl DHP Turnover Assay. An Fe(IV)═O DHP intermediate (Cpd II or Cpd ES) was formed to determine the rate of dehalogenation initiated from this species under steady-state conditions by addition of an excess of H_2O_2 (100−500 μM) to Fe(III) DHP (20 μM) in a 100 mM potassium phosphate solution (pH 5.5 or 7.0) at 4 °C (age time, ~1 min) before the reaction was quenched with TCP (10−500 μM). The formation of Fe(IV)═O DHP was confirmed by UV−vis absorption spectroscopy before addition of the TCP substrate, and the progress of the reaction was monitored at 272 nm.

[2]See Scheme 2, step iii-a, of ref *33*.

RESULTS

DHP from *A. ornata* catalyzes the oxidative dehalogenation of halophenols (*1*, *2*) and is unique in being a catalytically active globin peroxidase (*3*, *4*). The focus of this investigation is on whether oxidative halophenol dehalogenation by DHP proceeds by a normal peroxidase mechanism involving two consecutive one-electron steps with a dissociable radical intermediate and a catalytically active Fe(IV)=O (Cpd II or Cpd ES) state (Figure 1). The order of addition of organic substrate (TCP) versus oxidant cosubstrate (H_2O_2) has been analyzed in probing the recent proposal by Belyea et al. that organic substrate must bind before H_2O_2 (reverse order) to activate DHP (*25*). Our results suggest that multiple Fe(IV)=O DHP intermediates (Cpd I/ES and Cpd II) participate in the oxidative dehalogenation mechanism and that H_2O_2 must bind first (normal order) to generate the oxidizing equivalents.

UV−vis absorption spectra recorded during manual mixing experiments for the DHP-catalyzed oxidative dehalogenation of TCP (250 μM) initiated by H_2O_2 at 4 °C and pH 7.0 are shown in Figure 2. A higher concentration of DHP was used in this assay to monitor the Soret absorption band of DHP upon reaction with H_2O_2. One total equivalent of H_2O_2 (250 μM) was added to the reaction mixture in four equal aliquots (4 × 62.5 μM aliquots). Upon addition of H_2O_2, the intensity of the main absorption band for TCP (310 nm) decreased and that for 2,6-dichloro-1,4-benzoquinone (DBQ) (272 nm, 342 nm) increased. UV−vis absorption spectra were recorded ~2 min after addition of each H_2O_2 aliquot, resulting in the regeneration of Fe(III) DHP and the conversion of TCP to DBQ. The formation of the product required essentially 1 equiv of H_2O_2 per TCP molecule oxidized. A near-linear correlation of DBQ formed to H_2O_2 consumed is displayed (Figure 2, inset). In addition, the intensity of the Soret absorption band at ~406 nm decreases as a result of some heme decomposition seen over the course of the experiment; however, we propose that the integrity of the heme is mostly preserved once the distal histidine moves out of the active site upon binding of substrate (*3*). Recently, a pH study confirmed that both neutral 2,4,6-tribromophenol and anionic 2,4,6-tribromophenolate are converted to the quinone product (*26*). Thus, the stoichiometry of the DHP reaction using TCP as substrate was examined at pH 5.5 (data not shown) and pH 7 (Figure 2), and the stoichiometry was unchanged. As a control, TCP was incubated with excess H_2O_2 overnight, and no DBQ was detected.

As noted above, classical peroxidases typically use the Fe(IV)=O intermediates, Cpds I and II, to oxidize organic substrates, including phenols, by two consecutive one-electron transfers. DHP Cpd I has been reported to form transiently (*25*); however, DHP Fe(IV)=O species have not been fully characterized. Reactions of Fe(III) DHP with cumene hydroperoxide were examined to probe the nature of O−O bond cleavage en route to formation of Fe(IV)=O intermediates (Cpds I and II). Heterolytic O−O bond cleavage of cumene hydroperoxide yields Cpd I and cumyl alcohol, while homolytic cleavage generates Cpd II, acetophenone, and a methyl radical (Scheme S-1 of the Supporting Information) (*35*). The relative percentage of cumyl alcohol and acetophenone formation was analyzed by GC−MS. DHP appears, even with a neutral imidazole histidine proximal ligand, to undergo a substantial amount of heterolytic O−O bond cleavage, consistent with generating a Cpd I species upon reaction with peroxides. In fact, GC−MS analysis indicates that reaction of DHP with cumene hydroperoxide results in ~82%

