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Functional Switching of *Amphitrite ornata* Dehaloperoxidase from O_2-Binding Globin to Peroxidase Enzyme Facilitated by Halophenol Substrate and H_2O_2[†]

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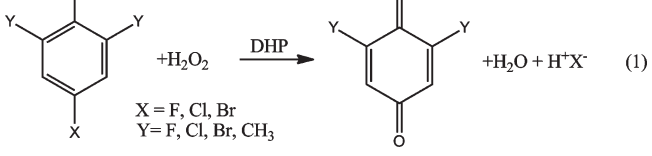
ABSTRACT: *Amphitrite ornata* dehaloperoxidase (DHP) is the first heme-containing globin possessing a native peroxidase enzymatic activity. DHP catalyzes the H_2O_2-dependent dehalogenation of halophenols. By possessing this detoxifying enzymatic activity, these organisms are able to thrive in an environment contaminated with toxic haloaromatics. It has been proposed that DHP evolved from a dioxygen carrier globin protein and therefore possesses dual physiological roles of O_2 carrier and dehaloperoxidase. Although DHP is isolated in the catalytically inactive oxyferrous state (oxy-DHP), we find that the combination of H_2O_2 and the substrate 2,4,6-trichlorophenol (TCP) brings about facile switching of oxy-DHP to the enzymatically active ferric state via a process likely involving substrate radicals (TCP·). In contrast, in the absence of TCP, H_2O_2 alone converts oxy-DHP to an inactive state (compound RH) instead of oxidizing the enzyme to the ferric state. Further, although the rate of autoxidation of oxy-DHP is somewhat enhanced by the presence of TCP, the effect is too small to be the functional switch. Instead, both substrate and H_2O_2 are needed to convert oxy-DHP to the catalytically active ferric state. These observations provide a physiological link between the O_2 carrier role of the ferrous protein and the peroxidase activity of the ferric enzyme in this bifunctional protein.

Dehaloperoxidase (DHP)[1], discovered from the terebellid polychaete *Amphitrite ornata*, is the first enzymatic heme-containing globin possessing a native peroxidase activity. The major physiological function of DHP is to detoxify halophenols in their living environment via the H_2O_2-dependent oxidative dehalogenation to yield the corresponding quinones (eq 1) (*1*). We have previously shown that an O_2 carrier, horse heart myoglobin (HH Mb), also catalyzes the same reaction in the ferric state (*2*). Both HH Mb and DHP appear to catalyze the reaction by a mechanism involving two consecutive one-electron steps via a phenoxy radical mediated by the high valent oxidants compound I (Cpd I) (Fe^{IV}=O porphyrin π-cation radical) or compound II−Y· (Cpd II−Y·)[2] (Fe^{IV}=O with a tyrosyl radical) and compound II (Cpd II) (Fe^{IV}=O state) (*2*, *3*).



(1)

As revealed by X-ray crystallography, DHP is composed of two identical heme-containing subunits (15.5 kDa) with the same protein fold as myoglobin (Mb) despite the relatively low sequence identity between these two proteins (*4*−*6*). Like the myoglobins, DHP is isolated primarily in the oxyferrous state, showing its stability in the ferrous state and ability to bind O_2 reversibly (*7*). Crude extracts of *A. ornata* contain DHP at about 3% of the soluble protein, indicating the protein expression level of DHP is very high (*1*). An O_2-carrying and transporting protein in *A. ornata* has not been fully studied so far. It has been reported that there is a monomeric Hb in the coelom of *A. ornata*; however, its sequence has not yet been determined (*8*). The 28 amino acid N-terminal sequence of the coelomic hemoglobin from another polychaete worm, *Enoplobranchus sanguineus*, a physiological oxygen carrier, is 32% identical to that of DHP (*8*). It is notable that most O_2 carrier proteins are globin-shaped (*9*). LaCount et al. proposed that DHP may have evolved from an oxygen carrier protein (*4*). The enhanced dehaloperoxidase activity of DHP was proposed to have arisen from gene codon duplication and divergence (*4*). If this hypothesis is true, DHP has two physiological functions, serving as an O_2 carrier in addition to its peroxidase detoxification activity. Kinetic studies have revealed that the rate of DHP-catalyzed reactions is over 1 order of magnitude slower than that of horseradish peroxidase (HRP) but 1−2 orders of magnitude faster than that of Mb (*2*, *10*). The requirement of carrying out two distinct physiological functions could be the reason why *A. ornata* has not evolved into a more efficient dehaloperoxidase.

