A pH Dependant Switch in DHP Oxidation Mechanism

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Abstract

Dehaloperoxidase (DHP) is a multifunctional enzyme found in *Amphitrite ornata*, a sediment-dwelling marine worm. This enzyme possess the structure of a traditional hemoglobin enzyme and serves as the primary oxygen carrier in *A. ornata;* however, it also possesses peroxidase and peroxygenase capabilities. These secondary oxidative functions provide a remarkable ability for *A. ornata* to resist the effects of toxic metabolites secreted by other organisms that cohabit its benthic ecosystem. This study will analyze the novel catalytic switching between peroxygenase and peroxidase oxidation mechanisms employed by DHP in response to pH changes.

A pH Dependant Switch in DHP Oxidation Mechanism

The terrebellid polychaete *Amphitrite ornata* is a sediment-dwelling marine worm that inhabits coastal seawaters (Chen, Woodin, Lincoln, & Lovell, 1996). This worm cohabits its benthic ecosystem with various other infaunal marine organisms, many of which have defense mechanisms that involve the secretion of toxic metabolites including halophenols and haloindoles (Lebioda et al., 1999). Examples of marine worms that contaminate benthic ecosystems include *Notomastus lobatus*, a polychaete, and *Saccoglossus kowalevskii*, a hemichordate. These toxic compounds affect other benthic organisms through contact while burrowing or through ingestion while feeding (Chen, Lincoln, Woodin, & Lovell, 1991; D'Antonio & Ghiladi, 2011). *A. ornata* itself does not have any known chemical defense mechanisms; however, it does possess a remarkable ability to overcome these volatile compounds secreted by other benthic organisms and thrive in its environment. The secret to *A. ornata*'s toxin resistance is found in a unique, multi-functional enzyme known as dehaloperoxidase (DHP) (Osborne, Taylor, Han, Ely, & Dawson, 2004).

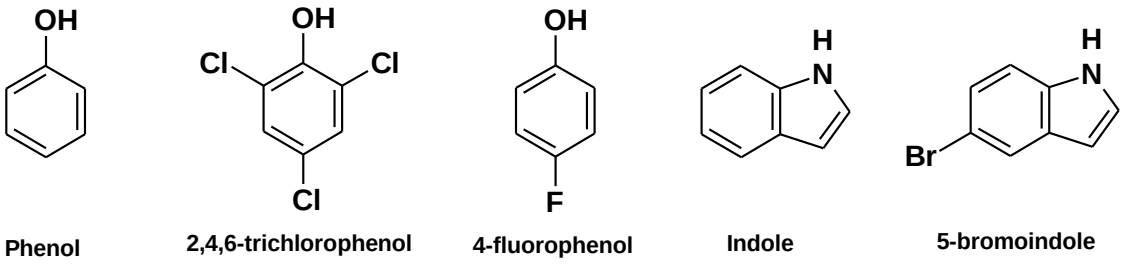

Figure 1. Phenol and Indole parent compounds and representative members of the halophenol and haloindole families of compounds. These are examples of some of the chemical defense compounds secreted by organisms that cohabit the benthic ecosystem where *A. ornata* is found.

Discovery of DHP

DHP was first discovered in the late 1970s as an intracellular coelomic hemoglobin (Weber et al., 1977). Its peroxidase and peroxygenase functions were unknown at the time, and therefore the enzyme was known only as *A. ornata*'s hemoglobin, with its lone physiological role consisting of transporting and storing oxygen molecules. At the time of its discovery, DHP was shown to possess an unusually high oxygen affinity compared to that of previously discovered hemoglobins, but relatively low compared to traditional myoglobin proteins (Weber et al., 1977). In 1995, the enzyme responsible for oxidizing toxic halogenated secondary metabolites in *A. Ornata* was purified and found to be this same heme containing enzyme now known as dehaloperoxidase (Chen et al., 1996). Through oxidative dehalogenation of toxic compounds, DHP is capable of converting these metabolites to forms that are no longer harmful to the organism and can be excreted from the body.

Initially, the oxidation of toxic metabolites by DHP was believed to be solely based on a peroxidase mechanism in which the oxygen atom to be incorporated into the metabolite was derived from water through a radical mechanism (Dumarieh et al., 2013;

Poulos & Kraut, 1980). A 2014 study found however that DHP can also perform oxidation reactions through peroxygenase or oxidase mechanisms (Barrios et al., 2014). This multifunctionality of the enzyme has allowed it to become of significant interest for research, particularly because of the seemingly contradictory conditions required for each type of reaction (Franzen, Thompson, & Ghiladi, 2012). This multifunctionality also brings to light the longstanding question regarding structure-function relationships and how proteins with very similar structures can serve very different roles in catalysis.

DHP as a Hemoglobin

DHP is classified primarily as a hemoglobin protein. Its physiological oxygen-carrying role appears to be mainly intracellular. *A. ornata* is a terebellid polychaete with a red-ringed body. The red color of the body is the result of DHP being the most abundant protein in the organism, as oxy-DHP itself possesses a bright red color. In the benthic coastal mudflats where *A. ornata* resides, the organism's body is generally buried in the mud with only its small, white tentacles extending above the mud to trap food particles and obtain oxygen. Another giant hemoglobin protein, known as erythrocruorin, is found in these tentacles (Chiancone, Ferruzzi, Bonaventura, & Bonaventura, 1981). Erythrocruorin is a multisubunit vascular hemoglobin that binds dioxygen in the tentacles, which are exposed to the water (Weber et al., 1977). This large extracellular hemoglobin is capable of moving through tissue with an oxygen molecule bound to it. Because of its ability to move between tissues, erythrocruorin has been compared to a miniature red blood cell (Franzen et al., 2012), although in reality it is a single protein. Once it has made its way to the coelom of the worm, erythrocruorin transfers the oxygen

molecule to DHP which is responsible for the storage and transport of oxygen within the cells of the worm's body.

The oxygen carrying capacity of DHP was the first function to be discovered (Weber et al., 1977). The structure of DHP has also been shown to very closely resemble that of a traditional globin, leading researchers to speculate that the oxidative functions of DHP may have arisen from the original oxygen-carrying function of the enzyme. The globin superfamily of proteins has a characteristic structure, including the 3/3 α-helical fold as found in the well-studied myoglobin (Mb) protein. DHP also possesses this typical globin folding pattern.

DHP as a Peroxidase Enzyme

Two genes (*dhpA* and *dhpB*) code for two distinct isozymes of dehaloperoxidase, known as DHP A and DHP B (Han, Woodin, Lincoln, Fielman, & Ely, 2001). DHP A and B differ from each other at only five amino acids: T9L, R32K, Y34N, N81S, and S91G (D'Antonio et al., 2010). Despite possessing a characteristic globin structure, DHP exhibits relatively little sequence homology with other globins. This however is not unprecedented considering that various proteins within the globin superfamily often possess as little as 10% sequence homology (de Serrano, D'Antonio, Franzen, & Ghiladi, 2010). Both isozymes of DHP are able to catalyze the oxidative dehalogenation of trihalophenols to dihaloquinones in the presence of hydrogen peroxide. This oxidation reaction has become known as the standard reaction for DHP; however, many other compounds, including various halophenol derivatives, have also been shown to act as substrates for DHP oxidation.

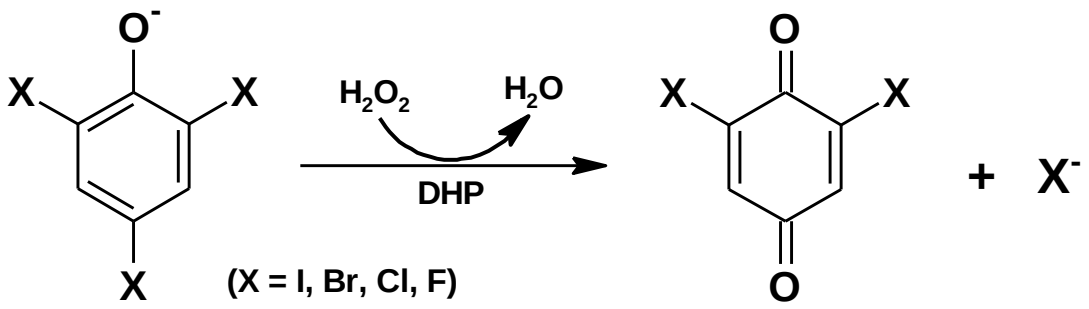

Figure 2. Peroxidase reaction converting a 2,4,6-trihalophenol to its corresponding 2,6-dihaloquinone. While various other halogenated substrates for DHP have been discovered, trihalophenols were the first to be discovered and were the substrates used in many of the studies described here.

