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Review

The dehaloperoxidase paradox

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ABSTRACT

The dual functions of the dehaloperoxidase-hemoglobin of Amphitrite ornata leads to a paradox. Peroxidase and hemoglobin functions require ferric and ferrous resting states, respectively. Assuming that hemoglobin function is the dominant function, the starting point for peroxidase activation would be the oxyferrous state. Activation of that state leads to the ferryl intermediate, followed by one-electron oxidation of the substrate, which results in the ferric state. Since no exogenous reductant is known, there is no return to the ferrous form or hemoglobin function. The observation that an internal binding site for 4-bromophenol leads to inhibition leads to a further paradox that the enzyme would be inhibited immediately upon activation under ambient conditions in benthic ecosystems where the inhibitor, 4-bromophenol is present in greater concentration than the substrate, 2,4,6-tribromophenol. In this review, we explore the unresolved aspects of the reaction scheme that leads to the apparent paradox. Recent data showing activation of the oxyferrous state, an extremely high reduction potential and exogenous reduction by the 2,6-dibromoquinone product present a potential resolution of the paradox. These aspects are discussed in the context of control of reactivity radical pathways and reactivity by the motion of the distal histidine, H55, which in turn is coupled to the binding of substrate and inhibitor.

1. Introduction

Hemoglobin cooperativity and associated allostery provide an excellent example of the dynamic response of proteins to their environment. The quaternary structure of hemoglobin changes in response to oxygen tension, and the binding constant of each heme iron in the $\alpha\beta$ heterodimer of human hemoglobin is modulated by the ligation state of the others. This regulation is characterized by changes in the iron coordination from six-coordinate (oxygen bound) to five-coordinate (deoxy) and an associated change in the interaction of protein subunits that lead to relaxed (R) to tense (T) structure change. The entry of oxygen into hemoglobin and myoglobin itself requires protein fluctuation to open a channel. In their 1990 article on the crystal structure of myoglobin expressed from a synthetic gene, Sligar and co-workers refer to a paradox in myoglobin function, which has been known since the time of the first myoglobin X-ray crystal structure. The oxygen is bound to the heme iron, but there is no path for the diatomic oxygen ligand to enter or exit the protein [1].

Several X-ray crystal structures of a unique hemoglobin, known as dehaloperoxidase-hemoglobin (DHP), clearly establish that molecules as large as 4-iodophenol (4-IP) can enter and exit a protein cavity [2–6]. In light of the discovery that 4-IP enters and exits from DHP, the requirement for protein conformational changes would appear to be greater in DHP than in other hemoglobins. This is certainly not a paradox in the modern thinking about protein dynamics since there are now crystal structures of a number of large molecules in the distal pocket of proteins. However, in globins large molecules that enter the distal pocket are always observed bound to the heme iron. We can compare the entry and exit of 4-BP in DHP to the binding of benzhydroxamide in type C horseradish peroxidase, which is not bound to the heme iron [7]. The difference between behavior typical of globins and peroxidases must be rationalized in a single protein when considering structure and function of dual function proteins such as DHP.

DHP, isolated from the marine annelid *Ampihtrite ornata* naturally functions as both a globin and a peroxidase and thus carries out two functions that are in opposition in a way that presents a paradox for both structure and function. On the physiological level, the conditions required for the dual function protein are apparently contradictory. Globin function, which comprises O_2 transport and storage, requires ferrous (Fe^{2+}) iron. Peroxidase function, which comprises oxidation of phenolic substrates, requires ferric (Fe^{3+}) iron. In hemoglobin, the oxy form is the active form that carries its cargo to tissues that need oxygen, but in a peroxidase, the oxy form is called compound III and is an inactive species.

A. ornata is classified as a terebellid polychaete. It has a red-colored ringed body that is buried in the mud flats common in benthic coastal ecosystems. The red color is attributable to DHP, the most abundant protein in the organism. Two genes encode for DHP in *A. ornata*, dhpA and dhpB [8]. The coelom is the center portion of the ringed body of the worm, where DHP A and/or B function as oxygen storage and transport proteins. In addition to its ringed body, *A. ornata*

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possesses small spaghetti-like white tentacles that extend above the surface of the mud that allow A. ornata to catch food particles. A second, giant Hb (3.6 MDa) known as the erythrocruorin is located in the tentacles [9]. Many organisms in benthic ecosystems produce brominated compounds as repellents to protect against predators. Specifically, the compounds 2,4,6-tribromophenol (2,4,6-TBP) and 4-bromophenol (4-BP) are prevalent in the shallow coastal waters where *A. ornata* is found. The function of DHP is the oxidation of 2,4,6-TBP to 2,6-DBQ to reduce the concentration of the toxic phenols in A. ornata as shown in Fig. 1. One of the motivations for this review was the discovery of an interaction between the two commonly found molecules, 2,4,6-TBP and 4-BP. 2,4,6-TBP is the known substrate for DHP, and we have recently shown that 4-BP is an inhibitor [10,11]. The ratio of the abundance of these molecules is such that the concentration of 4-BP is twice that of 2,4,6-TBP [12]. The inhibitor appears to be key to understanding DHP function. The mechanism of inhibition links back to the same protein dynamics that allow molecules like 4-IP and 4-BP to enter into DHP. Otherwise, the inhibitor, 4-BP, would be a substrate if it did not bind on the interior of the enzyme. When it binds inside it pushes the distal histidine to a solvent exposed position, which appears to reduce the affinity for substrate. This dynamic aspect of DHP appears to be linked to the peroxidase function or the switch between the hemoglobin and peroxidase functions.

In this review, we will first provide background on the structure and genetics of the two isoforms of DHP in Section 2. Then, in Section 3, we will state the DHP paradox, which derives from the incompatible redox states of the hemoglobin and dehaloperoxidase functions. Next we discuss the effect of inhibition of DHP by 4-BP on observed kinetics. We also justify the hypothesis that DHP is a peroxidase by consideration of its highly specific inhibition and show that the inhibitor and substrate play opposing structural roles in the protein (Section 4). In Section 5 we consider the key amino acid, the distal histidine (H55), that is affected by substrate and inhibitor binding. H55 is also the switch that governs the fate of protein radicals in DHP. Thus, Sections 6 and 7 discuss the fate of radicals in the protein and ultimate inactivation of DHP by a radical pathway, respectively. In Section 8 we combine all of the latest information to provide the most comprehensive mechanism of DHP activity available. Section 9 treats the pH dependence of this new mechanism. The fate of phenoxy radicals in solution, which are produced by peroxidase chemistry is discussed in Section 10. All of these radicals are inherently harmful to the cell, which accentuates the physiological aspect of the DHP paradox.

A hemoglobin is a protein designed to be an inert reversible binding site for oxygen and to carry out as little chemistry as possible. A peroxidase is an enzyme that catalyzes the cleavage of the O–O bond of bound H_2O_2 and oxidizes substrates, usually by a radical mechanism. Radicals do not need to be harmful to the cell. Indeed, radicals such as ascorbate reduce the burden due to radicals produced during photosynthesis. Ascorbate peroxidases oxidize ascorbate, which then functions as a radical scavenger that protects the cell from other radicals. However, the function identified for DHP is more like the secretory peroxidases, which generate highly reactive radicals with the potential for great harm to the cell. Thus, in Section 11 we conclude with a discussion that places the DHP mechanism in the context of other heme proteins and consider the possible roles that radical chemistry may play in the resolution of the DHP paradox.