Recently, the binding order of the organic substrate TCP versus the oxidant cosubstrate H_2O_2 to DHP in the catalytic cycle has been disputed. Belyea et al. proposed that the organic substrate must bind to the enzyme first to activate DHP to react with H_2O_2 and initiate catalysis (*11*). In contrast, we have presented data showing that DHP follows a normal order of addition with H_2O_2 binding first to generate the oxidizing equivalents (Cpd I/Cpd II−Y·, which is subsequently converted

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[1]Abbreviations: DHP, dehaloperoxidase; HH Mb, horse heart myoglobin; TCP, 2,4,6-trichlorophenol; compound RH, "reversible heme" state of dehaloperoxidase, formed from the decay of compound II−Y· in the absence of substrate; Cpd I, compound I; Cpd II, compound II; Cpd II−Y·, compound II−Y·; Mb, myoglobin; WT, wild type; HRP, horseradish peroxidase; UV−vis, UV−visible; oxy-DHP, oxyferrous DHP; DBQ, 2,6-dichloro-1,4-benzoquinone; LiP, lignin peroxidase.

[2]Previously termed compound ES (*3*).

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to Cpd II) followed by halophenol binding to initiate a dehalogenation mechanism involving two consecutive one-electron steps via a dissociable substrate radical intermediate and a catalytically active Fe^{IV}=O (Cpd II or Cpd II−Y·) state (*3*). We also proposed that substrate binding serves a protective role to avoid heme bleaching. In this study, we have further probed the role of organic substrate (TCP) binding in oxidative dehalogenation reactions starting from the oxyferrous DHP (oxy-DHP) state. It is notable that, in the presence of TCP substrate, oxy-DHP quickly switches to the enzymatically active ferric state following addition of just 0.5 equiv of H_2O_2[3] (relative to DHP). This functional switch, which may be directly facilitated by substrate TCP radicals, is physiologically essential for *A. ornata* to be able to survive in the presence of a toxic halophenol environment (*12*).

MATERIALS AND METHODS

Materials. All reagents and biochemicals were purchased from Aldrich, ACROS, or Fisher and used without further purification except for potassium ferricyanide, which was recrystallized from water. WT six-His-tagged DHP was expressed and purified as previously described (*11*). His-tagged DHP has the same enzymatic activity as recombinant dehaloperoxidase with the substrate 2,4,6-trichlorophenol (TCP) (*11*). UV−visible (UV−vis) absorption spectra were recorded in 0.5 cm cuvettes on a Cary 400 spectrophotometer interfaced with a Dell computer.

Preparation of Ferric and Oxyferrous DHP Samples. Isolated DHP exists as a mixture of ferric and oxyferrous states. Complete oxidation of the heme iron is accomplished by addition of a few crystals of potassium ferricyanide (Fluka) followed by Bio-Gel P6DG (Bio-Rad) desalting column (1 cm ×30 cm) chromatography using 100 mM potassium phosphate buffer (pH 7) at 4 °C (*13*). Protein concentrations were determined by the pyridine hemochromogen method (ε_{555} = 34.4 M^{-1} cm^{-1}) (*14*) and are the concentrations of the heme component. Oxyferrous DHP (oxy-DHP) was formed by addition of a slight excess of sodium dithionite to ferric DHP to generate the ferrous state followed either by bubbling with O_2 gas or by Bio-Gel P6DG (Bio-Rad) desalting column (0.5 cm ×15 cm) chromatography with 100 mM potassium phosphate buffer (pH 7) at 4 °C. Oxy-DHP formation was confirmed by UV−vis absorption spectroscopy before each experiment (Supporting Information Figure S1).

H_2O_2 stock (10 mM) in deionized water was prepared daily from a 30% stock solution, and H_2O_2 concentrations were confirmed spectrophotometrically (ε_{240} = 39.4 M^{-1} cm^{-1}) (*15*). 2,4,6-Trichlorophenol (TCP) stocks (10 mM) were freshly made in a 50/50 ethanol/deionized water mixture.

Peroxidase Activity Assay and Lag Time Measurements. UV−vis absorption spectroscopy was used to measure the peroxidase activity of ferric and oxyferrous enzyme ([DHP] = 6 μM). Substrate TCP and hydrogen peroxide were added together to initiate the reactions with the enzyme in a 0.5 cm cuvette. After quickly mixing the reagents with the protein sample (in about 5 s), an initial reaction rate and a lag time can be measured by monitoring the change in absorbance at 272 nm for the appearance of quinone products (ε_{272} = 12.0 ± 1.0 mM^{-1} cm^{-1}) (*16*). To ensure the reproducibility of the results, measurements were repeated five times for each run. All concentrations listed are after mixing.

Stopped-Flow UV−vis Spectrophotometric Studies. Transient kinetics and the detection of enzyme intermediates were monitored with a stopped-flow spectrophotometer (1.0 cm path length, model SF-61 DX2; Hi-Tech Scientific, Salisbury, U.K.). All of the stopped-flow experiments were carried out at 4 °C in 100 mM potassium phosphate buffer (pH 5.4 and 7.0). Data were collected and analyzed by using KinetAsyst3 software (Hi-Tech Scientific, Salisbury, U.K.).