The first oxidative dehalogenating function of DHP was found to occur via a peroxidase mechanism, making it the first hemoglobin identified to have a biologically relevant peroxidase function (Chen et al., 1996). While on the surface this appears to be a convenient multifunctionality, there exists an apparent paradox between the enzyme oxidation states required for these dual functions. The storage and transport of oxygen molecules in a hemoglobin protein requires that the iron atom in the heme group be in a ferrous (+2) state, while the oxidation of toxic metabolites via a peroxidase mechanism requires a ferric (+3) resting state for the heme iron. Until recently, there was no known mechanism by which an enzyme could alter the heme oxidation state on demand *in vivo*. Therefore, the way in which DHP carries out its dual functions remained a mystery for some time.

Several recent studies have shed light on DHP's catalytic mechanisms. A 2014 study analyzed the structural components of DHP that allow it to carry out dual hemoglobin / peroxidase functions by comparing it to a well-known hemoglobin, sperm whale myoglobin, and a well known peroxidase, horseradish peroxidase (Sun et al., 2014). The major difference between globins and peroxidases was found to be the

distance between the distal histidine and the heme group, with peroxidase activity being higher in enzymes with a longer distal histidine-heme distance (Fe-His $N^{\varepsilon 2}$). This distance is typically 5.5-6.0 Å in peroxidases and 4.1-4.6 Å in globins (Matsui, Ozaki, Liong, Phillips, & Watanabe, 1999). As expected, DHP exhibits an intermediate distance between these with the Fe-His $N^{\varepsilon 2}$ distance being 5.5 Å in ferric DHP B with H55 in the closed position (de Serrano et al., 2010), and 4.8-5.5 Å in ferric DHP A under the same conditions (de Serrano, Chen, Davis, & Franzen, 2007; LaCount et al., 2000). Further, oxyferrous DHP A has an Fe-His $N^{\varepsilon 2}$ distance of 5.1 Å, with His^{55}-O(1) (iron-bound) and His^{55}-O(2) (terminal) distances of 3.2 Å and 2.8 Å respectively (de Serrano et al., 2007).

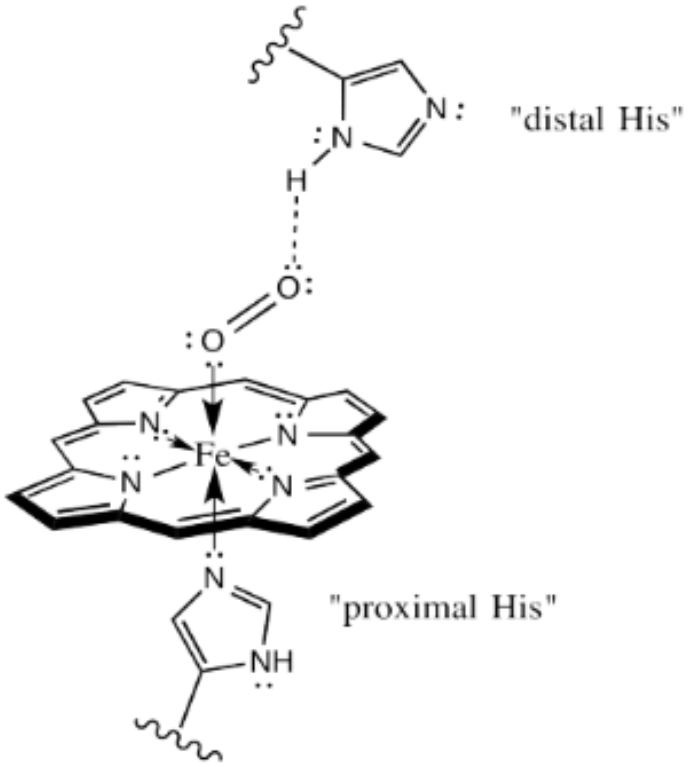

Figure 3. Histidine-Heme Configuration This structure shows a heme group with histidine amino acids on both sides of the heme face. The distal histidine is the active amino acid in the chemistry discussed for DHP. The distal histidine-heme distance (Fe-His $N^{\varepsilon 2}$) refers to the distance between the end of the imidazole side chain of the distal histidine and the iron atom at the center of the heme ring.

These Fe-His $N^{\varepsilon 2}$ distances suggest that the distal histidine of oxyferrous DHP interacts with both oxygen atoms of the head-on bound ligand in the same manner that myoglobin does. Thus the ferric state of DHP consists of a longer Fe-His $N^{\varepsilon 2}$ distance more similar to that of peroxidase enzymes, while the oxyferrous state possesses a shorter histidine-heme distance similar to oxygen-carrying proteins. This is consistent with the demonstrated multifunctionality of DHP. Peroxidase activity increases with increasing distal His distance while dioxygen affinity decreases. It is noteworthy that while the longer Fe-His $N^{\varepsilon 2}$ distance observed in ferric DHP is more similar to peroxidases than traditional hemoglobins, it is still relatively low compared to monofunctional peroxidase

enzymes. This also is consistent with observed enzyme kinetic data from DHP which shows that it is less catalytically efficient than a monofunctional peroxidase. While this does not fully address the issue regarding conflicting heme oxidation states for the two functions, it does give some insight into the way in which DHP structure reflects an intermediate between the characteristic structures of these two well known classes of enzymes (Sun et al., 2014).

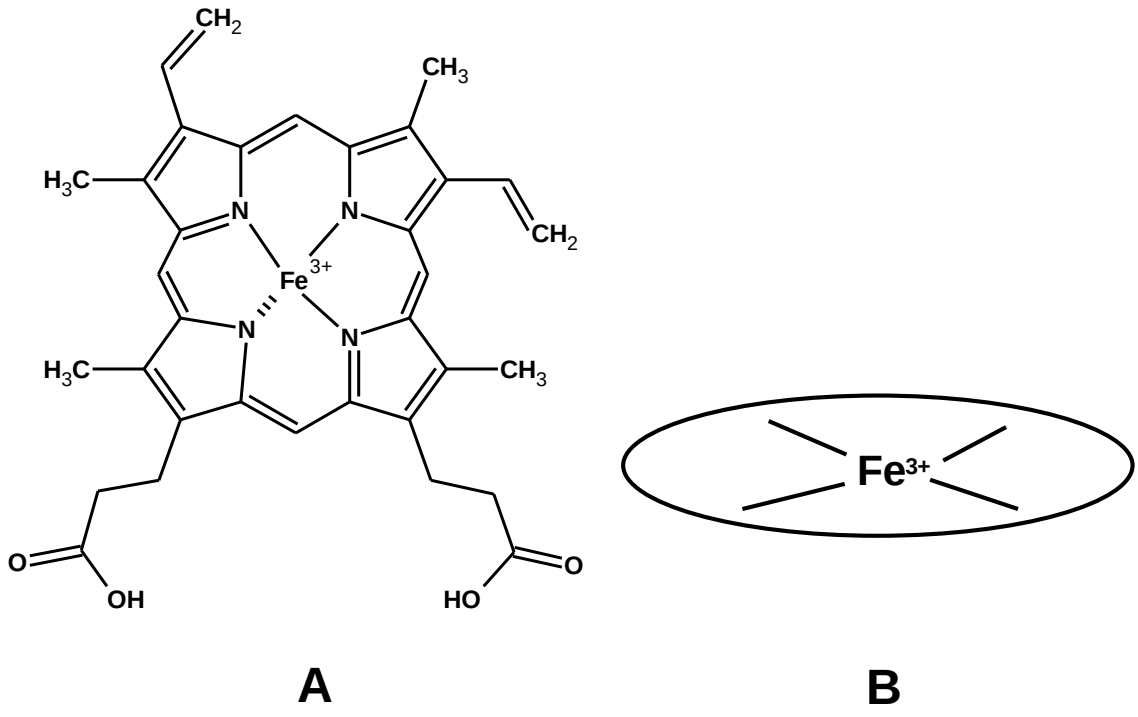

Figure 4. Ferric Heme. (A) A full heme structure is shown with a ferric (+3) iron in the center of the porphyrin ring. (B) A simplified representation of the heme porphyrin ring is shown. This representation will be used for subsequent figures to show the oxidation state and immediate environment of the heme iron.