2. Structure and function of the two isoforms DHP A and B and their mutants

A. ornata is able to oxidize a wide variety of mono-, di-, and trisubstituted halophenols that possess bromine, chlorine, or fluorine substituents [13] due to the production of dehalogenating enzymes that allow it to tolerate such environmental haloaromatic toxins. Both isoforms of DHP possess a broad substrate specificity for the oxidation of the trihalophenol [13,14], and are therefore the first globins identified to possess a biologically relevant peroxidase activity (Fig. 1) [13]. DHP binds dioxygen ($P_{50} = 2.8$ Torr) that is delivered to the coelom by the extracellular, multi-subunit vascular erythrocruorin ($P_{50} = 11$ Torr) [15]. Hb phylogeny reveals a common genetic ancestry across species from bacteria to plants and animals extending back 1.8 billion years [16,17]. However, despite DHP being categorized as a globin according to the Structural Classification of Proteins (SCOP) database [18], DHP has little sequence homology to other known Hbs. The X-ray data [2–5] show that DHP has a globin structure and a histidine proximal ligand in contrast to the cysteinate proximal ligand in cytochrome P450 (monooxygenase) and chloroperoxidase (dechlorination), two enzymes that have superficially similar functions [2,6,19–22].

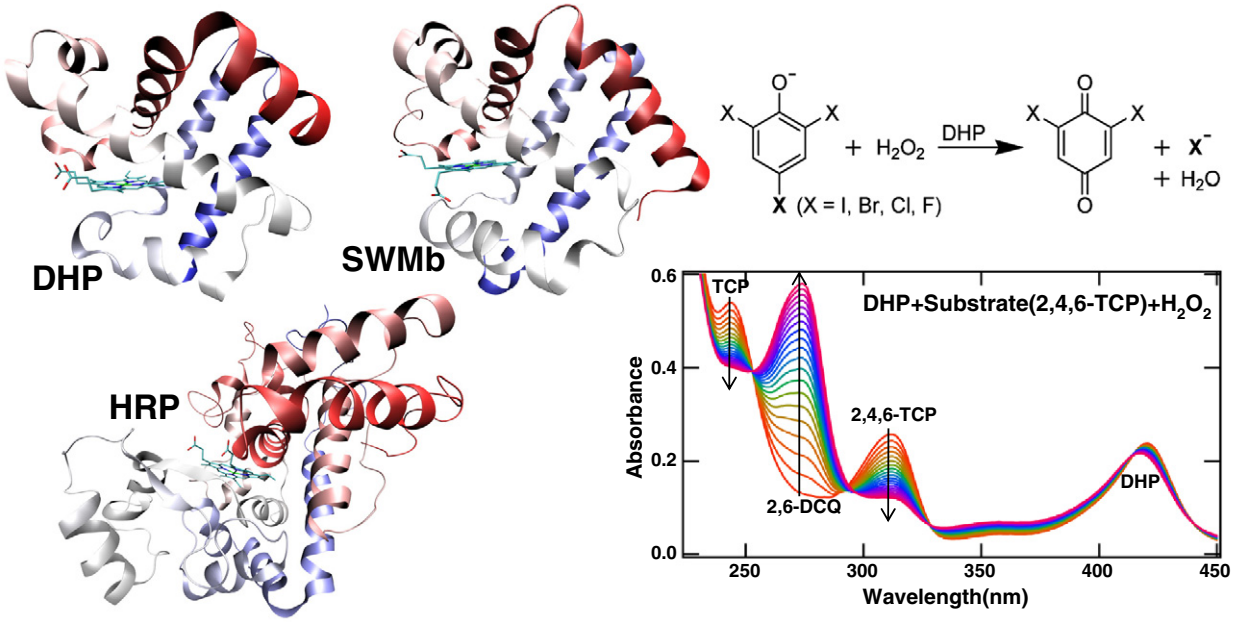

Fig. 1. Structures of globins DHP and Mb and horseradish peroxidase (HRP) from the CcP family. The biological oxidation reaction is shown with a typical spectrum that can be obtained using bench top mixing (from Ref. [10]).

Moreover, DHP bears little resemblance to the fold of cytochrome c peroxidase (CcP), the prototype for the heme peroxidase family as shown by the comparison with HRP in Fig. 1 [23]. DHP is an example of a bifunctional protein in which there is an economy of structural elements that carry out two disparate functions. Moreover, the fact that globins and peroxidases have ferrous and ferric resting states, respectively, poses a fundamental functional question whose resolution has general implications. Thus, as a representative of globins found in marine organisms, its relationship to other Hbs and peroxidases may aid in establishing the scientific foundation of protein structure–function relationships specific to bi/multi-functional proteins and further our understanding of marine peroxidases in relation to those of mammalian, plant, or bacterial origins.

The two DHP genes in *A. ornata*, dhpA and dhpB [8] share only moderate sequence homology with a handful of other marine organisms. However, many marine organisms have two types of hemoglobins similar to DHP. One is the intracellular storage hemoglobin, the other is a giant extracellular hemoglobin, also known as erythrocruorin. The giant hemoglobins are a little like miniature red blood cells, capable of moving through tissue and transporting oxygen. It is not yet clear how, if at all, the two intracellular storage hemoglobins (DHP A and B) relate to the giant, erythrocruorin. To begin the process of testing the various functions of the two hemoglobins, we have made DHP B by mutating the 5 amino acid residues in the DHP A gene that differ from DHP B, T9L, R32K, Y34N, N81S, and S91G [24]. An unanticipated result is that DHP B acts as an even better dehaloperoxidase than DHP A. Thus, both isoforms of dehaloperoxidase (DHP A and B) can function as both hemoglobins (Hbs) [15] and peroxidases [13,25,26], and both likely play the role of a protectant against the bromophenols secreted by other organisms as repellents in the benthic ecosystems [12,27,28].

The path that led to the discovery of the dehaloperoxidase function of DHP A justifies the physiological aspects of the hypotheses regarding DHP function. The oxy-DHP proteins are so abundant in A. ornata that they are responsible for the reddish color of the organism [9,15]. Independently, the dehaloperoxidase function was determined by fractionation of A. ornata to determine which component of the organism was capable of degrading the bromophenols [13]. Although DHP A was identified as a peroxidase in those experiments, the X-ray data show that DHP A and DHP B both have a globin structure. Neither DHP A nor DHP B bear resemblance to the fold of cytochrome c peroxidase (CcP). [23]. Thus, DHP A and B appear to be true bifunctional proteins that can both transport oxygen (globin function) and oxidize substrates like 2,4,6-TBP (peroxidase function). This combination of functions presents a mechanistic paradox since the activation of the DHP for peroxidase function by addition of H_2O_2 inherently alters its oxidation state, leading ultimately to loss of function. In the next section we pose the dehaloperoxidase paradox.

3. The DHP paradox

DHP was originally discovered as a hemoglobin and characterized most completely in 1977 as a monomeric coelomic oxygen carrier and storage protein [15]. Then, DHP was rediscovered in 1996 as a dehaloperoxidase [13,29]. It would appear that the requirement for ferrous iron in the globin function is at variance with the requirement for ferric iron in the peroxidase function because of the requirement for different redox states of the heme. DHP appears to be a stable ferrous heme protein. Aside from the abundance in the organism and the obvious role as an oxygen transport protein, the redox potentials of DHP A and B were determined to be +204 and +206 mV [24], respectively. This unusually high value for a globin represents a shift in the opposite direction of that required for peroxidase function. The autooxidation rate of DHP is correspondingly very low. The ferrous form of DHP is significantly more stable than in any other known hemoglobin or myoglobin, with the exception of the giant extracellular hemoglobin in Lumbricus. [30] These considerations appear to contradict the observation that originally led to the discovery of peroxidase function in DHP, namely a high rate of substrate oxidation in the ferric form in the presence of H_2O_2 and substrate, 2,4,6-TBP.