RESULTS AND DISCUSSION

Properties of Oxy-DHP Samples Prepared by O_2 Bubbling vs Desalting Column Chromatography Following Dithionite Reduction of the Ferric Enzyme. Oxy-DHP samples prepared by these two methods (see Materials and Methods) exhibited essentially indistinguishable spectral properties (Supporting Information Figure S1) and results from the experiments described below except for autoxidation tests in the presence and absence of the substrate TCP. Thus, all of the results reported herein were obtained with oxy-DHP that was generated by O_2 bubbling except for autoxidation experiments which were performed using oxy-DHP prepared chromatographically.

Dehalogenation Activity of DHP Starting with the Oxyferrous Form and Effects of Substrate on the Lag Time for Product Formation. Figure 1A shows the UV absorption spectral kinetic assays monitored at 272 nm (*11*) for formation of the product 2,6-dichloro-1,4-benzoquinone (DBQ) following oxidative dechlorination of TCP at pH 7 catalyzed by ferric DHP (6 μM) compared to that starting with oxy-DHP (6 μM). The organic substrate (TCP) (150 μM) and oxidant cosubstrate (H_2O_2) (600 μM) were added together to the cuvette to initiate the reaction. The initial rate of product formation with ferric DHP was 0.30 ± 0.04 [ΔAbs (272 nm)/min], which yields a turnover number of 8.3 ± 1.1 mol of product/(min·mol of enzyme). For the reaction starting with oxy-DHP, there was no product formation during a short lag time (~0.1 min). After the lag period, the rate of product formation was 0.40 ± 0.04 [ΔAbs (272 nm)/min], i.e., 11.1 ± 1.1 mol of product/(min·mol of enzyme), slightly faster than the ferric DHP-catalyzed reaction. Measured at pH 7.0, these apparent turnover numbers (8−11 min^{-1}) for the DHP-catalyzed dehalogenation of TCP are much lower than those at pH 5.4 [198 ± 0.8 mol of product/(min·mol of enzyme)] (*10*). The pH dependence of the DHP activity has been examined by Franzen et al., who found that the activity for substrate 2,4,6-tribromophenol at pH 5.0 is about 7.4-fold higher than that at pH 7.0 (*17*).

The effect of TCP concentration (40, 80, 120, and 160 μM) on the lag time for product formation without preincubation of the enzyme with TCP is shown in Figure 1B. Figure 1C illustrates the lag time as a function of concentrations of TCP (0−160 μM). The results demonstrate that the lag time is inversely correlated to the concentration of TCP. The lag time becomes shorter and almost undetectable by preincubation of oxy-DHP with TCP (150 μM) for 15 min at pH 7.0 and 4 °C. However, preincubation does not enhance the initial rate of product formation by either ferric or oxy-DHP. The lag time also almost disappears at pH 5.0 even without preincubation (data not shown). The turnover rate is much faster at pH 5.0 than at pH 7 (*17*) as mentioned above. To further investigate the effect of preincubation on the lag time for product formation, a mixture of oxy-DHP (6 μM)/TCP (150 μM)

[3]As a two-electron oxidant, 0.5 equiv of H_2O_2 provides 1.0 equiv of oxidant.