Spectroscopic methods, including stopped-flow UV-visible and rapid freeze-quench EPR, have provided strong evidence that ferric DHP can react with H_2O_2 to produced Compound ES. Compound ES is an iron(IV)-oxo heme center with an amino acid radical (Feducia et al., 2009), as shown in Figure 5. The highly reactive Compound

ES has been shown to oxidize 2,4,6-trichlorophenol (TCP) to 2,6-dichloroquinone (DCQ) through a peroxidase-like mechanism via two discrete one-electron steps (Osborne, Coggins, Raner, Walla, & Dawson, 2009).

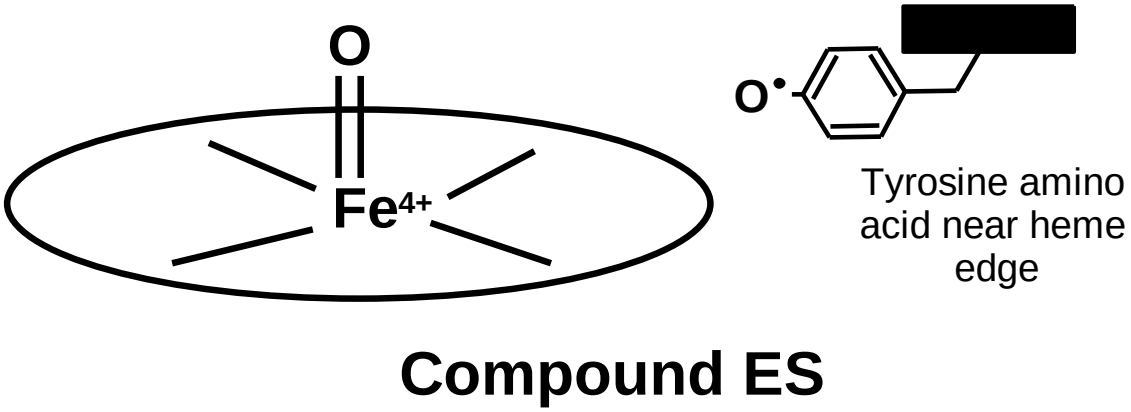

Figure 5. Compound ES. The oxidized iron is shown with its +4 charge in the center of a porphyrin heme structure. Compound ES is formally two electrons oxidized relative to the ferric state, with one oxidizing equivalent located on the heme iron and the other as a tyrosyl radical on an amino acid near the heme edge It is this amino acid radical that can be used to oxidize substrate molecules, as shown in the mechanism presented in Figure 6.

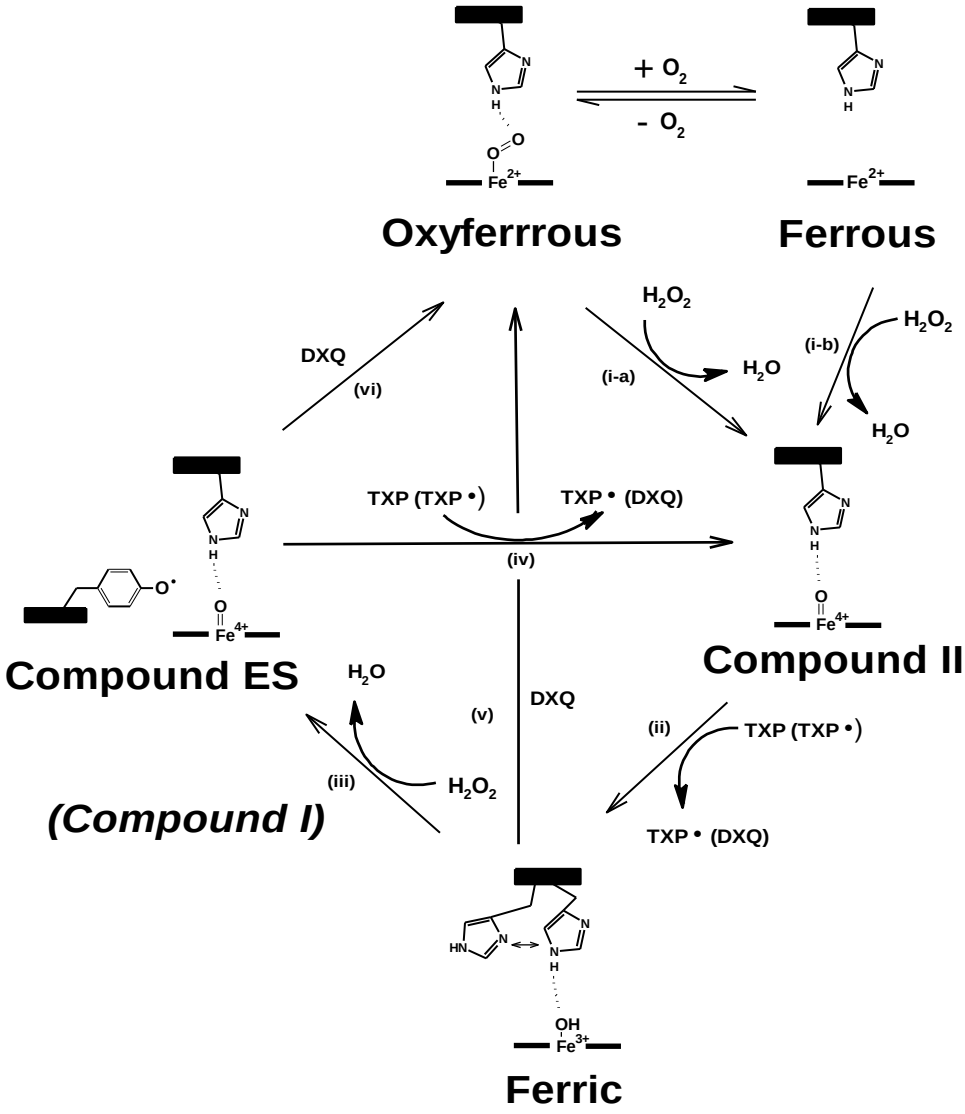

Figure 6. Proposed peroxidase catalytic cycle for Ferric and Oxyferrous Dehaloperoxidase. Peroxidase chemistry occurs in a three-step cyclical mechanism through steps ii, iii, and iv. Compound ES and Compound II are the active species which are responsible for oxidizing the substrate, and can themselves be obtained by oxidation of the ferric enzyme (step iii), or oxidation of the oxyferrous or ferrous species (step i). Product driven return to the globin-active ferrous enzyme state occurs through steps v and vi. The reaction scheme is described in detail in the text and is based on that proposed in (D'Antonio & Ghiladi, 2011).

It has further been shown that the product of trihalophenol oxidation, dichloroquinone, is itself capable of reacting with the heme center of DHP. Both ferric

DHP and Compound ES are capable of reacting with DCQ to yield oxyferrous DHP, the form capable of acting as a globin and transporting oxygen (D'Antonio et al., 2010). Spectroelectrochemistry studies have revealed that DHP has a reduction potential of +206 mV, which is remarkably high for a peroxidase. This is likely the characteristic that allows the heme group to be reduced to the ferrous state by DCQ (D'Antonio et al., 2010). This demonstrates that the product of the peroxidase reaction may act as one possible link between the oxidative and oxygen transport functions of DHP by returning the heme prosthetic group to the globin active oxyferrous state from the peroxidase active ferric state.

In the past, peroxidase catalytic cycles have always been thought to be initiated from the the ferric heme oxidation state. However, a 2011 study by D'Antonio and Ghiladi employed biochemical assays, stopped-flow UV-visible and rapid-freeze-quench electron paramagnetic resonance spectroscopies, as well as anaerobic methods to test whether the DHP catalytic cycle could possibly be initiated from the oxyferrous or deoxyferrous states under certain conditions. They found that DHP B, when combined with hydrogen peroxide, could be transformed to Compound II, a ferryl intermediate. This was found to occur independent of trihalophenol substrate when the enzyme was in the ferrous state. They also found that prior to Compound II formation, the heme species went through a ferrous-hydroperoxide precursor, which subsequently undergoes heterolysis to generate DHP Compound II. With this discovery the researchers published an updated catalytic mechanism, similar to the one shown in Figure 6 (D'Antonio & Ghiladi, 2011).