This contradiction was apparently resolved when it was discovered that DHP could be activated in the oxy form to carry out peroxidase oxidations [31,79]. While oxidation of the oxy form of myoglobin, Mb-O_2, has been demonstrated to yield compound II (Fe(IV)=O), there has not been a previous demonstration of a competent enzyme starting from the oxy form. While this finding apparently supports the hypothesis that a globin that normally carries oxygen could also function as a peroxidase, in practice, it leads to a paradox since activation of the oxy form ultimately ends in the ferric form. Thus the paradoxical scheme is as follows:

1. DHP is an oxygen transport and storage protein that reversibly binds O_2.

$$DHP + O_2 \leftrightarrow DHP{-}O_2$$

2. Peroxidase chemistry can be carried out starting from the oxy form.

$$DHP{-}O_2 + H_2O_2 \leftrightarrow CmpII + H_2O + O_2$$

3. The result of a peroxidase cycle is a ferric heme iron.

$$CmpII + X{-}A{-}OH + H^+ \leftrightarrow DHP(III) + X{-}A{-}O\cdot + +H_2O$$

4. A ferric heme iron cannot support globin function.

Once DHP is oxidized it would need to be reduced again by an exogenous agent if the paradox is to be resolved. As we ponder this paradox, we point out that ferric DHP presents a further corollary to this paradox, since it is inhibited in a unique manner by 4-bromophenol, 4-BP. The inhibitor only binds to the ferric heme form of DHP and thus, under native conditions, ferric DHP cannot function as a peroxidase in the presence of high concentrations of 4-BP. The corollary based on inhibition presents a true functional conundrum since the natural abundance of the inhibitor, 4-BP is two times greater than the substrate, 2,4,6-TBP in benthic ecosystems [12]. Since the inhibitor apparently does not bind to six-coordinate forms of DHP, such as DHP-O_2, but binds strongly to the ferric form, the corollary suggests that if DHP(III) was formed as a result of peroxidase chemistry under physiological conditions, inhibition would likely occur after a single turnover. It appears that DHP cannot function as both a peroxidase and a globin, without some additional factor that permits a reduction of the heme, but then if DHP is inhibited to a significant extent in the ferric form, it is not likely to function successfully as a peroxidase in any case.

As with any paradox, resolution is possible if more information is presented that can place the logical dead end in the appropriate context. One resolution to the physiological paradox would be the existence of a reductant that returns DHP to the ferrous state. The extremely high redox potential of DHP appears consistent with easy reduction back to the ferrous state. However, at present there is no known reductant capable of effecting a reversible conversion of the ferric enzyme back to the ferrous state. The class of flavohemoglobins and cytochrome P450s provide examples of this kind of cycling between ferric and ferrous forms [32,33]. However, it may also be relevant that the product of oxidative catalysis, 2,6-DCQ can reduce DHP B(III) to DHP B(II)[79]. Since it is not known how reduction occurs or if it occurs in nature, we can state that the paradox stands given the current state of knowledge, and DHP function requires an understanding of the significance of the protein interactions.

4. The significance of the inhibition of DHP by 4-bromophenol

It might be objected that the peroxidase function is not proven for DHP, and perhaps the aforementioned logic is based on an artifact observed in the laboratory that is found under physiological conditions. This objection would state further that all globins have some limited peroxidase activity [34], and it is therefore possible that DHP is just an extreme case of an adventitious reaction that has no relevance in the organism. Although DHP was originally discovered by biologists who isolated the fraction from *Amphitrite ornata* in search of the enzyme with the greatest capacity for oxidation of the naturally occurring toxin, 2,4,6-TBP [13], this is not a proof that DHP has a physiological peroxidase function.

Aside from the fact that DHP A and B have the highest peroxidase activity in any naturally occurring globin, the recent discovery of the highly specific inhibitor binding site in the interior of DHP provides support for a true oxidative function. The ratio of 4-bromophenol (4-BP) to 2,4,6-tribromophenol (2,4,6-TBP) is known to be approximately 2:1 in the coastal estuary environment where *A. ornata* is found [12,27], and at that ratio, 2:1, the peroxidase function of DHP is almost completely inhibited under in vitro conditions in a kinetic assay [10,11]. Fig. 2 shows the mechanism whereby 4-BP acts as an inhibitor that binds at the internal site and pushes the distal histidine, H55, to the external (open) conformation. Substrate binding has the opposite effect [10,11,35], which also suggests specificity of binding on the exterior as well. Originally, it was suggested by Lebioda et al. that the internal binding site was the substrate binding site [2]. This was a reasonable suggestion based on the X-ray crystal structure obtained on the native enzyme in 1996, which showed a 4-iodophenol molecule positioned above the heme iron, but not bound to it. However, this view does not explain much of the reactivity of DHP, and has now been replaced with extensive data showing that the internal binding site is an inhibitor site [10,11].

We have shown that the native substrate, 2,4,6-TBP, is too large to fit in the inhibitor site, but 4-BP does fit in the pocket. Other molecules such as imidazole and 2,4,6-trifluorophenol can fit in the pocket, but with lower affinity than 4-IP or 4-BP [36]. For example, the parahalophenols fit in the pocket with decreasing affinity in the order, 4-IP > 4-BP > 4-CP > 4-FP [10]. The greater affinity of the larger halogen suggests that there is an internal cavity deep inside the DHP A protein. We have tested this hypothesis using high pressure Xe derivatization [37]. The only dominant Xe binding site in DHP A is coincident with the position of the Br atom in the 4-BP structure [37], which was solved to 1.06 *Å* resolution. [10] Thus aside from the functional inhibition, all the structural data suggest strongly that 4-BP fits very well in an internal binding site immediately above the heme iron. This realization of the internal site resolves the previous DHP paradox when the internal binding site was thought to be the substrate binding site, and yet the substrate, 2,4,6-TBP, was never observed to bind in that site. Furthermore, the 4-BP, which binds internally, has extremely low activity since it is a self-inhibitor.

5. Unusual flexibility of the distal histidine in DHP

The distal histidine is the most important determinant of reactivity in globins and peroxidases. Despite its peroxidase function, DHP does not have the well-defined hydrogen bond network that is attributed to the distal histidine in peroxidases. Instead, the combination of resonance Raman [10,38], EPR [24,39–41] and X-ray crystallography [3–5,42] studies shown in Fig. 2 suggests that the distal histidine (H55) of DHP exhibits an unusual degree of conformational flexibility. As observed in other globins the conformation of the distal histidine is pH-dependent. The distal histidine more favors the 'open' conformation at pH 5 (Fig. 3) and the 'closed' or internal conformation at pH 7. H55 hydrogen bonds to a heme-bound water molecule or oxygen molecule in the X-ray crystal structure only when it is in the closed conformation [3]. While the conformations in Sperm Whale myoglobin (SWMb) and DHP are similar, the pH-dependence is quite different in DHP. The open conformation of H64 in SWMb is observed in an X-ray structure at pH 4.5, but not at higher pH, suggesting both that protonation is an important factor in stabilizing the open conformation and that the pKa of the distal histidine is lower than the nominal pKa of 6.0 [43]. In DHP, the open form is formed far more readily and is observed in the X-ray crystal structure at pH 6.0. There is no proof that histidine is protonated in this form, but this would be the most reasonable hypothesis considering the comparison with SWMb. In fact since the pKa of free histidine is 6.0 the observation of an equal population of internal (closed) and external (open) histidines would potentially mean that the pKa of histidine in DHP is closer to the free value than in SWMb.