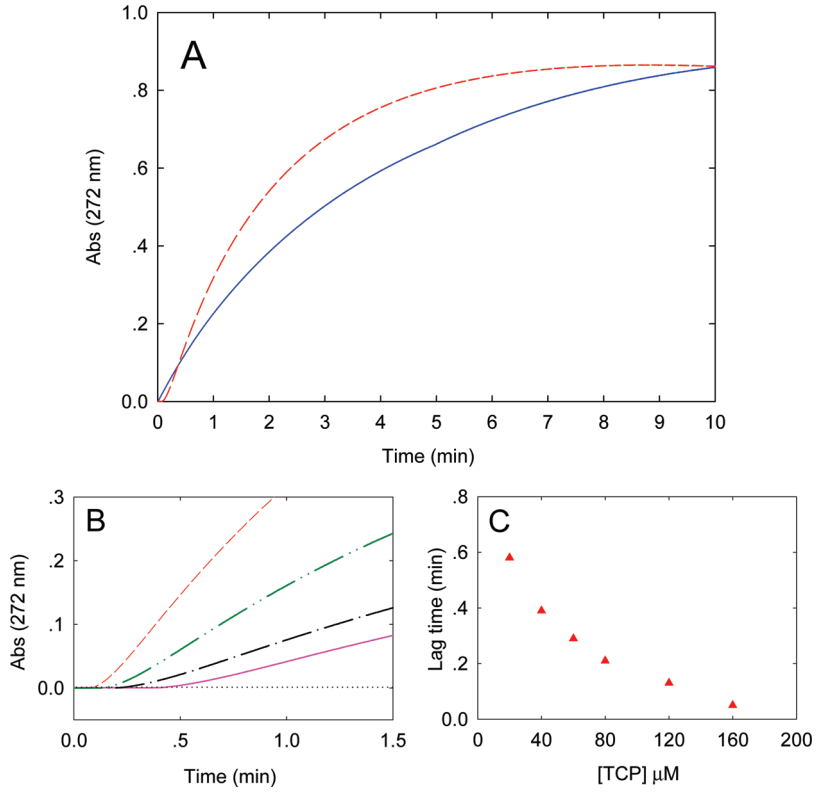

FIGURE 1: (A) UV absorption kinetic assays monitored at 272 nm for formation of DBQ initiated with the ferric DHP (6 μM) (blue solid line) or with oxy-DHP (6 μM) (red dashed line) vs TCP (150 μM)/H_2O_2 (600 μM). (B) Results of additional kinetic assays (only for 0−1.5 min) initiated with the oxy-DHP (6 μM) vs H_2O_2 (600 μM) and different concentrations of TCP (40 μM, pink solid line; 80 μM, black dash-dotted line; 120 μM, green dash-dot-dotted line; 150 μM, red dashed line). (C) Lag times for product (DBQ) formation monitored at 272 nm seen in absorption kinetic assays (shown in (B)) initiated with oxy-DHP (6 μM) and H_2O_2 (600 μM) at different concentrations of TCP (0−160 μM). All of the data were recorded in 0.1 M potassium phosphate buffer (pH 7.0) at 4 °C. The concentrations listed were after mixing.

was preincubated for 15 min, and then the reaction was initiated with H_2O_2 at various concentrations of (0.2, 0.4, 0.6, 0.8, 1.2, and 1.6 mM). The lag time was not observed except at the lowest concentration of H_2O_2 (200 μM) where a lag time of ~0.16 min was seen (data not shown).

Hydrogen Peroxide Alone Converts Oxyferrous DHP to Compound RH and Causes Heme Destruction in the Absence of TCP. What is happening during the lag time? To determine whether H_2O_2 alone could directly convert oxy-DHP to the active states of DHP (ferric or ferryl), oxy-DHP was reacted with different stoichiometric amounts of H_2O_2 with respect to the enzyme in the absence of TCP. Oxy-DHP was stable over 20 min at pH 7.0 and 4 °C when 1 equiv of H_2O_2 was added; however, when oxy-DHP (5.0 μM) was reacted with 10, 20, or 50 equiv of H_2O_2 (50, 100, or 250 μM) under the same conditions over a period of 2000 s, it was gradually converted to another species that has a Soret peak around 411 nm. Figure 2 shows the result of reacting oxy-DHP (5.0 μM) with 10 equiv of H_2O_2 (50 μM) in the absence of TCP. The intensity of the Soret peak of oxyferrous DHP gradually decreased with time upon mixing with H_2O_2 with an apparent rate constant of k = 0.0021 s^{-1} ($t_{1/2}$ = 315 s). No Cpd I/II−Y· and/or Cpd II intermediates were observed. The final absorption spectrum has a Soret peak around 411 nm. Similar results were obtained with 2, 20, or 50 equiv of H_2O_2 (10, 100, or 250 μM) with a nearly identical rate constant k = 0.0022 ± 0.0001 s^{-1}. However, the total absorbance change (ΔAbs (417 nm)) became slightly larger (by 6−11% vs 10 equiv of H_2O_2) for higher concentrations of H_2O_2, likely due to partial degradation of the heme. This enzyme state was first reported by Osborne et al. (*10*) and was recently

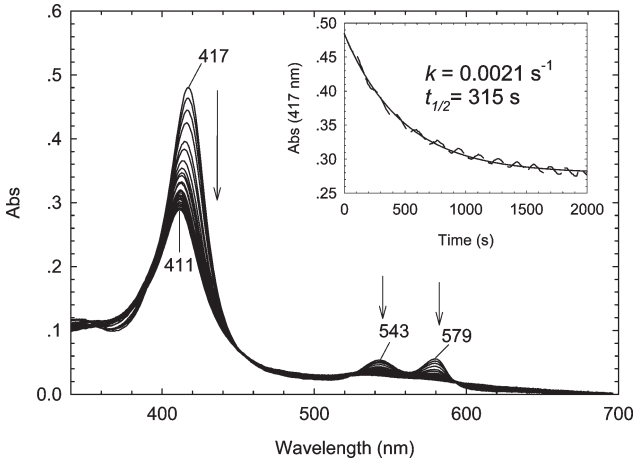

FIGURE 2: Spectral change upon addition of 10 equiv of H_2O_2 to oxy-DHP. Rapid scan stopped-flow spectrophotometry was used in the single-mixing mode. In the absence of substrate TCP, oxyferrous DHP (5 μM) was mixed with 10 equiv of H_2O_2 (50 μM) and allowed to react for 2000 s. The inset shows the result of absorption change (k = 0.0021 s^{-1}) monitored at 417 nm. The reactions were conducted in 100 mM potassium phosphate buffer (pH 7.0) at 4 °C. The concentrations listed were after mixing.