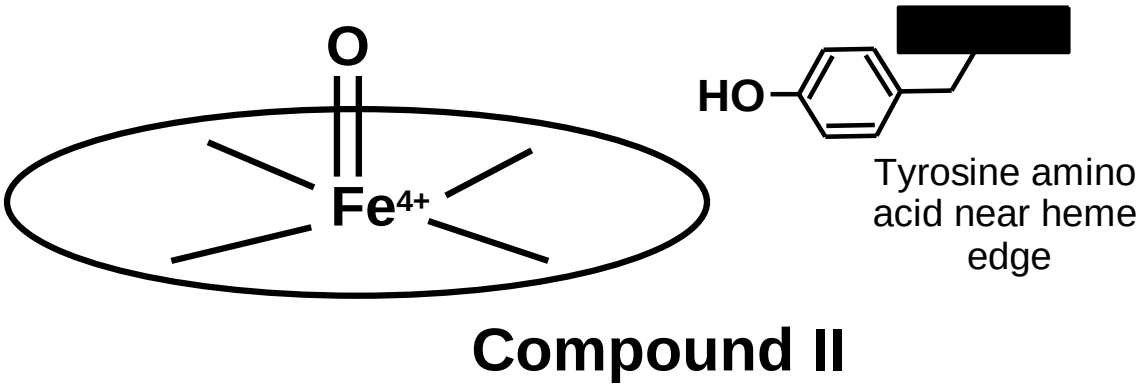

Figure 7. Compound II. The oxidized iron is shown with its +4 charge in the center of a porphyrin heme structure. The heme group of Compound II is at the same oxidation state as Compound ES however the tyrosyl amino acid does not contain the radical that is present in Compound ES. As a result, Compound II is one electron reduced compared to the Compound ES state and one electron oxidized compared to the ferric state.

As previously mentioned, the 2009 study by Osborne et al. found that the DHP peroxidase catalytic cycle could be initiated when Compound ES was intentionally introduced (Osborne et al., 2009). The results of the 2011 study by D'Antonio and Ghiladi suggest that Compound II may be transiently formed from Compound ES and thus the catalytic cycle can be initiated from Compound II. Compound II itself can also be formed from ferrous DHP by the addition of hydrogen peroxide. The oxidation of trihalophenol then occurs through the reduction of Compound II to ferric DHP (D'Antonio & Ghiladi, 2011).

When starting from the ferric heme oxidation state, DHP oxidizes its trihalophenol substrates in a manner similar to that of other monofunctional peroxidase enzymes, following a Poulos-Kraut type mechanism (Poulos & Kraut, 1980). Here, H_2O_2 reacts with the ferric heme group, oxidizing the heme and forming Compound I, an iron(IV)-oxo porphyrin π-cation radical species which is two electrons oxidized relative to the ferric state. Compound I is the principal reactive species in most peroxidases

(Dunford, 1999) and other hemoproteins (Matsui et al., 1999). In wild type DHP however, Compound I rapidly converts to Compound ES (Davydov et al., 2010). Essentially Compound I and Compound ES are both species which are two electrons oxidized compared to the ferric state, but differ in that Compound I stores both electrons on the porphyrin ring while Compound ES carries the second electron as a radical on a tyrosine amino acid near the heme (Dumarieh et al., 2013; Feducia et al., 2009). Compound ES then is a peroxidase active form of DHP and can return to the ferric form through two discrete one electron transfers, consistent with the mechanism shown in Figure 6, with the substrate being oxidized in the two step process. The following is a orderly description of the DHP peroxidase catalytic cycle combining many of the discoveries previously mentioned.

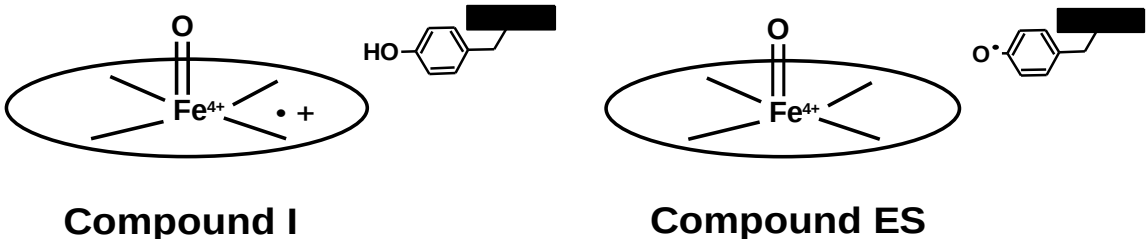

Figure 8. Compound I vs. Compound ES. A comparison between Compound I and Compound ES is shown. As described in the text, Compound I is the peroxidase active form in most peroxidase enzymes, however studies have suggested that Compound I exists only transiently in DHP and is rapidly converted to Compound ES. Both forms of the heme group are two electrons oxidized relative to the ferric state but differ in that Compound ES contains the second oxidizing equivalent as an amino acid radical near the heme edge.

Peroxidase Catalytic Cycle

One of the most influential discoveries in explaining how DHP overcomes the aforementioned paradox and serves its dual functions was provided by the 2011 study from D'Antonio and Ghiladi when they found that the peroxidase catalytic cycle could be

initiated from either the ferrous or oxyferrous state (D'Antonio & Ghiladi, 2011). The following is a plausible reaction sequence which begins with the enzyme starting in the globin-active ferrous state. Reaction numbering in the text refers to that shown in Figure 6.

Ferrous DHP reacts with H_2O_2 to produce water and in the process oxidizes the ferrous heme to Compound II, a peroxidase active species (step i). Compound II is then reduced to the ferric state, oxidizing the substrate in the process (step ii). This provides one of the two single-electron oxidations that forms a dihaloquinone (DXQ) product from the trihalophenol (TXP) substrate. Through the traditional peroxidase pathway, the ferric enzyme is again oxidized by H_2O_2, this time producing Compound ES through the transient Compound I state as previously described (step iii). Compound ES can itself provide one of the two single-electron oxidations requried to convert TXP to DXQ (step iv). The substrate for this step can either be the TXP molecule or the TXP• radical depending whether it's the first or second oxidation step for that particular substrate molecule. This oxidative step converts Compound ES back to Compound II. The catalytic cycle can then continue around through those three steps (ii-iv), fully oxidizing one substrate molecule per cycle; however, the enzyme can also return to the globin active oxyferrous state from either the ferric state (step v) or Compound ES (step vi). In these cases the product of the initial reaction, DXQ, acts as a co-substrate and returns the enzyme to the ferrous oxidation state where it can serve its oxygen transport function.

DHP as a Peroxygenase Enzyme

In 2014 another oxidative mechanism of DHP was discovered. Prior to this it was known that DHP served as an oxygen transporter as well as a peroxidase, however one

particular study was able to conclusively demonstrate for the first time that the enzyme could also oxidize substrate molecules through a peroxygenase mechanism (Barrios et al., 2014). This study used haloindoles rather than halophenols as substrates for DHP. Haloindoles are also produced by infaunal organisms living within the same benthic ecosystems as *A. ornata* (Gribble, 2000), and therefore the oxidation of haloindoles is likely another protective physiological adaptation for the organism. Isotope labeling studies showed conclusively that the oxygen atom being incorporated into the monooxygenated product was derived exclusively from hydrogen peroxide. The oxidation reactions proceeded under anaerobic conditions as well as in the presence of superoxide dimutase and radical quenchers, providing further proof that the oxidation was proceeding through a direct transfer peroxygenase mechanism rather than a radical-mediated peroxidase mechanism (Barrios et al., 2014). This study also suggested that the switch between peroxidase and oxygenase activity could be based on the steric limitations of the particular substrate molecule being used (Barrios et al., 2014). The switch between peroxidase and peroxygenase mechanisms is the focus of our present study and will be analyzed further in the following sections.

Peroxygenase Catalytic Cycle

In their report, Barrios et al. proposed a reaction scheme for DHP when peroxygenase chemistry is taking place, similar to that shown in Figure 9. The substrate used in their study was 5-bromoindole, with the major peroxygenase products being 5-bromo-2-oxindole and 5-bromo-3-oxindolenine, as shown in Figure 10.