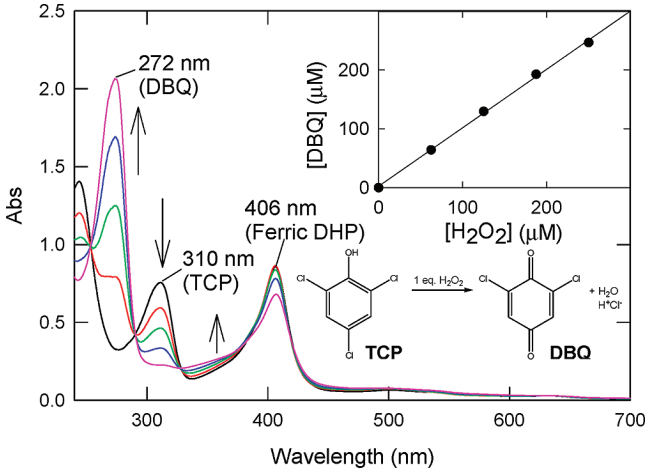

FIGURE 2: UV−visible absorption spectrophotometry of the reaction of ferric recombinant *A. ornata* dehaloperoxidase (15 μM) with 2,4,6-trichlorophenol (250 μM) initiated by H_2O_2 (four 62.5 μL aliquots, total concentration of 250 μM) in 100 mM potassium phosphate buffer (pH 7.0) at 4 °C. Each spectrum was recorded 2 min after H_2O_2 addition. The inset shows the formation of 2,6-dichloro-1,4-benzoquinone (micromoles) plotted as a function of H_2O_2 consumed (micromoles).

heterolytic cleavage and ~18% homolytic cleavage at 20 °C and pH 5.5 (Table S-1 of the Supporting Information). In contrast, cleavage of the O−O bond upon reaction of cumene hydroperoxide with Mb involves nearly equal amounts of heterolytic and homolytic cleavage (*36*).

A DHP Fe(IV)=O species quickly forms upon reaction with H_2O_2, but the intermediate is not stable enough at ambient temperatures during the time required to acquire a high-quality MCD measurement. Therefore, to characterize this species, we have generated and spectrally examined it at −40 °C using antifreeze solvents (Figure 3) (*37*). This DHP intermediate has UV−vis absorption and MCD spectra very similar to those of Mb Cpd II (*14*). The Soret absorption peak is blue-shifted by ~2 nm in comparison to that of Mb Cpd II (*14*), but the Soret intensities are nearly identical. Because the electronic spectral properties of Cpd II and Cpd ES are expected to be nearly identical (*38*), the species observed in Figure 3 is likely a mixture of the two. While the DHP Fe(IV)=O intermediate is relatively unstable, Mb Cpd II is stable enough to be examined spectroscopically at ambient temperatures. The difference is likely due to the positioning of the distal histidine in DHP to activate peroxide (vide supra), a structural arrangement that also promotes heme decomposition.

Rapid scan stopped-flow spectrophotometry was used to probe the order of addition of TCP versus H_2O_2 and examine if a preformed DHP Fe(IV)=O complex can be an active oxidant during the oxidative dehalogenation mechanism. First, Fe(III) DHP was reacted with H_2O_2 in the single-mixing mode at 4 °C and pH 5.5 to determine the amount of time required to form Fe(IV)=O DHP (~750 ms) (Figure S-1 of the Supporting Information). Next, this intermediate was generated in the first mix of a double-mixing experiment and then reacted with TCP in the second mix, also at 4 °C and pH 5.5. Upon reaction with TCP (Figure 4), Fe(III) DHP is re-formed within 3.5 s (Figure 4, inset). A clean isosbestic point at 414 nm (Figure 4) indicates that Fe(IV)=O DHP is directly reduced to Fe(III) DHP upon reaction with TCP, likely with formation of the phenoxy radical as previously suggested for the same reaction catalyzed by Mb