Fig. 2 shows that the histidine position is correlated with the presence of a water molecule bound to the heme iron. Specifically, when water is bound to Fe in the metaquo form, H55 is observed in the internal (closed) conformation [42]. When the heme Fe is 5-coordinate (water is absent), H55 is in the external (open) conformation. This is observed in a correlation of resonance Raman spectroscopy and X-ray crystallography (Fig. 2), but can also be observed at a deeper level in low temperature EPR and HYSCORE data where the 6-coordinate metaquo form is observed at pH 7 and the 5-coordinate form is observed at pH 5 [40]. This observation is correlated with the pathway for radicals that we have identified based on rapid-freeze quench (RFQ) EPR spectroscopy shown in Fig. 3. The correlation of pH with the radical pathway is likely also due to the conformation of the distal histidine [41]. These observations suggest that the distal histidine may respond to binding of H_2O_2. According to this idea, when H_2O_2 binds, the histidine moves to a conformation where it can act as an acid–base catalyst necessary for peroxidase function.

The conformation of the histidine is clearly a determinant of a radical pathway in DHP. Ultimately, the fate of the tyrosyl radical at low pH appears to produce a heme crosslink to the protein [41]. While such crosslinks are known in other globins, the pH-dependent yield of the radical pathway in DHP is quite different. At pH 7 the protein radical yield is nearly 100%, which is why the radical intermediate has the appearance of a true enzymatic intermediate, a compound ES, such as is observed in cytochrome c peroxidase [41]. In keeping with the open conformation the radical yield is much lower at pH 5. The fate of the radical is also quite different since protonation of ferryl DHP leads to a heme radical that can combine with the protein radical to crosslink the heme to the protein. This may be part of a protective function in DHP that limits reactivity at low pH.

6. The correlation of the radical pathway with both structure and kinetics

The formation of radical intermediates in peroxidases was first observed in cytochrome c peroxidase (CcP), where a tryptophan radical is an on-pathway electron transfer intermediate leading to oxidation of the bound cytochrome c. DHP also produces protein radicals when the enzyme reacts with H_2O_2 in the absence of substrate. However, DHP has a more complex behavior. Fig. 3 shows a correlation between the pH dependent pathway for radicals and the catalytic rate. As can be seen in Fig. 3, the EPR lineshape for the radicals varies with pH indicating the formation of more than one radical species. The primary radical species in DHP, i.e. the tyrosyl radical associated with the compound ES ferryl intermediate, is located on Y34 and is the dominant radical at all values of pH studied. A secondary radical species, located on Y38, has also been observed at lower pH and likely occurs as a result of the protonated ferryl. We have used both calculations and mutagenesis to assign the RFQ-EPR signals shown in Fig. 3A and relate those to X-ray crystal structures shown earlier. We know that the pH 5 and pH 7 structures have the open and closed conformations, respectively, from a combination of resonance Raman [10,38], EPR

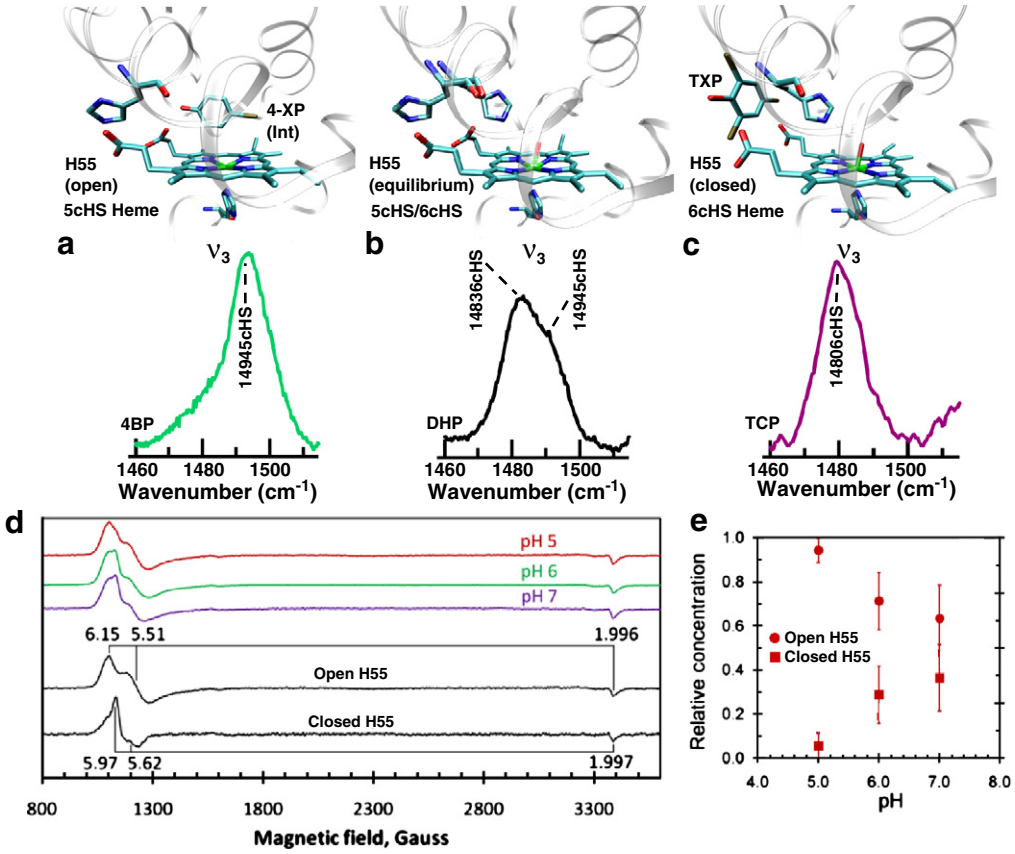

Fig. 2. (a–c) Model representations of internal and external binding in DHP. (a) The distal H55 is forced into the solvent exposed (open) position by the internally bound 4-XP inhibitor. (b) The room temperature X-ray crystal structure of WT DHP the H55 open (5cHS) and closed (6cHS) states in equilibrium. (c) Depiction of tri-halogenated phenol forcing the distal H55 into the closed position. PDB accession numbers are 2QFK, 3DR9, 3BL1, 3BL2, 3BL3, and 3BL4. (d) The EPR spectra of ferric DHP at different pH can be deconvoluted into two species. They have been correlated to the two conformations of the distal H55 [41]. (e) Stoichiometric ratio of the two H55 conformations determined from the EPR spectra in panel d [41].

[24,39–41], and X-ray crystallography [3–5,42]. A water molecule is bound to the heme iron in the pH 7 (closed) conformation of the ferric enzyme. Activation requires binding by H_2O_2 which would need to displace H_2O in the pH 7 form, which may explain the slower kinetics observed at pH 7 (Fig. 3). Despite the slower kinetics, the yield of product formation is much greater at pH 7, i.e. the total number of turnovers is greater [26].