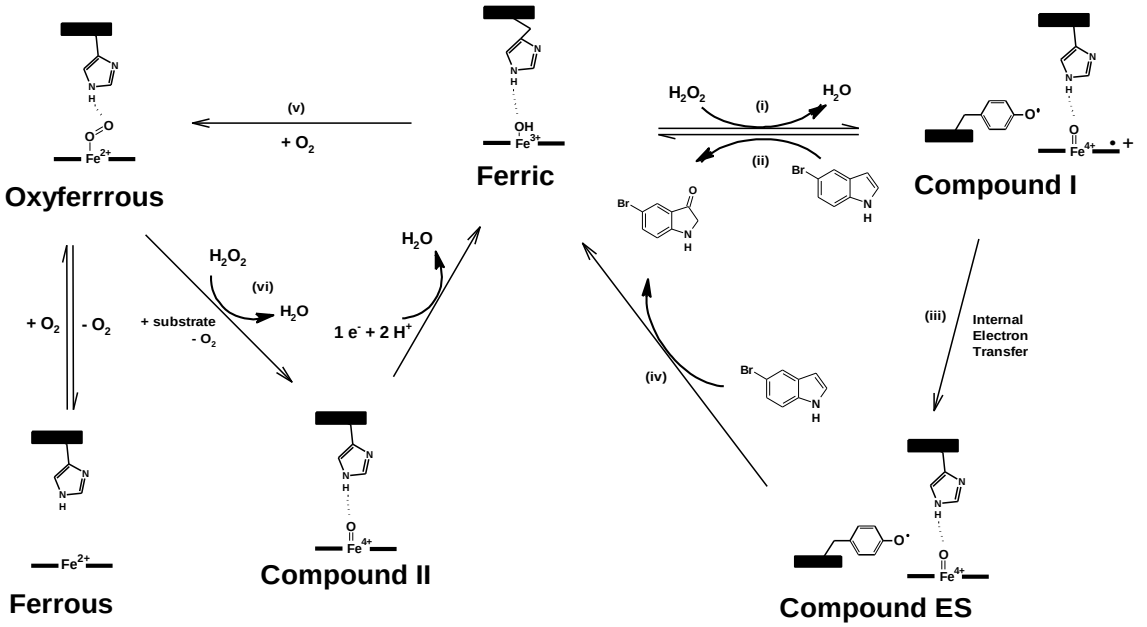

Figure 9. Proposed peroxygenase catalytic cycle for Ferric and Oxyferrous Dehaloperoxidase. Oxidation of the substrate can occur from either Compound I (step ii) or Compound ES (step iv). Both can be formed from the ferric state by the addition of H_2O_2 (step i / step i + iii). The ferric enzyme can be formed from the globin active oxyferrous enzyme through Compound II (step vi), and product-driven return to the oxyferrous state occurs in the presence of O_2 (step v). The reaction scheme is described in detail in the text and is based on that proposed in (Barrios et al., 2014).

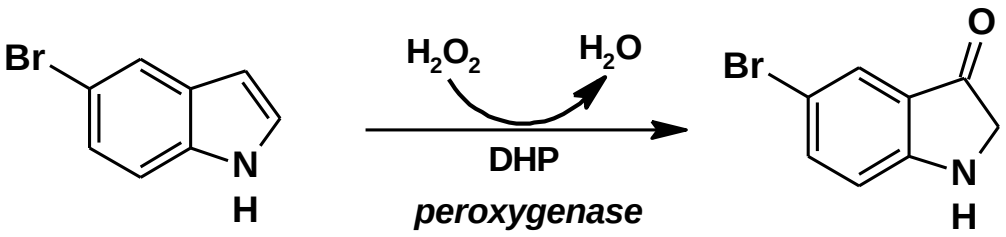

Figure 10. Peroxygenase reaction converting 5-bromoindole to 5-bromo-3-oxindolenine. This was the first DHP oxidation reaction shown to occur via a peroxygenase mechanism with the oxygen atom in the product derived from H_2O_2 as confirmed by isotopic labeling.

The scheme begins with the heme iron in the ferric state, the standard oxidation state for peroxygenase chemistry. The ferric heme is first oxidized to Compound I by hydrogen peroxide (step i). As in the peroxidase mechanism, Compound I can be modified to Compound ES through an internal electron transfer (step iii). As described previously, Compound I and Compound ES possess the same formal oxidation state, both two electrons oxidized above the ferric state, but differ in that in Compound ES the second oxidizing equivalent is present on an amino acid as a tyrosyl radical rather than on the porphyrin ring (Dumarieh et al., 2013; Feducia et al., 2009). Both DHP Compound I (step ii) and DHP Compound ES (step iv) were shown to react with and transfer an oxygen atom to the haloindole substrate forming a halo-oxindole product. In the process the ferryl heme group is reduced back to the ferric oxidation state. Similar to the product-driven switch observed in the DHP peroxidase mechanism, the major halo-oxindole product, 5-Br-3-oxindole in this case, was shown to be capable of reacting with the ferric heme leading to formation of the oxyferrous species (step v) (Barrios et al., 2014). Further, (step vi) and (step vii) show the same substrate driven activation of oxyferrous DHP to the ferric form proceeding through Compound II that was observed with TCP in the peroxidase mechanism (D'Antonio & Ghiladi, 2011).

Therefore, while peroxygenase chemistry occurs directly from the ferric enzyme oxidation state according to this mechanism, it seems that the overall resting state for DHP may be the ferrous or oxyferrous states. It has been demonstrated that any sort of oxidation chemistry, whether occurring through a peroxidase or peroxygenase mechanism, can be initiated from the ferrous heme with the enzyme going through the Compound II intermediate. Both mechanisms presented also feature cyclical pathways

whereby the oxidation chemistry, either peroxidase or peroxygenase, can continue without returning to the globin active state; however, the fact that product driven oxidation of the enzyme is observed in both mechanisms suggests that the enzyme converts back to the ferrous or oxyferrous forms relatively quickly, and that oxidative chemistry is generally initiated from the ferrous or oxyferrous state. This agrees with the notion that the oxygen carrying capacity of DHP is the primary physiological role in vivo.

A more recent study has expanded the scope of substrates which DHP is capable of oxidizing through a peroxygenase mechanism. In particular it has been shown that various deactivated phenols, particularly nitrophenols, can act as peroxygenase substrates for DHP (McCombs, D'Antonio, Barrios, Carey, & Ghiladi, 2016). While these compounds are not known to occur naturally in the habitat where *A. ornata* resides, they nevertheless present promising pathways for potential human applications, particularly with respect to the degredation of other persistent organic pollutants (POPs). For example, nitroaromatic compounds are commonly used in the synthesis of dyes, herbicides, and explosives, and are extremely toxic (Ju & Parales, 2010). As such, infaunal organisms like *A. ornata*, as well as enzymes such as DHP, represent potential solutions for oxidation and detoxification of these POPs. It seems likely that the range of substrates which DHP can oxidize is quite large, and expanding the known substrates has potential value in these applications. The study presented here uses 4-fluorophenol as a substrate for DHP oxidation. This also is a previously unreported substrate for DHP oxidation.

Structure of DHP

Many of the kinetic models discusses in the preceeding sections have been developed with the aid of supporting detailed structural studies. The first complete amino acid sequence of DHP, along with the first x-ray crystal structure, was released in 2000 (LaCount et al., 2000). It was found that DHP possess a folding pattern characteristics of globins, again suggestive of oxygen transport being its primary function in vivo. However, this and many subsequent crystal structures also present a further solution to the previously mentioned paradox between the ferrous heme oxidation state required for oxygen transport and the ferric heme state required for peroxidase and peroxygenase activity. This solution results from the discovery of an internal substrate binding site that DHP possesses, which suggests that substrate binding close to the heme edge may play a role in the switch between ferrous and ferric functional heme states. The presence of an internal substrate binding site is in contrast to most peroxidase enzymes which possess only external active sites. This suggestion makes sense given the previously described mechanisms for both peroxidase and peroxygenase activity, as it seems likely that the binding of the substrate and/or H_2O_2 initiates the switch from globin-active to peroxidase/peroxygenase active states. In terms of the efficiency with which a substrate can access the internal catalytic active site, both electrostatic (Schkolnik et al., 2013; Zhao, Rowe, Franzen, He, & Franzen, 2012) and steric effects have been observed (Nienhaus, Deng, Belyea, Franzen, & Nienhaus, 2006; Nienhaus, Nickel, Davis, Franzen, & Nienhaus, 2008).

Further, time-resolved x-ray crystallography studies have revealed a minor structural difference that DHP possesses compared to traditional hemoglobin or

myoglobin proteins. This difference is an open distal pocket that allows more than one molecule to simultaneously enter the distal pocket, allowing H_2O_2 to enter the pocket and displace an O_2 molecule being carried by the protein. The minor structural difference that facilitates this open distal pocket appears to be a small protein valve present in other globins that is lacking in DHP, thereby opening up the distal pocket enough for substrate molecules to enter (Zhao, Srajer, & Franzen, 2013).