named Cpd RH[1] by Feducia et al. (*18*) for a species that is formed by addition of excess H_2O_2 (0.5−1.5 mM) to ferric DHP in the absence of halophenol (*3*, *10*, *18*). Our previous data showed that, in the absence of substrate, this DHP entity could not be converted back to the ferric state even after extremely long incubation times (3000 s) and heme destruction is evident upon addition of excess H_2O_2 (*10*).

TCP Promotes the Slow Autoxidation of Oxy-DHP to the Ferric State and Protects the Enzyme from Denaturation at Room Temperature. In order to determine whether substrate affects the rate of autoxidation, oxy-DHP was incubated with and without TCP (150 μM) in 100 mM phosphate buffer, pH 7.0, at 4 °C as well as at 25 °C. Oxy-DHP sample prepared by bubbling O_2 gas into dithionite-reduced enzyme exhibited unusually fast initial spectral change (for ~15% of the total change expected for complete oxidation) during the first hour of incubation, followed by a slower rate of autoxidation (i.e., biphasic kinetics). A possible cause for such a behavior of oxy-DHP could be formation of certain peroxide species associated with this oxy-DHP preparation method. Oxy-DHP samples prepared by the desalting column chromatography (see Materials and Methods) gave monophasic kinetics in the autoxidation experiments described herein.

At 4 °C, the presence of substrate had very little effect on the absorption spectrum and stability of oxy-DHP for at least 1 h (Supporting Information Figure S1). At room temperature (25 °C), addition of TCP (150 μM) increased the autoxidation rate of oxy-DHP to the ferric state by about 2.6-fold, but the effect was too small to account for the facile conversion of oxy to ferric DHP (Figure 3A). The autoxidation rate of oxy-DHP in the presence of TCP at 4 °C was 1.9-fold slower than that at 25 °C. During the autoxidation process, the Soret peak blue shifted from 417 to 408 nm, and the intensity of visible peaks around 500 and 630 nm increased with time. The final spectrum was similar, but not identical, to that of ferric DHP that was prepared separately (Supporting Information Figure S1, panel B) because oxy-DHP was not converted to 100% ferric state under these conditions even after 72 h (Figure 3A). In the absence of TCP (Figure 3B), the oxy-DHP started to denature after 26 h of incubation at 25 °C. Thus, we conclude that although the substrate TCP does increase the rate of autoxidation of oxy-DHP, the effect is too small to be physiologically relevant for the functional switch to the enzymatically active ferric state. On the other hand, TCP does serve a protective role by preventing degradation of oxy-DHP during autoxidation.

Hydrogen Peroxide Switches Oxyferrous DHP to the Functionally Active Ferric State and Then to Cpd II/II−Y· in the Presence of TCP. To further clarify the reaction mechanism of oxy-DHP-initiated dehalogenation, oxy-DHP in the presence of TCP was mixed with a stoichiometric amount of H_2O_2, and the reaction was monitored by rapid scan stopped-flow spectroscopy (Figure 4). The concentrations of oxy-DHP, TCP, and H_2O_2 after mixing were 5, 150, and 5 μM, respectively. The starting oxy-DHP has a Soret peak at 417 nm, which blue shifted to 407 nm at the end of the conversion indicating the formation of the ferric state. The absorbance change at 407 nm as a function of time (0−400 s) is shown as the black solid line in the inset of Figure 4. We found that in the presence of substrate TCP, even 0.5 equiv of H_2O_2[3] was able to convert oxy-DHP to the enzymatically active ferric state (blue dashed line in the inset of Figure 4). In both cases (with 0.5 and 1 equiv of H_2O_2), the conversion rates progressively become faster at the initial stage before reaching maximal values and the absorbance changes eventually reach plateaus. Thus, the overall shape of the absorbance (417 nm) vs time plot looks like a sigmoid curve, especially for the 0.5 equiv of H_2O_2 case. Similar conversion from oxy-DHP to the ferric state can be achieved with 1 equiv of H_2O_2 at pH 5.4 with significantly faster initial rate (Supporting Information Figure S2) compared with the corresponding result at pH 7