The lower yield of radical at pH 5 can be correlated to a higher population of the open H55 conformation preventing activation of the bound H_2O_2. At this low pH, the ferryl can be protonated leading to

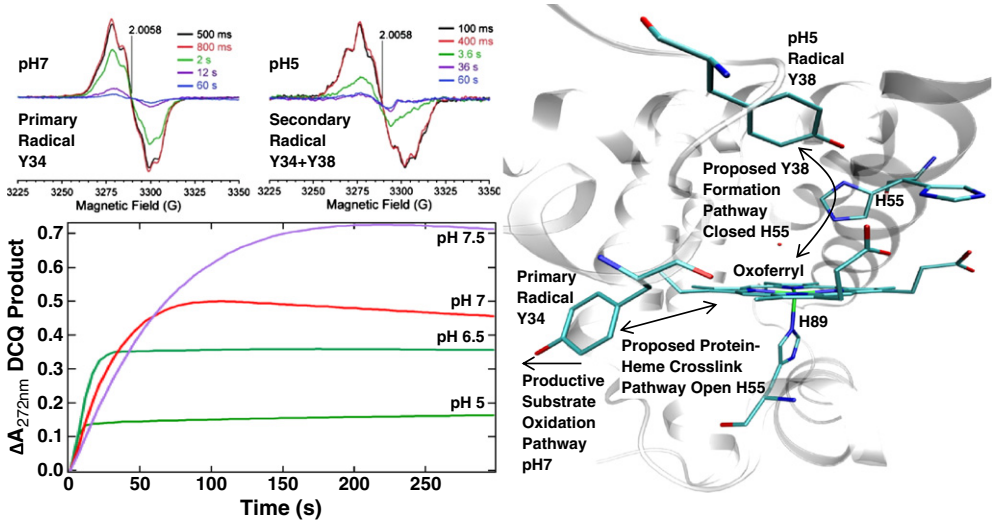

Fig. 3. (Upper left) RFQ-EPR data showing pH-dependent radical lineshapes [39]. (Lower left) Kinetic data showing pH-dependence of rate. (Right) (PDB 1EW6 [6]) Proposed radical pathways for productive and non-productive substrate oxidation [41].

the formation of a hydroxyl radical in the interior of DHP. Thompson et al. demonstrated that the hydroxyl radical decays via two alternative pathways that also depend on the conformation of the distal H55 [41]. When H55 is "closed" (minor population at pH 5), the hydroxyl radical is transferred to Y38, whereas when H55 is "open," a protein–heme crosslink forms known as compound RH. Both of these alternative decay processes contribute to the small product yield observed at pH 5. In line with this reasoning, only a small fraction of the Y38 radical or protein–heme crosslink was observed at pH 7 [41].

The protonation state of ferryl oxygen may play a role in determining the fate of the radical. This has been investigated in the nominal compound II form using ENDOR [44]. Results on the compound II form are valid for compound ES in DHP since both consist of ferryl heme. Davydov and co-workers showed that the Fe(IV)=O oxygen atom is not protonated in DHP at pH 7, but has a strong hydrogen bond. Although one cannot be certain, the most logical hydrogen bond partner is H55. One possible reason for a pH-dependent difference in the fate of the radical in compound ES could be protonation of the Fe(IV)=O at pH 5 [41].

7. The inactivation of DHP: formation of compound RH

At pH 5, the catalytic rate is greater, but the enzyme is rapidly inactivated under conditions where $[H_2O_2]>[2,4,6\text{-TBP}]$. As stated earlier, we hypothesize that this inactivation occurs due to a protein–heme crosslink observed by HPLC [41] similar to those observed in myoglobin (Mb) and cytochrome c peroxidase (CcP) [45]. Compound RH is observed as a species with a Soret band at 411 nm in both DHP A and B. However, in DHP B compound RH may be reduced to another distinct form known as compound P_{426} [24]. These observations are consistent with the crosslink hypothesis since a crosslinked heme would not necessarily be completely inactive. Compound RH is not observed in other heme proteins. It appears that compound RH forms as an endpoint when H_2O_2 co-substrate is present, but substrate is not present. This form may protect the organism A. ornata from deleterious oxidation chemistry by rapidly shunting DHP to an inactive form. While there is still some amount of speculation about the function of compound RH, it is clear that it is an important part of the mechanism as discussed in the next section.

8. Mechanism of DHP

Although DHP is clearly a structural member of the globin family, the pH-dependent radical pathway, inhibition by 4-BP, and formation of compound RH are unique features not observed in other members of this family. The distal histidine, H55 in DHP, is central to these observations. We have suggested based on the data that the distal histidine of DHP is unusually flexible. However, the conformations of the distal histidine have played an important role in all hemoglobins, and thus, the conformational space accessed by the histidine in DHP may differ only subtly from that of other globins. Even so, the functional role played by the histidine in DHP is apparently unique given that it is an excellent acid–base catalyst, it is a switch that gives rise to inhibition, and it is likely to be the key determinant of the radical pathways.

Fig. 4 shows a mechanistic scheme that accounts for the all of the pathways discussed earlier based on a number of studies [3–5,10,11,14,24,25,29,31,35,36,38–42,46–54,79]. The non-classical competitive inhibition is illustrated above the cycle in terms of the binding of substrate (right side, step i) and inhibitor (left side, step ii). The action of each in displacing the distal histidine is represented. Addition of H_2O_2 leads to transient formation of compound I (step iii), which has been observed experimentally using cryoreduction techniques [44]. In the absence of a substrate, compound I subsequently undergoes internal electron transfer to yield compound ES (step iv) [39,41]. With two oxidizing equivalents (one on the ferryl and the other on the tyrosyl radical), reaction of compound ES with trihalophenol proceeds through two one-electron oxidations of the substrate that return DHP to the resting state via a compound II intermediate (steps v-a,b) [52]. We can refer to this as typical peroxidase chemistry. However, Fig. 4 shows two other possibilities for compound ES. If no substrate is present, the peroxidase-inactive compound RH species is formed (step vi) [25,39,79]. Surprisingly, if product 2,6-DCQ is present then compound ES can be reduced to oxy-DHP (step vii) [79]. We have recently shown that the oxyferrous form of DHP can be activated for exchange of O_2 by H_2O_2, which results in formation of compound II (step viii) followed by a one-electron oxidation of substrate [79]. The details of the activation of oxyferrous DHP by substrate are discussed in (see Section 12).

The mechanistic scheme shows both classic peroxidase chemistry in a cycle starting from the ferric form and an unusual activation of oxyferrous DHP. Given that reversible oxygen-binding is only mediated via a ferrous heme in globins and that peroxidase activity is initiated from ferric centers and to the exclusion of the oxyferrous oxidation state from the peroxidase cycle [55], the bifunctional nature of DHP as a globin-peroxidase appears to be at odds with the traditional starting oxidation state for each individual activity. Recently, however, in a peroxidase reaction that is unique to DHP, both we [79] and Dawson and co-workers [31] have reported that dehaloperoxidase activity has been observed when the catalytic cycle is initiated from the oxyferrous state. Activation of the oxyferrous form is physiologically relevant since the red color of the coelom of A. ornata is due to the presence of oxy-DHP. However, in the absence of a reductase or reducing agent the peroxidase cycle cannot reversibly return to globin function. The DHP paradox arises from the fact that the presence of halophenols in their natural abundance leads to inhibition as soon as DHP starts turnover and appears to create the conditions for a frustrated peroxidase. DHP can only function as a peroxidase in nature if 2,4,6-TBP and H_2O_2 are present, but the 2,4,6-TBP is only observed in nature in the presence of inhibitor 4-BP. The inhibitor only binds when oxygen is not present in the pocket, and thus DHP appears to be activated perhaps only for a single turnover from the oxy form, after which it would remain in the inhibited state and unable to function to degrade the toxin that triggered its action in the first place. Activation of the oxyferrous form likely also depends on the conformation of the distal histidine, as is evident from the effect on H55 when substrate binds [10,11]. Thus, substrate binding may force the distal histidine into close proximity to iron-bound H_2O_2. The inhibition paradox exists in tandem with the more obvious fact that the DHP mechanism has no return to globin function and therefore the cycle does not have closure. One can conclude that peroxidase function in DHP appears to be a suicide function for the protein. In the aquatic ecosystem, activation of oxyferrous DHP would lead to formation of the ferric form, which could be inactivated by formation of compound RH or inhibited by 4-BP leading to relatively few turnovers of 2,4,6-TBP prior to losing all function. However, both of these aspects would be explained if there is a natural reductant or reductase that returns DHP to the ferrous state. The cycle could be closed by return to ferrous iron and the binding of the inhibitor is significantly reduced in the deoxyferrous state.