Another characteristic of DHP that plays a role in its multifunctionality is the flexibility of the distal histidine amino acid (H55). The critical importance of this particular histidine has been demonstrated through experiments that test peroxidase activity when the histidine amino acid has been mutated to an aspartate (H55D) or asparagine (H55N) amino acid. In these cases, observed peroxidase activity in the presence of H_2O_2 decreased ~6-fold and ~11-fold for the H55D and H55N mutants respectively compared to the wild type enzyme, with 2,4,6-trichlorophenol used as a substrate (Zhao, de Serrano, et al., 2012). Thus the properties of the distal histidine, H55, in DHP are a central focus in determining the structure-function relationships that allow for its unique multifunctionality.

There are no polar amino acids aside from H55 in the distal pocket of DHP. This also is in contrast to other peroxidases which typically have at least one other polar amino acid which participates in the activation of H_2O_2 for peroxidase catalysis (Plummer, Thompson, & Franzen, 2013; Poulos & Kraut, 1980). This means that in DHP, the distal histidine is the only polar residue available to participate in the heterolytic bond cleavage of peroxide to initiate peroxidase function. The absence of other polar groups in the distal pocket allows for the internal substrate binding that has been observed. The abundance of

hydrophobic amino acids in this pocket stabilize internal substrate binding, while in other peroxidases polar amino acids in this region destabilize and prevent internal binding.

Figure 11. Crystal structure of DHP internal active site shows substrate binding in close proximity to the heme edge allowing direct transfer peroxygenase oxidation to occur. (A) View from the β-edge with 4-nitrocatechol substrate bound. Distances are shown in Å. (B) Same view as (A) after conversion of the substrate to 4-nitrocatechol. (C) and (D) show alternate views of the 4-NC product in the active site. Figure taken from (McCombs et al., 2016).

The multifunctionality of DHP does come at a cost, with catalytic efficiency being lower than in monofunctional peroxidase enzymes. A 2013 study sought to increase the catalytic efficiency of DHP for potential use as a bioremediation enzyme. The study sought to prevent the internal binding of monohalogenated phenols, which can act as inhibitors of DHP when binding internally. By blocking internal binding they would force all of the catalytic activity to be concentrated on the external active site where monohalogenated phenols such as *p*-bromophenol, are no longer inhibitors but act as substrates for oxidation. This would increase the catalytic efficiency of DHP as well as broaden the range of substrates, which are two important features of an effective bioremediation enzyme. The researchers found that they could inhibit internal binding by changing a hydrophobic leucine amino acid near the back edge of the heme, L100, to a polar amino acid, thereby creating electrostatic interference and preventing substrate or inhibitor binding at the internal site (Plummer et al., 2013). Therefore, the fact that H55 is the only polar residue in the distal pocket of native DHP shows that DHP is uniquely suited for internal binding, and that the distal histidine is sufficient for initiation of peroxidase catalysis. Further work, including our own study presented here, aims to reveal the role that the distal histidine plays in the peroxidase and peroxygenase capabilities of DHP.

Experimental Section

The aim of this study is to examine an apparent pH dependant switch of the DHP oxidation mechanism between a peroxidase and a peroxygenase mechanism. High Pressure Liquid Chromatography (HPLC) was used to preliminarily identify the ratio of products formed under different reaction conditions. The products in question likely

represent reaction mechanisms that differ in the source of the oxygen that is incorporated into the product. Future analysis including gas chromatography-mass spectrometry (GC/MS) will also be used to further identify reaction products. Isotope labeling, including the use of ^{18}O water and ^{18}O hydrogen peroxide, will also be used to determine the origin of the oxygen atom used in the oxidation of the substrate. The substrate being analyzed is 4-fluorophenol.

Materials and Methods

Production of DHP enzyme. The enzyme used in this study was produced using a bacterial expression system. *E. Coli* cells overexpressing DHP were grown and harvested. Frozen cell extract was combined with 0.1M monobasic potassium phosphate buffer in a 1:10 ratio and lysozyme powder (0.5g/100 µL cell extract) was added to break open the cells. The extract was then vortexed and mixed with a pipette before being left to stand for 1 hour. The tube containing the extract was then centrifuged at 12,500 rpm for 10 minutes, and the supernatant containing the DHP enzyme was extracted, transferred to a new tube and frozen until needed.

Enzymatic reactions. Reactions were set up with DHP enzyme, 4-fluorophenol substrate, and 100 µM potassium phosphate buffer. Reactions were completed at room temperature. Hydrogen peroxide (2.5 mM) was added to initiate the timed reactions, and 0.2 M hydrochloric acid was added to quench the reactions after a set time. Samples were then vortexed and placed on ice immediately following the reaction to ensure that enzymatic activity had completely ceased. Parameters such as substrate concentration, pH, and reaction time were adjusted in various trials. A 2-minute reaction was determined to be the optimal duration and was used for other subsequent trials.

Product identification and quantification. Following the reactions, the samples were run on a high performance liquid chromatography (HPLC) column to generate a chromatogram from which peak location and area were used to identify and quantify reaction products. The HPLC detector was set to a detection wavelength of 290 nm and a reference wavelength of 360 nm. The HPLC protocol consisted of a 10 minute run with a mobile phase of 60% deionized water (with 0.1% TFA) and 40% acetonitrile (with 0.1% TFA). The column / stationary phase was a 150 mm BDS HYPERSIL C-18 HPLC column. When available, standards such as 4-fluorocatechol were also run separately using the same protocol to aid in correctly identifying the product peaks.

Results

pH dependence of DHP reactions with 4-fluorophenol as a substrate. Figure 12 shows the HPLC chromatogram with varying pH values from 5.0-7.5. All reactions consisted of a 3:80 ratio of DHP to total volume, 100 mM phosphate buffer, and 5mM 4-fluorophenol substrate. Reactions at varying pH were controlled by using different phosphate buffers at the appropriate pH. These buffers were prepared from calculated ratios of monobasic potassium phosphate buffer and dibasic potassium phosphate buffer. Reaction were initiated by the addition of 2.5 mM H_2O_2 and allowed to run for 2 minutes before being terminated by the addition of 0.2 M HCl. The product distribution of each reaction was then analysed by running the sample through a 150 mm C-18 HPLC column with a mobile phase consisting of 60% deionized water (with 0.1% TFA) and 40% acetonitrile (with 0.1% TFA).

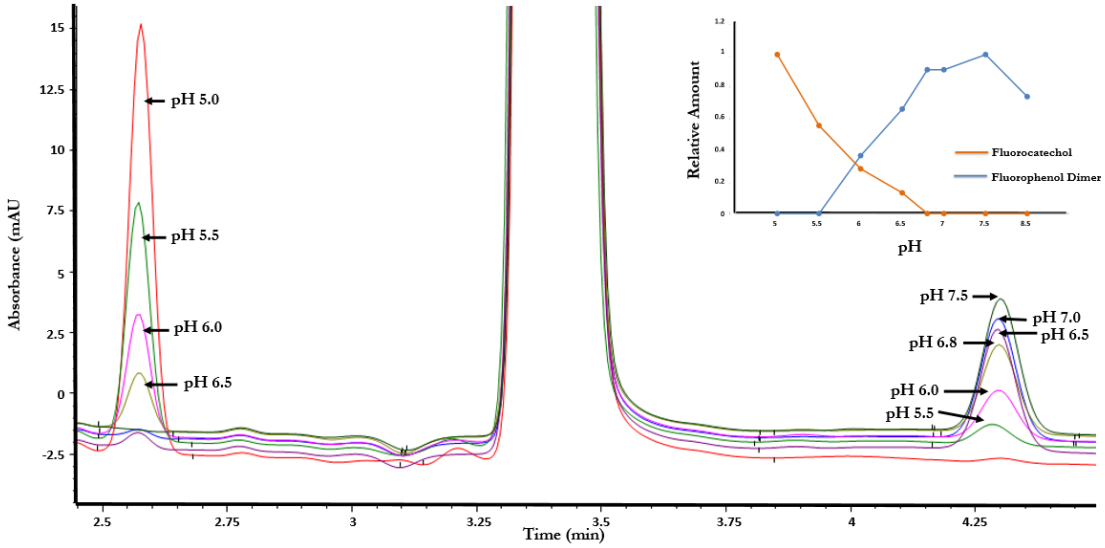

Figure 12. pH dependant change in DHP oxidation product with 4-fluorophenol as a substrate. HPLC chromatogram showing an overlay of 7 different reactions with pH values ranging from 5.0-7.5. There is a distinct decrease in 4-flurocatechol production as pH increases, and a second product, presumably a fluorophenol dimer, as pH increases. The inset shows relative peak areas for each product as a function of pH, and suggests that the pKa for the enzyme residue responsible for the catalytic switching is approximately 6.