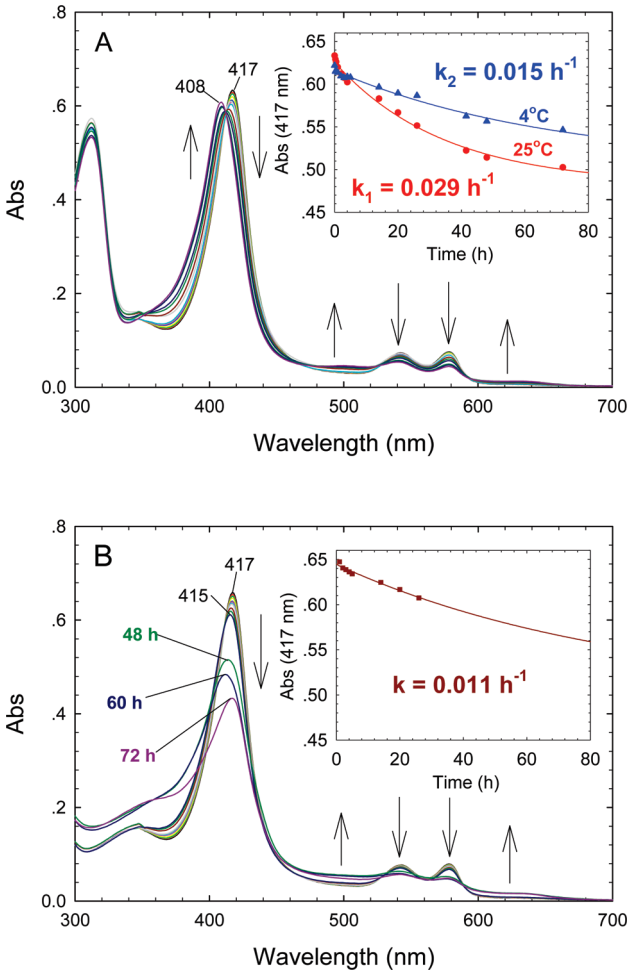

FIGURE 3: UV−vis absorption spectral change during the autoxidation of oxy-DHP (11 μM) (A) in the presence of TCP and (B) in the absence of TCP (150 μM) during 0−80 h incubation at 25 °C. The insets show the results of absorption changes monitored at 417 nm for oxy-DHP disappearance for autoxidation of oxy-DHP (10 μM) in the presence of TCP (150 μM) at 25 °C (red dot) or at 4 °C (blue triangle) and in the absence of TCP at 25 °C (brown square, only plots up to 26 h, because of the enzyme denaturation). The rate constant k is calculated based on the fit to a single exponential decay curve. The spectra were obtained in 100 mM potassium phosphate buffer (pH 7.0) at 25 or 4 °C.

(black solid line in Figure 4 inset). These results indicate that, in the presence of the organic substrate TCP, H_2O_2 is able to convert oxy-DHP to the enzymatically active ferric state.

We have previously shown that ferryl DHP species are active oxidants during the oxidative dehalogenation mechanism involving two consecutive one-electron steps (*3*). When 20 equiv of oxidant cosubstrate (H_2O_2) was reacted against a mixture of oxy-DHP in the presence of TCP (5 and 150 μM, respectively, after mixing), DHP Cpd II/II−Y· was the only intermediate observed (~40 s reaction time) (Figure 5). The spectrum of this species (Figure 5B, dashed line) closely matches our previously published data for Cpd II/II−Y· (*3*). This indicates that, in the presence of TCP and relatively high concentrations of H_2O_2 (100 μM after mixing), the conversion of ferric DHP to form Cpd II/II−Y· is much faster than the initial reaction of oxy-DHP with H_2O_2 to generate the ferric enzyme. Consequently, the ferric enzyme is not observed as an intermediate between oxy-DHP and Cpd II/II−Y· under these conditions. Upon completion of the reaction, ferric DHP was formed as a final species after 300 s reaction time (Figure 5).