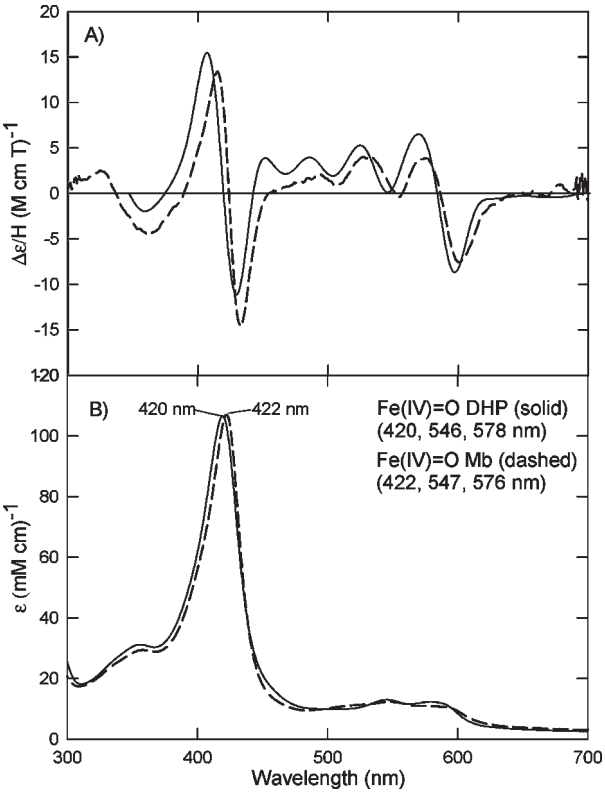

FIGURE 3: (A) Magnetic circular dichroism spectrum of ferryl [Fe(IV)=O] recombinant *A. ornata* dehaloperoxidase (DHP) generated by addition of H_2O_2 (25 μM) to ferric [Fe(III)] DHP (20 μM) in 50/50 glycerol/100 mM potassium phosphate buffer (pH 5.5) at −40 °C (—) and incubated for ~5 min and magnetic circular dichroism spectrum of horse heart myoglobin (Mb) Compound II (Cpd II) in 100 mM potassium phosphate buffer (pH 7.0) at 4 °C (– – –) (*14*). (B) UV−visible absorption spectrum of Fe(IV)=O DHP generated as described above via addition of H_2O_2 to Fe(III) DHP in 50/50 glycerol/100 mM potassium phosphate buffer (pH 5.5) at 4 °C (—) and UV−visible absorption spectrum of horse heart Mb Cpd II in 100 mM potassium phosphate buffer (pH 7.0) at 4 °C (– – –) (*14*).

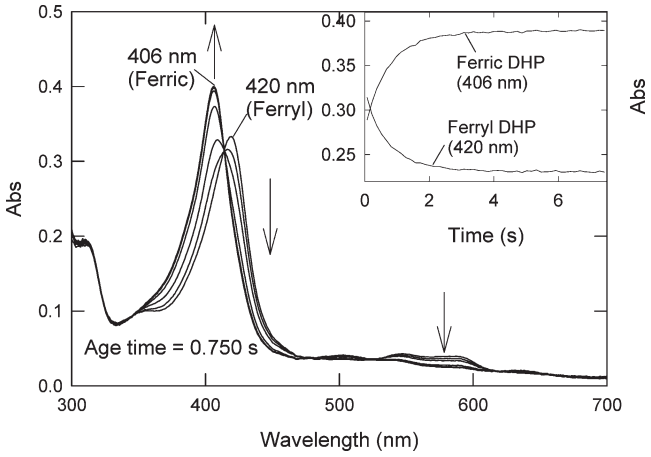

FIGURE 4: Reaction of ferryl [Fe(IV)=O] recombinant *A. ornata* dehaloperoxidase (DHP) with 2,4,6-trichlorophenol (TCP) (reaction time, 7.5 s). The Fe(IV)=O DHP intermediate was formed in the first mix by reacting ferric [Fe(III)] enzyme with H_2O_2 for 750 ms. The reactions were conducted in 100 mM potassium phosphate buffer (pH 5.5) at 4 °C. The concentrations after the final mix are 3.3 μM DHP, 50 μM H_2O_2, and 125 μM TCP. The inset shows kinetic traces at 406 and 420 nm for this reaction showing the generation of Fe(III) DHP and the disappearance of Fe(IV)=O DHP, respectively.