9. pH-dependence of kinetics, product yield and radical pathway in DHP

The pH-dependence of the radical pathway also appears to be correlated with a pH-dependence of the rate of substrate oxidation as shown in Fig. 3. The kinetics are faster at pH 5 than at pH 7, which can be explained in light of the closed H55 and consequent heme-bound water molecule. Since activation requires binding by H_2O_2, the need to displace the H-bonded H_2O in the pH 7 'closed' conformation leads to slower kinetics when compared with the water-free (distal histidine

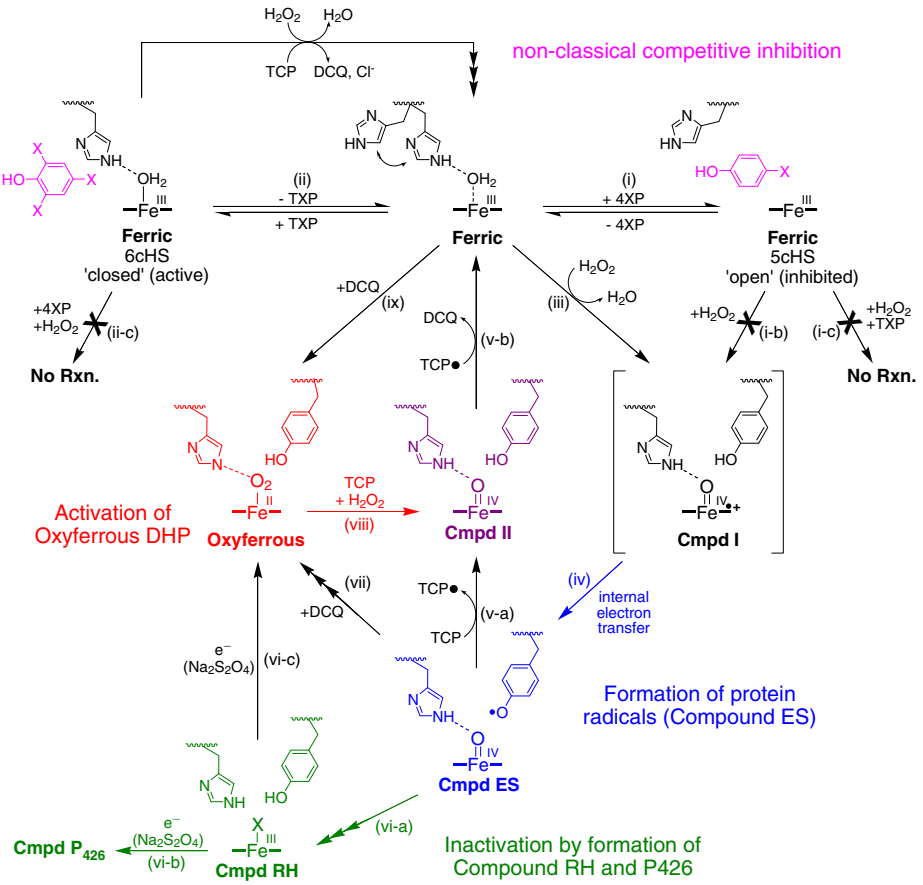

Fig. 4. Proposed catalytic cycle for DHP with unique features noted in the figure.

open) scenario at pH 5. It is very surprising, however, that while the kinetics are slower, the yield (or total number of turnovers) of product formation is much greater at pH 7, i.e. kinetics and yield are inversely correlated with each other under conditions where [2,4,6-TCP]> [H_2O_2] [56]. Here, we note that turnover number has two definitions. In the chemical literature, turnover number is defined as the number of enzyme cycles that can be completed before the enzyme is inactivated. This is called the number of turnovers in the biochemical literature, in which turnover number is synonymous with the catalytic rate with units of Ms^{-1}. We have used the biochemical definitions in this review.

The role played by the distal histidine appears to depend on whether there is a sixth ligand bound to the heme iron as well as the relative concentration of substrate and inhibitor. The conformations are linked with pH since the histidine is almost certainly protonated in the external conformation. One might assume that protonation drives the conformational switch, but that is not necessarily the case. Instead protonation could be a consequence of the fact that H55 is a switch that is regulated by substrate and inhibitor binding, as well as heme–iron ligands, Fe(III)–H_2O, Fe(III)–H_2O_2, and Fe(II)–O_2. Clearly, that switch results in vastly different mechanistic outcomes at high and low pH. Thus, the pH-responsiveness is clearly connected to the switching of the histidine. However, the question of which process is the key determinant of histidine conformation is not resolved at present.

10. The fate of radical formation in the catalytic oxidation of phenols by peroxidases

Radicals are unavoidable side products in oxidative chemistry that affect both the efficiency and longevity of enzymatic catalysts. The initiation of catalysis also generates radicals in solution, which affects living cells as well. Since the radicals inherent in catalytic peroxidase oxidation can be harmful to a living cell and can also react with the enzyme by suicide inhibition, peroxidases that generate reactive radicals are usually either secretory peroxidases or part of an immune response that has the expressed purpose of degrading an invader in a controlled biological structure that protects the organism, e.g. myeloperoxidase. These aspects are regulated by 4-BP inhibition and compound RH formation. We consider the inherent radical chemistry of substrate and inhibitor that form the background for this regulation.

The standard peroxidase mechanism consists of substrate activation by Cmp I (k_1) via two different substrate oxidation steps (k_2 and k_3), each of which creates radical intermediates in solution [55] (ping-pong mechanism). The substrate radical intermediate formed following process k_2 (or k_2 and k_3 in the ping-pong mechanism) can be toxic because of its propensity to form dioxin [57]. Chlorinated phenoxy radicals are stable for long periods of time in the environment [58], which increases the risk to human health associated with these compounds.

The product of 2,4,6-TCP oxidation has been shown to be 2,4,6-TCP radical by computational modeling of kinetics [50,52] and more directly by flow EPR [59]. In solution two of these radicals can dismutate to form the product 2,6-DCQ and substrate 2,4,6-TCP [50,52]. We have also recently shown that 2,6-DCQ reacts with hydroxide, and possibly hydrogen peroxide anion to give 3-hydroxy-2,6-dichloroquinone (3-OH-2,6-DCQ) [60]. Our recent study [60] shows the first evidence for radical formation in a non-photochemical process [61]. Peroxidase catalysis involving 4-CP and 4-BP radicals has been shown to produce dimers [52], and even polymeric products [60]. The nature of the difference in products of the two similar reactions involving 4-XP and 2,4,6-TXP (X=Cl or Br) is related to the ortho-position halogen. While the solution phenoxy radicals produced in

catalytic peroxidase oxidations can be harmful to a living cell and even react with the oxidizing enzyme to inhibit it, polymerization products from 4-BP oxidation can be even more deleterious in vivo.