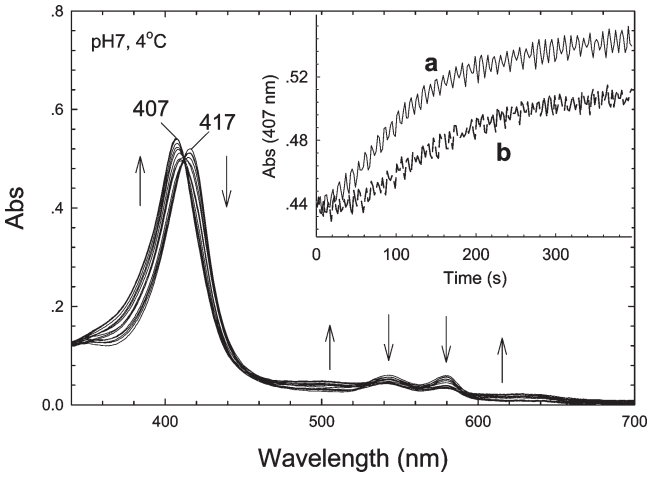

FIGURE 4: Reaction of oxy-DHP in the presence of excess TCP with 1 equiv of H_2O_2 (reaction time: 400 s). The concentrations after the mix were 5 μM DHP, 5 μM H_2O_2, and 150 μM TCP. The inset shows the results of absorption changes monitored at 407 nm for formation of ferric DHP after mixing of oxy-DHP (5 μM)/TCP (150 μM) with 1 equiv of H_2O_2 (5 μM) (solid line, upper trace a) and with 0.5 equiv of H_2O_2 (2.5 μM) (dashed line, lower trace b). The reaction was conducted in 0.1 M potassium phosphate buffer (pH 7) at 4 °C. The concentrations listed were after mixing.

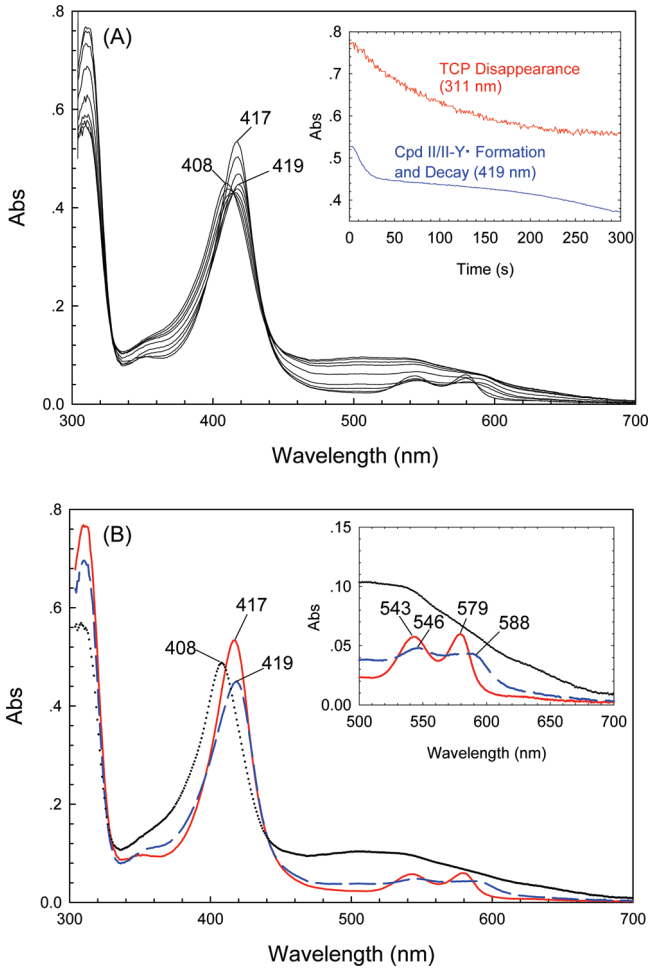

FIGURE 5: Spectral change upon addition of 20 equiv of H_2O_2 to a mixture of TCP and oxy-DHP. Rapid scan stopped-flow spectrophotometry was used in the single-mixing mode. (A) Oxy-DHP (5 μM) with TCP (150 μM) was mixed vs 20 equiv of H_2O_2 (100 μM) and allowed to react for 300 s. The concentrations listed were after mixing. The inset shows the results of absorption changes monitored at 311 nm for TCP disappearance and at 419 for Cpd II formation and decay after mixing of oxy-DHP (5 μM)/TCP (150 μM) with 20 equiv of H_2O_2 (100 μM). The reactions were conducted in 100 mM potassium phosphate buffer (pH 7.0) at 4 °C. (B) UV−vis absorption spectra of the starting oxy-DHP (red solid line), the intermediate DHP Cpd II (blue dashed line), and the final ferric DHP (black dotted line) extracted from (A). The inset shows the enlarged visible region of UV−vis absorption spectra. The concentrations listed were after mixing.

Lignin peroxidase (LiP), a well-studied heme-containing peroxidase, showed similar behavior in its oxyferrous form (*19*) to that reported herein for DHP. In fact, only 1 equiv of H_2O_2 rapidly converted oxy-LiP to the active ferric enzyme in the presence of the substrate, veratryl alcohol (*20*). Radicals of the natural substrate or analogous compounds were shown to directly cause this conversion (*21*). In those experiments, oxy-LiP was prepared by addition of excess (~50-fold) H_2O_2 with (*19*, *20*) or without (*21*) removal of the excess H_2O_2 after formation of the oxyferrous complex by desalting column chromatography. Although oxy-LiP is not involved in the normal catalytic cycle with a natural substrate, i.e., the oxyferrous state of LiP does not occur naturally, it can be formed when certain compounds such as phenols are used as substrate (*22*). Thus, the published data with oxy-LiP provide excellent precedence for our results with oxy-DHP. An important distinction for our current work is that the switch from the oxyferrous state of DHP to the ferric form serves a physiological purpose.