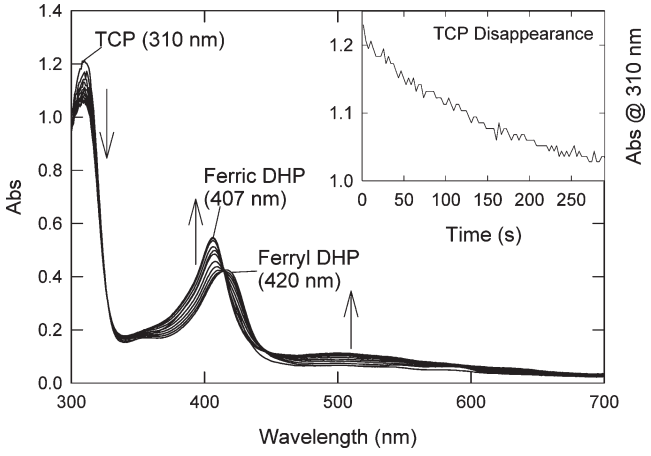

FIGURE 5: Reaction of ferryl [Fe(IV)=O] recombinant *A. ornata* dehaloperoxidase (DHP) with 2,4,6-trichlorophenol (TCP) (reaction time, 300 s). The Fe(IV)=O DHP intermediate was generated in the first mix by reacting ferric enzyme with H_2O_2 for 750 ms. The reactions were conducted in 100 mM potassium phosphate buffer (pH 7.0) at 4 °C. The concentrations after the final mix are 4.7 μM DHP, 125 μM H_2O_2, and 500 μM TCP. The inset shows a kinetic trace at 310 nm following the disappearance of TCP during the course of this reaction.

and CCPO (*13*, *30*, *31*). Furthermore, these results are consistent with the recent report of Cpd ES reactivity by Feducia et al. (*33*). In contrast, in the absence of substrate, Fe(IV)=O DHP is not reduced back to the Fe(III) state even after extremely long reaction times (3000 s), and heme destruction is evident (Figure S-2 of the Supporting Information). Formation of this intermediate species, recently named Compound RH (*33*), was first reported by Osborne et al. (*12*). In the absence of substrate, the distal histidine is not likely positioned properly (*3*) to prevent heme decomposition, and the reducing equivalents are not available to regenerate Fe(III) DHP. The proposed role of the distal histidine is based on the crystal structure of native DHP. The crystal structure of DHP revealed the distal histidine to be in two different conformations, one of which places this residue away from the heme active site (*3*). The heme destruction seen upon addition of excess H_2O_2 in the absence of organic substrate (Figure S-2 of the Supporting Information) likely explains why Belyea et al. (*25*) did not see product formation when Fe(III) DHP premixed with excess H_2O_2 was reacted with organic substrate.

As the disappearance of TCP cannot be easily monitored in the visible region at acidic pH, the reaction was examined at neutral pH where the characteristic absorption peak of TCP at 310 nm could be observed (Figure 5). Fe(IV)=O DHP was generated in the first mix of a double-mixing experiment and then reacted with TCP in the second mix at 4 °C and pH 7.0. An increase at 407 nm after reaction of TCP with Fe(IV)=O DHP is evidence that the Fe(III) enzyme is regenerated, and a decrease in the TCP absorption peak at 310 nm (Figure 5, inset) is indicative of concomitant substrate oxidation upon reaction with Fe(IV)=O DHP (Figure 5). The reaction is slower at neutral pH (reaction time, 300 s).

Turnover studies were carried out to further probe the order of addition of organic substrate (TCP) versus oxidant cosubstrate (H_2O_2) and whether the mechanism of oxidative dehalogenation involves two consecutive one-electron steps (*13*, *30*−*32*) or a single two-electron oxidation (*25*, *33*). Fe(III) DHP was reacted

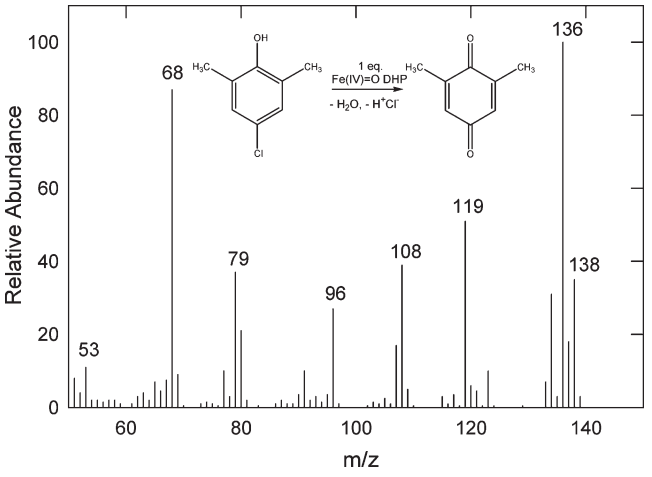

FIGURE 6: Mass spectrum of 2,6-dimethyl-1,4-benzoquinone extracted with ethyl acetate from the reaction of ferryl [Fe(IV)=O] recombinant *A. ornata* dehaloperoxidase (DHP) (50 μM) with 4-chloro-3,5-dimethylphenol (250 μM) in 100 mM potassium phosphate buffer (pH 5.5) at 4 °C. The Fe(IV)=O DHP species was generated by adding a slight excess of H_2O_2 (60 μM) to ferric DHP (50 μM).