11. DHP mechanism in the context of other heme enzymes

Typical class III peroxidases do not have protein radical intermediates since the heme cation radical is a stable species known as compound I (Cmp I). However, CcP, a class I peroxidase, forms a Trp radical intermediate, which was the first Cmp ES species identified [62]. Cmp ES in CcP is thought to be a functional radical that provides a conduit for electron transfer from the substrate, cytochrome c, to the Trp residue [63]. The name Cmp ES implies that the radical plays a productive role in catalysis, and ES refers to the enzyme substrate complex in Michaelis–Menten kinetics in accord with this implication. Tyrosyl radicals are also observed in the oxygen transport proteins, hemoglobin and myoglobin [64–69]. Those radicals lead to protein–protein and protein–heme crosslinking. One might think of these as simply accidental reactions, since the normal function of Hbs and Mbs is not thought to include oxidation. However, as with all proteins, one may consider whether there is a protective aspect to this reactivity. If reactive oxygen species (here mainly H_2O_2) are encountered in cells, it may be useful for the protein to consume the H_2O_2 without generating a radical species in solution that can produce further reactions that are potentially harmful. DHP, which is both an Hb and a peroxidase may have an aspect of this protective reactivity as well. The productive and non-productive reaction pathways are shown in Fig. 3. Compound RH is on the non-productive pathway, although it may be possible to regenerate a ferric heme with limited reactivity from compound RH. Further study has led to the conclusion that compound RH is a heme to protein crosslinked species [41], probably involving Y34. This specific hypothesis will be tested as part of the more general investigation of the fate of protein radicals in various mutants of DHP.

Radicals are highly reactive species, and there are many possible side reactions, both within the protein and involving radical intermediates in solution. In addition to the on-pathway Trp191 radical [70], radicals are observed at Tyr39 and Tyr15 in CcP [71]. Myoglobins and hemoglobins have limited peroxidase activity, but Mb mutants have been made that have increased peroxidase activity [72–74]. Although, Tyr103 radicals have been reported to form protein–protein crosslinks in Mb [64], the Y103F mutant of Mb has been observed to have increased peroxidase activity [75]. These observations are analogous to the findings for DHP. Mb has a very slow peroxidative rate, but the total number of turnovers may be higher than DHP [52,59]. In Sperm whale (SW) Mb, there are three protein radicals, including two tyrosyl (Y103 and Y151) and one tryptophanyl (W14) radical [76,77]. In human Hb, four radicals have been trapped, including two tyrosyl (Y24 and Y42), one cysteinyl (C93), and one histidyl based radical (H20), using the 5,5-dimethyl-1-pyrroline N-oxide (DMPO) spin trap and subsequent MS analysis [76,78].

12. Resolution of the paradox: 2,4,6-Trihalophenol as a trigger for oxyferrous activation

The bifunctional nature of DHP as a globin-peroxidase appears to be at odds with the traditional starting oxidation state for each individual activity. Namely, reversible oxygen-binding is only mediated via a ferrous heme in globins, and peroxidase activity is initiated from ferric centers and to the exclusion of the oxyferrous oxidation state from the peroxidase cycle. Additionally, an implicit problem in the paradox outlined in Section 3 is that reactivity of H_2O_2 in the absence of the substrate would lead to inactivation of DHP. This issue was identified in studies of order of addition of H_2O_2 and 2,4,6-TXP [25,39,52]. In a previous study we observed that DHP is inactivated and forms compound RH if H_2O_2 is added more than 30 s prior to the addition of substrate [25]. We noted that inactivation is complete within about 1–2 s at pH 5 and within 60 s at pH 7 in the absence of substrate, but that this effect is not significant when substrate is present [39,79]. Dawson and co-workers conducted a similar investigation and concluded that H_2O_2 can bind first in normal DHP functioning even if substrate is absent [52]. In that study it was shown that product can be formed if it is added less than 1 s following H_2O_2 addition. However, that study did not report on the effect of longer exposure times of H_2O_2 in the absence of substrate as we did in our work [39,79]. The control experiment of adding H_2O_2 without substrate to produce compound RH, which has significantly less activity, raises a physiological question regarding normal DHP function. Thus, a dual resolution to the dehaloperoxidase paradox is needed, wherein (a) the incongruity of the oxidation states for the globin-peroxidases is addressed, and (b) the inactivation of DHP in the absence of substrate can be avoided.

One possibility, akin to a trigger mechanism, has been found in the oxyferrous form. Both we [79] and Dawson and co-workers [31] have recently demonstrated that dehaloperoxidase was able to catalyze the oxidative dehalogenation of 2,4,6-trihalogenated phenols to their corresponding 2,6-dihalo-1,4-benzoquinones in the presence of H_2O_2 when the catalytic cycle was initiated from the oxyferrous state. This is of critical importance given that the oxyferrous state is normally a catalytically incompetent species for monofunctional peroxidases [55], and represents a partial solution to the aforementioned paradox. Namely, it is possible that dehaloperoxidase may have evolved its peroxidase function to begin from the oxyferrous state, which is the normal coordination and oxidation state for this hemoglobin in A. ornata. Furthermore, in the absence of 2,4,6-TXP, oxyferrous DHP was unreactive towards H_2O_2 with respect to the formation of observable high-valent iron-oxo intermediates (i.e., Compound II) [31,52,79]. Therefore, 2,4,6-TXP apparently plays the role of activator in catalysis initiated from the oxyferrous state. Dawson and co-workers have suggested that the observed conversion of oxyferrous DHP to Compound II/ES was due to the presence of a trace amount of ferric DHP that undergoes a traditional peroxidase cycle, thereby generating TCP radicals that oxidize bulk oxyferrous DHP to the ferric enzyme [31]. Subsequent reaction of the resulting ferric DHP with H_2O_2 generates the observed ferryl-containing species. However, from anaerobic studies we have observed the formation of Compound II upon the reaction of deoxyferrous DHP B with H_2O_2 in the absence of 2,4,6-TXP [79]. The conversion of deoxyferrous hemoproteins to compound II in what is generally regarded as a single-step, two-electron oxidation has been noted and/or postulated in a number of other systems, including horseradish peroxidase [80], leghemoglobin [81], lactoperoxidase [82,83], myeloperoxidase [84], and KatG [85]. Importantly, none of aforementioned systems invoked substrate radicals as mechanistically relevant to account for their observed chemistry. Additionally, stopped-flow UV–visible and rapid-freeze-quench EPR spectroscopic measurements confirmed that in the presence of as little as one equivalent of 2,4,6-TCP substrate, the first intermediate observed upon reaction of oxyferrous DHP B with H_2O_2 was the peroxidase-active intermediate compound II, and not compound ES.

Thus, an alternative role for 2,4,6-TXP binding in oxyferrous DHP activation that does not require 2,4,6-TXPO• radical formation is needed to describe the reaction of deoxy- and oxyferrous DHP with hydrogen peroxide yielding compound II. The distal histidine, H55, is observed in distinct 'open' and 'closed' conformations. This conformational switching appears correlated with O_2 displacement from the heme in the presence of substrate, which suggests that the histidine serves either as a trigger for the conversion to peroxidase function or as a mediator of the conformational change induced by substrate binding that activates DHP for peroxidase function [42]. Consequently, we have considered the role of the distal histidine as integral to the substrate-dependent activation of oxyferrous DHP B, and propose the

mechanism in Fig. 5 in which 2,4,6-TXP binding plays a role in the activation of oxyferrous DHP to the deoxy state when H_2O_2 is present. Upon binding of 2,4,6-TXP to oxyferrous DHP, tautomerization of the distal H55 occurs, forming a hydrogen-bond between the His-N^{δ} and the phenolic hydrogen of the co-substrate, and disrupting the H-bond between the His-$N^{\varepsilon 2}$ and the bound oxygen ligand that had stabilized the oxyferrous species [10], thus shifting the equilibrium towards deoxyferrous DHP. Evidence for the destabilization of oxyferrous DHP upon 2,4,6-TXP binding comes from Dawson and co-workers who observed a 2.6-fold increase in the rate of autoxidation of the oxyferrous enzyme in the presence of TCP when compared to its absence [31]. The deoxy state is therefore activated for reaction with H_2O_2, generating an Fe(II)–OOH intermediate that was observed in the deoxyferrous studies (Fig. 5). Heterolysis of this ferrous-hydroperoxide intermediate, facilitated by the tautomer that now positions the His-$N^{\varepsilon 2}$ as a general base, yields compound II, which can now initiate a peroxidase-cycle. It is important to note that although the 2,4,6-TXP binding site is believed to be external to the heme active site [40,42,46], it is not known whether bound 2,4,6-TXP can directly interact with the distal histidine. Thus, tautomerization may result from either a direct or indirect (allosteric) binding event. Histidine tautomerization has been postulated to play an important role during enzymatic function for both heme and non-heme proteins [86–91].