Based on the present findings with oxy-DHP as well as the above-mentioned reports with oxy-LiP (*17*) and related studies with oxy-HRP (*23*), Figure 6 illustrates plausible reaction mechanisms for the switch of oxy-DHP to the ferric enzyme (part A) and subsequent organic product (DBQ) formation catalyzed by ferric DHP via the normal peroxidase reaction cycle (part B, blue arrows). The switch of oxy-DHP to ferric DHP (part A) is initiated by reaction of a trace amount of the ferric enzyme, present in the oxy-DHP sample, with H_2O_2 to form Cpd I/II−Y· (part A), which oxidizes TCP to generate TCP radical (TCP·). Then, as previously seen for oxy-LiP (*19*−*22*), TCP· oxidizes oxy-DHP to the ferric enzyme (part A, red arrows) and O_2 with concomitant reduction of TCP· back to TCP without forming the product (DBQ). This process continues until oxy-DHP is completely converted to the ferric state. Thus, similar to the case of LiP (*21*), a lag time is seen for conversion of oxy-DHP to ferric DHP before product (DBQ) formation can occur (part B). The substrate (TCP) concentration-dependent lag time (Figure 1B) likely reflects the affinity of TCP to bind and form a catalytically significant complex with DHP (K_d = ~75 μM for the ferric DHP−TCP complex at pH 7.0 and 4 °C; unpublished result).

Factors that accelerate the rate of TCP radical formation such as an increase in [H_2O_2] (5 μM in Figure 4 vs 100 and 600 μM in Figures 5 and 1, respectively) are expected to accelerate the conversion of oxy-DHP to the ferric enzyme, thus shortening the lag time for product formation. To test this hypothesis, a fractional amount of exogenous ferric DHP with oxy-DHP (10% ferric + 90% oxy, in the presence of TCP) was included in a reaction against excess H_2O_2. A shorter lag time was observed for product formation (Supporting Information Figure S3, blue solid line vs red dashed line), presumably due to the generation of TCP· by the exogenous ferric DHP in the presence of H_2O_2.

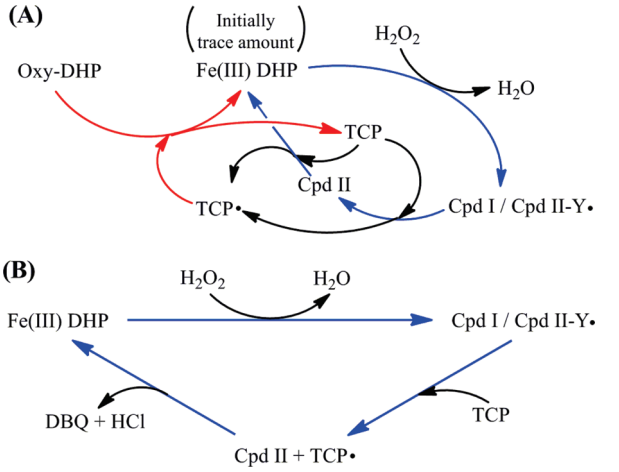

FIGURE 6: A schematic presentation of a plausible reaction mechanism for (A) substrate radical-mediated conversion of oxy-DHP to ferric DHP in the oxy-DHP/TCP/H_2O_2 system that is accountable for the lag time for (B) product DBQ formation through the normal peroxidase catalytic reaction cycle. The TCP radical-mediated conversion of oxy-DHP to ferric DHP is shown with red arrows in (A) and the normal peroxidase catalytic cycle is shown with blue arrows in (A) and (B).

CONCLUSIONS

In this report, we have provided compelling evidence that, in the presence of TCP substrate *and*, minimally, a stoichiometric amount of H_2O_2 (relative to DHP), oxy-DHP is rapidly converted to the enzymatically active ferric state. For *A. ornata* to survive in a toxic halophenol environment (*12*), it needs the ability to convert oxy-DHP to the enzymatically active ferric state. LiP has previously been shown to exhibit a similar state switching behavior directly promoted by substrate radicals (*21*), but *this is the first case where this conversion may play a physiological role*. The present findings with oxy-DHP link the O_2 carrier role of the ferrous globin protein with the peroxidase activity of the ferric enzyme in this bifunctional protein.

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SUPPORTING INFORMATION AVAILABLE

Additional experimental and results. This material is available free of charge via the Internet at http://pubs.acs.org.

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