with varying excess amounts of H_2O_2 to generate an Fe(IV)=O DHP species, followed by organic substrate addition (i.e., normal peroxidase order of addition: H_2O_2 first followed by organic substrate). After addition of TCP, the formation of DBQ was monitored at 272 nm as a function of time (Figure S-3 of the Supporting Information). The increase in absorbance at 272 nm suggests that under steady-state conditions, DHP Cpd II or Cpd ES (*33*) is an active oxidant for conversion of TCP to DBQ.

A stoichiometric amount of Fe(IV)=O DHP was generated by addition of a slight (1.2-fold) excess of H_2O_2 to Fe(III) DHP. Following treatment with the less reactive 4-chloro-3,5-dimethylphenol, the product was extracted and identified to be 2,6-dimethyl-1,4-benzoquinone by GC−MS analysis (Figure 6). The parent ion peak (*m*/*z* 136) and fragmentation pattern of the product are in agreement with authentic 2,6-dimethyl-1,4-benzoquinone standards. Furthermore, the spectral characteristics are similar to those previously reported for the quinone product identified by GC−MS for similar reactions catalyzed by horse heart Mb (*13*). This clearly demonstrates that Fe(IV)=O DHP is an active oxidant that can catalyze the oxidative dehalogenation of halophenol substrates.

The oxidative dehalogenation mechanism catalyzed by several heme-containing peroxidases is currently under investigation (vide supra). The data consistently support a mechanism involving both Cpd I (or ES) and Cpd II (i.e., two consecutive one-electron steps). This conclusion is based in part on the use of *p*-halophenols as mechanistic probes of the dehaloperoxidase activity of CCPO and similar studies with Mb and HRP (*13*, *30*, *32*). Both 1,4-benzoquinone and a dimeric biphenyl product were detected after reaction of CCPO or Mb with 4-chlorophenol. We have hypothesized that formation of a dimeric product, consistent with the involvement of radicals during the reaction, is possible when the 2- and 6-positions are not blocked as is the case with TCP as the substrate and only the quinone product is observed. The same benzoquinone and biphenyl products (Figure S-4 of the Supporting Information) were identified by GC−MS for the DHP-catalyzed reaction with 4-chlorophenol as we have previously reported with CCPO and Mb (*13*, *30*). A mass spectral library search for a structure(s) corresponding to the observed mass spectrum (Figure S-4) resulted in a biphenyl chlorophenol dimer (*m*/*z* 254). Similar biphenyl coupling products have been reported by Celik et al. for the oxidation of *p*-cresol by ascorbate peroxidase (*39*). Detection of 1,4-benzoquinone was limited by its inherent instability during workup. Thus, an accurate quantitative comparison of dimer to quinone formation could not be determined. Consequently, we cannot rule out some product formation occurring via a tightly coupled mechanism by which Cpd I/ES initiates the reaction and Cpd II is reduced back to the Fe(III) state by the phenoxy radical intermediate prior to release of the radical. However, we consistently observed formation of the biphenyl product, and this clearly shows that the radical intermediate formed during the full oxidation of halophenol to quinone is able to dissociate from the active site to dimerize. The formation of dimer, along with the numerous experiments demonstrating the reactivity of Fe(IV)=O DHP intermediates, supports a mechanism of oxidative dehalogenation involving two consecutive one-electron steps (*1*).