Importantly, the lack of reactivity observed for oxyferrous DHP B may have a physiological role when 2,4,6-TXP substrate is absent [31,79]. First, we note that DHP functions primarily as the intracellular (coelomic) hemoglobin in A. ornata and hence functions in the ferrous form as an oxygen transport protein. In the case of the different reactivities observed for deoxy- vs. oxyferrous DHP B in the absence of 2,4,6-TXP, it is likely that the bound oxygen molecule acts as an inhibitor of the reaction by blocking H_2O_2 from binding to the heme iron. One way that a dehaloperoxidase-hemoglobin may minimize the deleterious effects of unwanted peroxidase activity in the absence of a reducing substrate is to function in a cycle starting and ending in the ferrous state, rather than the ferric state. Initiation of the catalytic cycle in the ferrous state would avoid the problem of protein radicals (compound ES) and inactivation to compound RH when exposed to H_2O_2 for short reaction times (<60 s at pH 7). The ferric state likely has some physiological relevance since DHP would have to transit through this oxidation state in any mechanism. Moreover, inhibition is mainly observed in the ferric state, and this may be a role played by 4-BP to prevent harmful activity in the ferric state. In line with this reasoning, DHP catalysis that begins in the ferrous state and then ends in the ferric state may be ultimately shut down by 4-BP, possibly to prevent further reaction to form radicals.

A final outstanding question in the field is the determination of the reducing agent that returns DHP to a ferrous resting state. The high redox potential of both DHP A and B facilitates the reduction of the enzyme and this may present a clue as to the nature of the reductant. One remarkable observation that may have physiological relevance is that the product, 2,6-DCQ, either due to its inherent instability or as an alternative co-substrate for the enzyme, leads to the reduction of the ferric state to the ferrous, generating oxyferrous DHP B in the presence of dioxygen (Fig. 4, step ix) [79]. Alternatively, compound ES may directly react with 2,6-DCQ as a co-substrate, leading to the generation of the (de)oxyferrous species (Fig. 4, step vii). It is also possible that there is a naturally occurring flavoprotein that provides reducing equivalents to DHP and either initiates the reactive cycle or completes it by electron transfer. Flavohemoglobins are known in bacteria and in the chloroperoxidase from Notomastus lobatus, which must have some common features with DHP although it catalyzes the formation of 2,4,6-TBP rather than its oxidation [12,54,92].

13. Conclusion

The mechanisms proposed in Figs. 4 and 5 resolve the dehaloperoxidase paradox wherein the bifunctional nature of a globin-peroxidase does not logically follow from the traditional starting oxidation state for either individual activity. Specifically, (de)oxyferrous DHP is a catalytically competent state for initiating a peroxidase catalytic cycle, and is the first such example that has a relevant biological function. Moreover, 2,6-DCQ generated from the peroxidase cycle is itself a substrate for both ferric and compound ES DHP B, leading

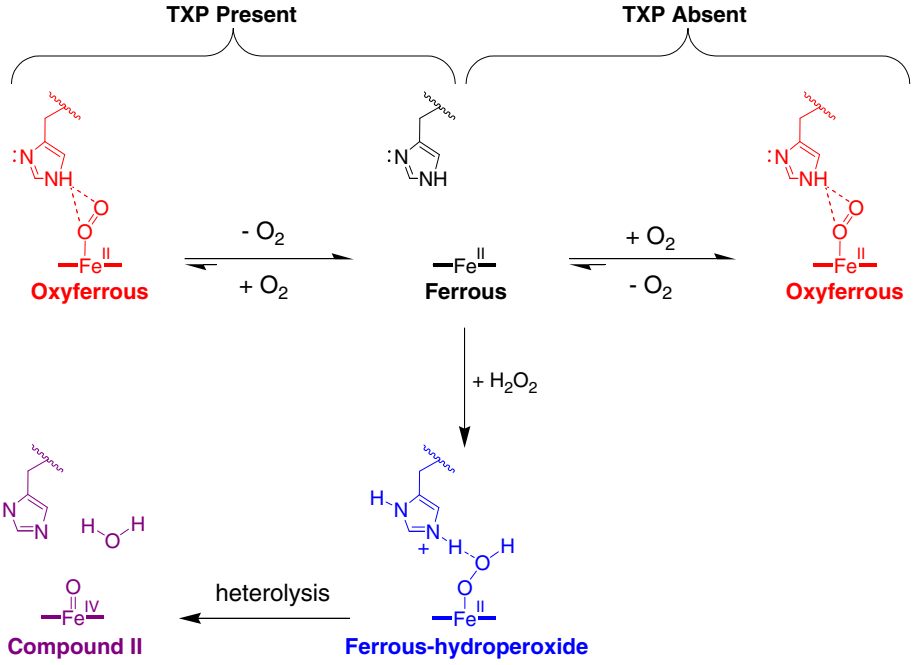

Fig. 5. Proposed mechanism for the activation of oxyferrous DHP (red) leading to compound II (purple) via the ferrous hydroperoxide intermediate (blue) and in equilibrium with the ferrous state (black). Although the ferrous state is not directly observed in experiments it is likely that O_2 dissociation is rate limiting. The figure shows that O_2 dissociation is relatively slow in the absence of TXP, but is accelerated when the substrate TXP binds to DHP.

to formation of oxyferrous DHP B [24]. Thus, the product of the peroxidase cycle regenerates the globin-active species, which is also a viable oxidation state for initiating peroxidase chemistry. Taken together, these two reactivities observed here highlight the unique chemistry of DHP which enables both globin and peroxidase functions to coexist in an environment with a limited set of resources.

DHP exhibits a remarkable number of functional states for a globin. Not only does it function as a hemoglobin and peroxidase, but it also exhibits non-classical competitive inhibition, and other unusual reactivities such as a H_2S oxidation reaction [93], which is a consequence of the high redox potential of DHP [4]. Perhaps the most significant aspect of the present research is that it points to the fact that even a relatively simple class of proteins that has long been regarded as a "solved problem" in biophysics holds many new surprises and levels of function. Rather than complexity in the form of multiple conformational states, the complexity of DHP is a mapping of many functions onto a few states. It is an apparent economy of conformational states that gives rise to multiple interactions with the chemicals in the environment. Fundamentally, the ability of this simple protein to cycle between two oxidation states must be similar to many other heme proteins, including oxidases and cytochrome P450s. However, while our present state of knowledge permits us to complete the cycle, the significance of the dehaloperoxidase paradox (and its resolution) suggests that our understanding of the nuances in protein structure–function relationships, even for globins, is still in its infancy.

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