The product formed optimally at pH 5.0 is 4-fluorocatechol (retention time ~ 2.6 min), as determined by running a 4-fluorocatechol standard under identical conditions. The production of 4-fluorocatechol decreases as pH increases, while a second product elutes at approximately 4.3 minutes and increases in quantity as pH increases with an optimum production at pH 7.5. For clarity, trials run at pH 8.0 were omitted from the figure as the production of the second product actually decreases relative to the pH 7.5 trials. This suggests that the 4.3 minute product is produced optimally at higher pH values, however enzyme activity as a whole likely decreases above pH 7.5. While the identity of this second product has not been confirmed by running a standard, it is presumed to be a fluorophenol dimer for reasons that will be discussed in the following section.

The inset in Figure 12 shows the relative amounts of each product as a function of pH as calculated from the peak areas in the chromatogram. This shows once again how the relative amounts of each product varies with the pH of the reaction. The trial at pH 8.0 is included in the inset and shows that the relative amount of product decreases relative to the pH 7.5 trial. It seems likely that the enzyme begins to denature at this high pH. The relative amounts of the two products are equal at approximately pH 6.0, suggesting that the enzyme residue responsible for the catalytic activity, presumably the shifting of H55, has a pKa around 6.

Kinetic data for the two products of DHP catalysis with 4-fluorophenol substrate. A Michaelis-Menten curve for the production of 4-fluorocatechol was generated using substrate concentrations ranging from 2-15 mM (Figure 13A). Reactions were run using a 3:80 ratio of DHP, 100 mM phosphate buffer at pH 5.0, and varying concentrations of 4-fluorophenol substrate. Reactions were again initiated by the addition of 2.5 mM H_2O_2, allowed to run for 2 minutes, and terminated by the addition of 0.2 M HCl. Product distribution was again monitored by running the sample through a 150 mm C-18 HPLC column with a mobile phase consisting of 60% deionized water (with 0.1% TFA) and 40% acetonitrile (with 0.1% TFA). Product quantity was plotted and analyzed. The Michaelis-Menten curve for the production of 4-fluorocatechol showed a K_m value of 6.0 mM and a V_{max} of 82 AU/min. A similar analysis for the production of the second product at pH 7.5 was also performed. A Michaelis-Menten curve for this product was generated using substrate concentrations ranging from 0.5-20 mM (Figure 13B), and showed a K_M value of 1.9 mM and a V_{max} of 59 AU/min.

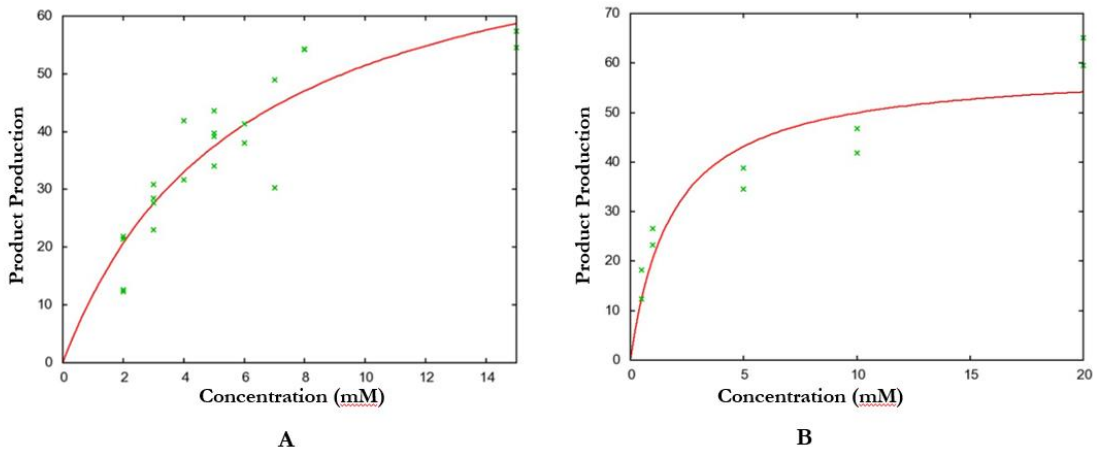

Figure 13. Michaelis-Menton Curves for DHP with 4-fluorophenol as a substrate. The Michaelis-Menton curves were generated by performing reactions with varying substrate concentration. (A) 4-fluorocatechol was produced at pH 5.0 using 10 μL DHP. The K_M value was determined to be 5.94 mM and the V_{max} 82 AU/min. (B) A second product was produced at pH 7.5 using 15 μL DHP. The K_M value was determined to be 1.86 mM and the V_{max} 59.2 AU/min.

Discussion

It is clear that the formation of each product is pH dependent; however, the chromatogram and kinetic data on its own does not show why exactly this occurs. We predict that this represents a pH dependent switch in oxidation mechanism. It has previously been shown that DHP is capable of oxidizing substrates through either a peroxidase or a peroxygenase mechanism; however, this has never been tested using 4-fluorophenol as a substrate. We believe that the formation of 4-fluorocatechol at pH 5.0 is the result of a peroxygenase mechanism, while the formation of the second product at a higher pH is the result of a peroxidase mechanism. If true, this would represent a new substrate which DHP can oxidize through a peroxygenase mechanism. Further, while both peroxidase and peroxygenase mechanisms have previously been shown for DHP, it has yet to be shown that the enzyme can switch between the mechanisms for a single

substrate. Further, DHP appears to be the first enzyme which can switch between catalytic mechanisms based solely on the pH of its environment.

In their 2016 study demonstrating that nitrophenol can act as a substrate for the peroxygenase oxidation mechanism of DHP, McCombs et al. reported higher catalytic turnover at pH 5, with decreasing nitrocatechol production at higher pH values (McCombs et al., 2016). Their study used isotope labeling to show conclusively that the reaction converting nitrophenol to nitrocatechol was the result of a peroxygenase mechanism with the oxygen atom incorporated into the product being derived from H_2O_2. This suggests that our own reaction converting fluorophenol to fluorocatechol is proceeding through the same mechanism. The 2016 study did not offer a suggestion as to why production decreased at higher pH values or that there might be a pH dependant switch between peroxygenase or peroxidase mechanisms, which is what we intend to show here.

Figure 14 presents our proposed reaction sequence for both mechanisms of fluorophenol oxidation. As described above, it seems likely that the production of flurocatechol at pH 5 is the result of a direct transfer peroxygenase mechanism with the oxygen atom used to oxidize the substrate derived from hydrogen peroxide (Figure 14 - reaction A). Future work will include isotope labeling studies using O^{18} water and hydrogen peroxide, followed by GC/MS analysis of the fluorocatechol product to conclusively determine the origin of the oxygen atom incorporated into the product.

We believe that the reaction at higher pH values is resulting in the production of a fluorophenol radical through a peroxidase mechanism (Figure 14 – reaction B). It is very plausible that the unstable fluorophenol radical produced at pH 7.0 can combine to form a

more stable fluorophenol dimer, which we believe we are isolating as our 4.3 minute product (Figure 14 – reaction C). The reaction of the radical with water to produce a quinone product is also characteristic of peroxidase mechanisms, and this would be the true oxidation product we are seeking (Figure 14 – reaction D). However, the quinone product likely breaks down very rapidly and therefore is not isolated in our chromatograms. We predict that the addition of glutathione to the reaction mix could produce a quinone-glutathione adduct which we could then isolate and identify using HPLC (Figure 14 – reaction E), as described by (Harkey, Kim, Kandagatla, & Raner, 2012).