DISCUSSION

The experiments conducted during this study were designed to probe the order of addition of substrates (i.e., halophenol and H_2O_2) to DHP and whether oxidative halophenol dehalogenation proceeds by two consecutive one-electron steps or by a direct two-electron oxygen atom insertion mechanism. The results presented here clearly support a mechanism requiring H_2O_2 to bind to the heme active site before the organic substrate (Figure 1). Since the oxidation of TCP to DBQ is a two-electron process and H_2O_2 is a two-electron oxidant, the observed stoichiometry in Figure 2 strongly suggests that two DHP Fe(IV)=O species (i.e., Cpd I or Cpd ES and Cpd II) participate in the reaction. Cumene hydroperoxide is often used to probe the nature of O−O bond cleavage during reaction of peroxides with heme enzymes. The product distribution upon reaction of Fe(III) DHP with cumene hydroperoxide (Table S-1 of the Supporting Information) shows that a large percentage of O−O bond cleavage occurs heterolytically en route to formation of Cpd I. This clearly indicates that DHP Cpd I is initially formed upon reaction of the Fe(III) enzyme with peroxides. Our results are consistent with an earlier report of formation of a transient Cpd I species (*25*). Recently, Cpd ES has been detected for DHP (*33*), and it is reasonable to assume in our studies that one or both form upon reaction with H_2O_2 since DHP is a His-ligated enzymatic globin (*3*). Cpd ES, which has been observed to form rapidly in Mb and cytochrome *c* peroxidase as a result of transfer of the radical on the porphyrin of Cpd I to an adjacent amino acid residue (*15*, *36*, *40*, *41*) before being reduced to Cpd II, could participate in the reaction in parallel with or in place of Cpd I (Figure 1). While Cpd ES is a two-electron oxidized intermediate, no evidence has ever been published to suggest that a Cpd ES species can perform a concerted two-electron, P450-like oxygen atom insertion mechanism.

The ability of a preformed Fe(IV)=O DHP intermediate to oxidize TCP as indicated in the double-mixing experiments (Figures 4 and 5) provides evidence supporting a normal order of oxidant cosubstrate (H_2O_2) addition before organic substrate (TCP). The Fe(IV)=O DHP intermediate formed could be Cpd II, Cpd ES (*33*), or a mixture of both, and the ability of either species to react with organic substrate (TCP) supports an electron transfer mechanism involving two consecutive one-electron steps. In addition, being able to initiate the reaction from

the Cpd II/ES state indicates that Fe(IV)=O DHP is an active oxidant even if it formed before binding of organic substrate (*25*).

A single-turnover experiment with Fe(IV)=O DHP and 4-chloro-3,5-dimethylphenol yielded the more stable 2,6-dimethyl-1,4-benzoquinone, which was isolated and identified by GC−MS (Figure 6). This clearly rules out the previous proposal that Fe(IV)=O DHP species can participate in the oxidative dehalogenation mechanism only if substrate binds before H_2O_2 (*25*) or in a tightly coupled reaction whereby Cpd II cannot initiate the reaction (*26*). Additionally, the formation of a biphenyl product upon reaction of DHP with 4-chlorophenol clearly shows that a radical intermediate that forms during the full oxidation of halophenol to quinone is able to dissociate from the active site to dimerize. Observation of the same biphenyl product led us to reach the identical conclusion in our earlier work on oxidative halophenol dehalogenation by CCPO and horse heart Mb (*13*, *30*). The ability of DHP Cpd II, a one-electron oxidant, or Cpd ES (also known to be only capable of one-electron oxidations) to oxidize halophenols and the formation of dimeric products argues strongly for the traditional peroxidase mechanism involving two consecutive one-electron steps via a dissociable substrate radical intermediate. Since an Fe(IV)=O DHP intermediate clearly is able to oxidize halophenols by the normal order of substrate addition (H_2O_2 first followed by organic substrate), it would be extremely unlikely that DHP Cpd I would function by the opposite (reverse) order of addition as proposed by Belyea et al. (*25*).

The proposal that oxidative halophenol dehalogenation by DHP may start from a substrate-bound state (*25*) is intriguing but incorrect. Furthermore, the fact that substrate binding moves the distal histidine out of the position necessary for H_2O_2 activation (*3*, *4*) draws into question how DHP would activate H_2O_2 from the substrate-bound state. Instead, we propose that substrate binding serves a protective role to actually prevent H_2O_2 activation, thus avoiding heme bleaching by preventing Cpd I from forming. Such a mechanism is very similar to the cytochrome P450 peroxide shunt where substrate also protects against heme bleaching, but the active site cannot accommodate both the artificial oxidant and substrate so substrate must be released to initiate the shunt reaction (*15*).

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SUPPORTING INFORMATION AVAILABLE

Additional rapid scan stopped-flow and GC−MS data and a table comparing the percentages of heterolytic and homolytic O−O bond cleavage. This material is available free of charge via the Internet at http://pubs.acs.org.

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