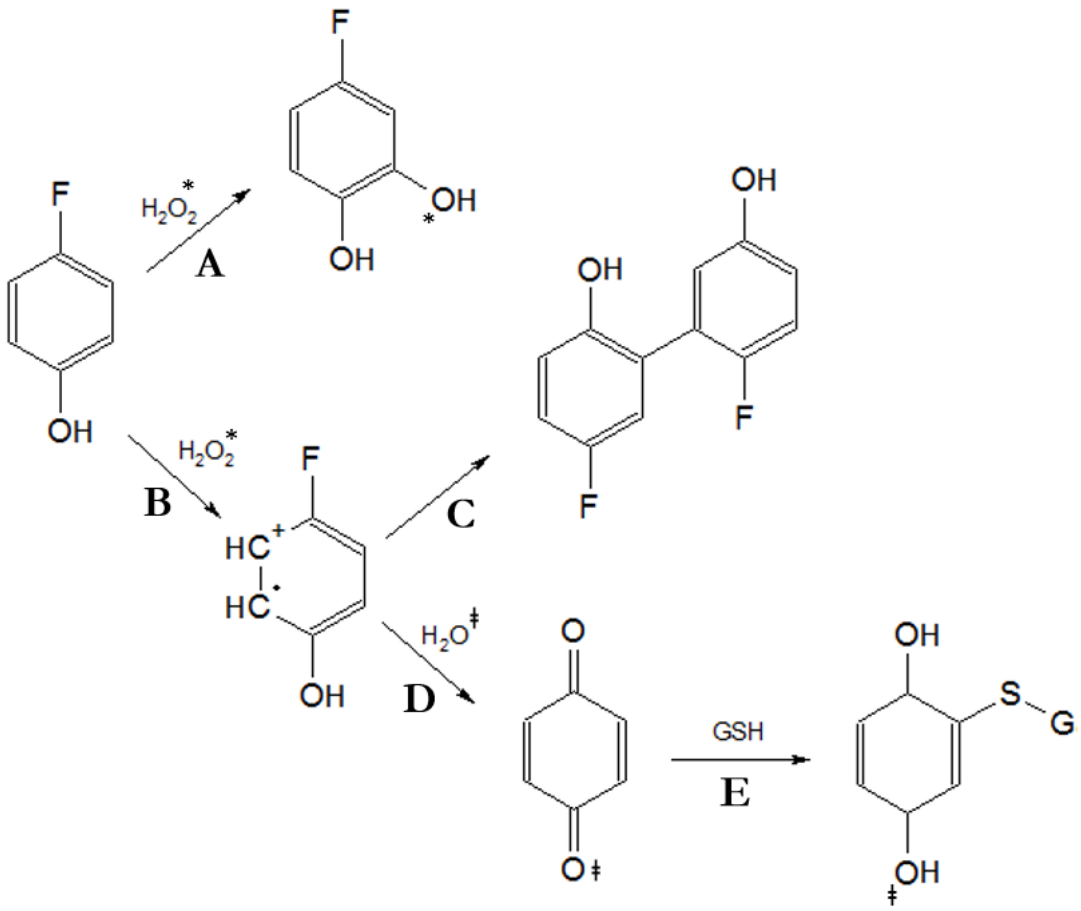

Figure 14. Proposed reaction products when 4-fluorophenol is oxidized by DHP. (A) The product favored at pH 5.0 has been shown to be 4-fluorocatechol. (B) We predict that at pH 7.0 the favored mechanism involved the formation of a fluorophenol radical. (C) The product we see in our HPLC chromatograms at pH 7.0 is believed to be a fluorophenol dimer. This formation can be easily explained by the combination of two of the fluorophenol radicals produced in step B resulting in the stable dimer. (D) We predict that a quinone is also produced at pH 7.0, however it is unstable and breaks down rapidly and thus is not identifiable in the chromatogram. (E) Future work will attempt to stabilize the quinone intermediate by adding glutathione (GSH) to the reaction. We predict that this will result in the product shown here.

Figure 15 presents some of the reasoning behind the proposed dual mechanisms of DHP oxidation. These crystal structures show the distal histidine residue near the heme group that can flex inward or outward. We believe that at a lower pH, this histidine becomes protonated and positively charged, causing it to be attracted to the negatively

charged propionate groups surrounding the active site. This outward flexing of the histidine then allows the required space for the substrate to directly access the heme center and be oxidized through a direct transfer mechanism (peroxygenase). By contrast, at higher pH values, H55 loses its proton and adopts a neutral charge. In this condition the histidine amino acid tends to swing back over the heme edge, interacting with the iron atom at the heme center. This is a much more typical position for the distal histidine in peroxidases and oxygen transporters. The substrate molecules can then no longer directly access the heme edge, eliminating the possibility of direct transfer peroxygenase oxidation. In this case DHP seems to proceed to oxidize the substrate through a radical-mediated peroxidase mechanism. The suggestion that the histidine amino acid is responsible for the switch in catalytic mechanism also makes sense given the data shown in Figure 12, where the catechol product is produced optimally at pH 5.0 and the secondary product is produced optimally at pH 7.5, with the switch in the relative amounts of each product occurring at pH 6.0. The pKa for the imidazole side chain of histidine is generally around 6.0, explaining why it becomes protonated around this pH and swings to its outward conformation in DHP.

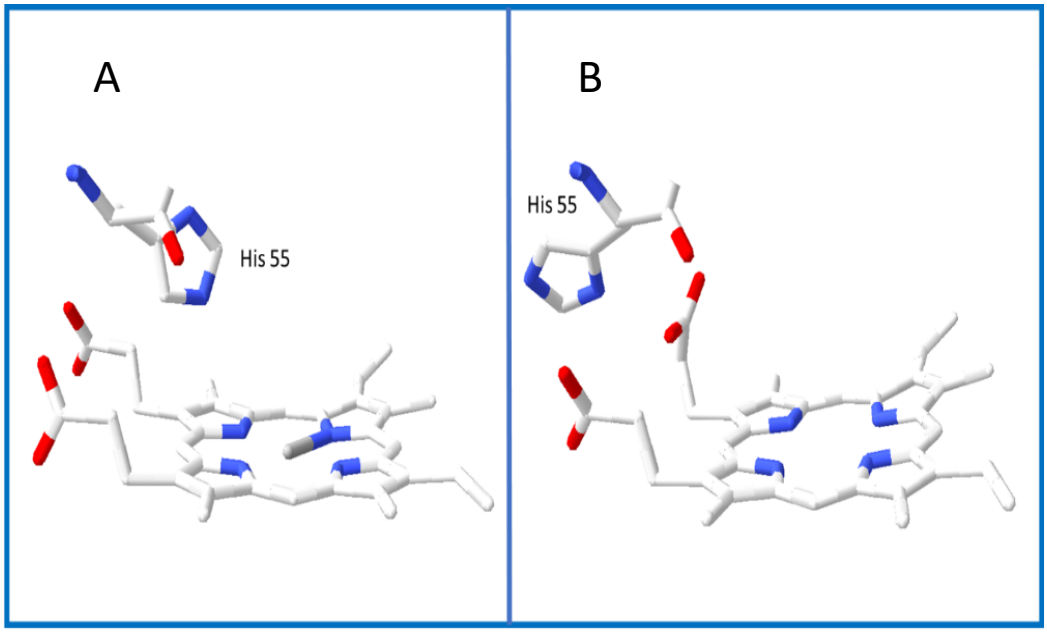

Figure 15. Active site His-55 with respect to the heme prosthetic group. This image is generated from dehaloperoxidase crystal structures (A) with 4-nitrophenol bound (5CHQ) and (B) in the substrate free ferric or oxyferrous forms (2QFK and 2QFN).

Conclusion

In conclusion, various studies have demonstrated the unique multifunctionality of DHP. The globin protein found in the benthic marine worm *Amphitrite ornata* is the primary oxygen transporter within the coelom of the worm. It has also been shown to possess oxidation capabilities that allow the organism to survive in an environment contaminated by toxic metabolites secreted by other organisms. The oxidation of these toxins can proceed through either a radical-based peroxidase mechanism with the oxygen atom incorporated into the product derived from water, or a direct transfer peroxygenase oxidation mechanism with an oxygen atom from hydrogen peroxide incorporated into the product.

Our study presented here has expanded the substrate scope for which DHP oxidation is possible by demonstrating that 4-fluorophenol is converted to 4-fluorocatechol by DHP at pH 5.0. Comparison to previous studies suggests that this is the result of a peroxygenase mechanism. A second product is produced in increasing quantity as pH increases which we predict to be the result of peroxidase oxidation of 4-fluorophenol. Future work, including mass spectrometry, will be necessary to fully confirm this. If true, this represents a change in catalytic mechanism based on the pH of the environment that has yet to been shown for any enzyme. Potential applications would include catalysis within the fields of bioremediation and the management of environmental toxins. The potential to control the mechanism of substrate oxidation on the basis of pH is attractive because it increases the precision with which we can complete the desired chemistry. For example, peroxidase catalysis is inherently unpredictable due to the fact that it proceeds through radicals, while peroxygenase catalysis avoids this issue. Overall, DHP represents a very intriguing possibility for many applications, and future work to better understand the structure-function relationships that allow for its unique multifunctionality would be advantageous.

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