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14. ABSTRACT

Electrochemical investigations of the heme protein dehaloperoxidase (DHP) were undertaken. The motivation for this work lies in the unusual bifunctional nature of DHP, a globin-type protein found in the marine worm Amphitrite ornata. DHP is a monomeric hemoglobin that not only binds and transports dioxygen but that features a high level of peroxidase activity for the enzymatic detoxification of halogenated phenolic compounds such as 2,4,6-tribromophenol. Direct electrochemistry of DHP in the absence of mediators was realized for both

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Report Title

Generation and Characterization of SAM Immobilized Enzyme Films in Ionic Liquids

ABSTRACT

Electrochemical investigations of the heme protein dehaloperoxidase (DHP) were undertaken. The motivation for this work lies in the unusual bifunctional nature of DHP, a globin-type protein found in the marine worm Amphitrite ornata. DHP is a monomeric hemoglobin that not only binds and transports dioxygen but that features a high level of peroxidase activity for the enzymatic detoxification of halogenated phenolic compounds such as 2,4,6-tribromophenol. Direct electrochemistry of DHP in the absence of mediators was realized for both diffusional and non-diffusional voltammetric cases using mixed OH/COOH self-assembled monolayer modified electrodes of alkanethiolate/gold construction. It appears that DHP interacts with anionic electrodes via a lysine patch situated in the vicinity of the heme edge. The interfacial voltammetry, however, suffers from instability problems that have not been completely resolved. Accordingly, mediated thin-layer spectroelectrochemistry (rather than cyclic voltammetry) was utilized to characterize the Fe(III)/Fe(II) redox thermodynamic properties of DHP. The reduction potential at pH 7 was determined under anaerobic conditions to be +202 mV vs SCE, the most positive value for any known intracellular globin. This unusual value was rationalized in terms of a redox-coupled conformational behavior involving the distal histidine (H55) by analyzing the Gibbs free energy contributions to the potential. The impact of enzyme substrate interactions on redox properties was also characterized and gave evidence for both internal and external binding depending upon the extent of halogen substitution of the phenolic substrate. A cubic thermodynamic cycle was proposed that accounted for both conformational changes and substrate interactions. Finally, several methionine-86 mutants of DHP were prepared to investigate the electron push effect concept for peroxidase activity. The first successful installation of a peroxide-like aspartate-histidine-iron triad into a globin protein was achieved (M86D mutant).

List of papers submitted or published that acknowledge ARO support during this reporting period. List the papers, including journal references, in the following categories:

(a) Papers published in peer-reviewed journals (N/A for none)

J.D. D'Antonio, E.L. D'Antonio, M.K. Thompson, E.F. Bowden, T. Smirnova, S. Franzen, and R.A. Ghiladi, "Spectroscopic and Mechanistic Investigations of Dehaloperoxidase B from Amphrite ornata", Biochemistry, 2010, 49, 6600-6616.

V.S. de Serrano, M.F. Davis, J.F. Gaff, Qi Zhang, Z. Chen, E.L. D'Antonio, E.F. Bowden, R. Rose, and S. Franzen, "X-ray structure of the metcyano form of dehaloperoxidase from Amphitrite ornata: evidence for photoreductive dissociation of the iron-cyanide bond", Acta Crystallogr. D, 2010, 66, 770-782.

Number of Papers published in peer-reviewed journals: 2.00

(b) Papers published in non-peer-reviewed journals or in conference proceedings (N/A for none)

Number of Papers published in non peer-reviewed journals: 0.00

(c) Presentations

Number of Presentations: 0.00

Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Number of Non Peer-Reviewed Conference Proceeding publications (other than abstracts): 0

Peer-Reviewed Conference Proceeding publications (other than abstracts):

Number of Peer-Reviewed Conference Proceeding publications (other than abstracts): 0

(d) Manuscripts

Number of Manuscripts: 0.00

Patents Submitted

Patents Awarded

Awards

Graduate Students

NAME	PERCENT_SUPPORTED
Edward D'Antonio	1.00
Thomas Chen	0.25
FTE Equivalent:	**1.25**
Total Number:	**2**

Names of Post Doctorates

NAME	PERCENT_SUPPORTED
FTE Equivalent:	
Total Number:	

Names of Faculty Supported

NAME	PERCENT_SUPPORTED	National Academy Member
Edmond Bowden	0.00	No
FTE Equivalent:	**0.00**	
Total Number:	**1**	

Names of Under Graduate students supported

NAME	PERCENT_SUPPORTED
FTE Equivalent:	
Total Number:	

Student Metrics

This section only applies to graduating undergraduates supported by this agreement in this reporting period

The number of undergraduates funded by this agreement who graduated during this period:	0.00
The number of undergraduates funded by this agreement who graduated during this period with a degree in science, mathematics, engineering, or technology fields:.....	0.00
The number of undergraduates funded by your agreement who graduated during this period and will continue to pursue a graduate or Ph.D. degree in science, mathematics, engineering, or technology fields:.....	0.00
Number of graduating undergraduates who achieved a 3.5 GPA to 4.0 (4.0 max scale):	0.00
Number of graduating undergraduates funded by a DoD funded Center of Excellence grant for Education, Research and Engineering:	0.00
The number of undergraduates funded by your agreement who graduated during this period and intend to work for the Department of Defense	0.00
The number of undergraduates funded by your agreement who graduated during this period and will receive scholarships or fellowships for further studies in science, mathematics, engineering or technology fields:	0.00

Names of Personnel receiving masters degrees

NAME

Total Number:

Names of personnel receiving PHDs

NAME
Edward D'Antonio

Total Number: 1

Names of other research staff

NAME PERCENT_SUPPORTED

FTE Equivalent:

Total Number:

Sub Contractors (DD882)

Inventions (DD882)

Scientific Progress

See attachment

Technology Transfer

Final Report

Introduction

The work completed during the period of this proposal was directed at developing and understanding electrochemical aspects of an unusual heme protein, dehaloperoxidase, (DHP), which features a unique enzymatic detoxification caability in addition to a conventional dioxygen-binding globin function. DHP is found in *Amphitrite ornata*, a marine annelid that thrives in estuarine mud flats cohabited by species such as *Saccoglossus kowalewskii* and *Notomastus lobatus* that produce significant amounts of 2,4,6-tribromophenol (TBP) and other haloaromatic repellents. Although *A. ornata* itself does not produce haloaromatic repellents, it has evolved an interesting enzymatic detoxification mechanism based on DHP, which evolved from a hemoglobin protein. In the presence of H_2O_2, DHP is known to catalyze the overall 2-electron oxidation of various 2,4,6-substituted trihalophenols (including fluoro-, chloro-, and bromo- substituents) to less toxic 2,6-dihaloquinone products (1,2), which is an unusual catalytic activity for a protein that has the characteristic 3-over-3 globin helix fold. Site-directed mutagenesis has shown that both the proximal histidine (H89) and distal histidine (H55) are crucial for function. There are two DHP genes in *A. ornata*, which have the designations *dhp A* and *dhp B.* Although research published on DHP since the 1990's nearly always refers to DHP A, a recent mutagenesis study has shown that DHP B also has enhanced peroxidase activity (3).

The unusual globin/peroxidase dual function of DHP A has stimulated a number of recent studies aimed at understanding its structure, enzymatic mechanism, and evolution. The close structural homology between myoglobin and DHP A has been known for 10 years. The initial structural work showed that a substrate analog, 4-iodophenol (4-IP) will bind in the distal pocket of DHP A close to the heme iron, a finding that led to the hypothesis that this internal site is the

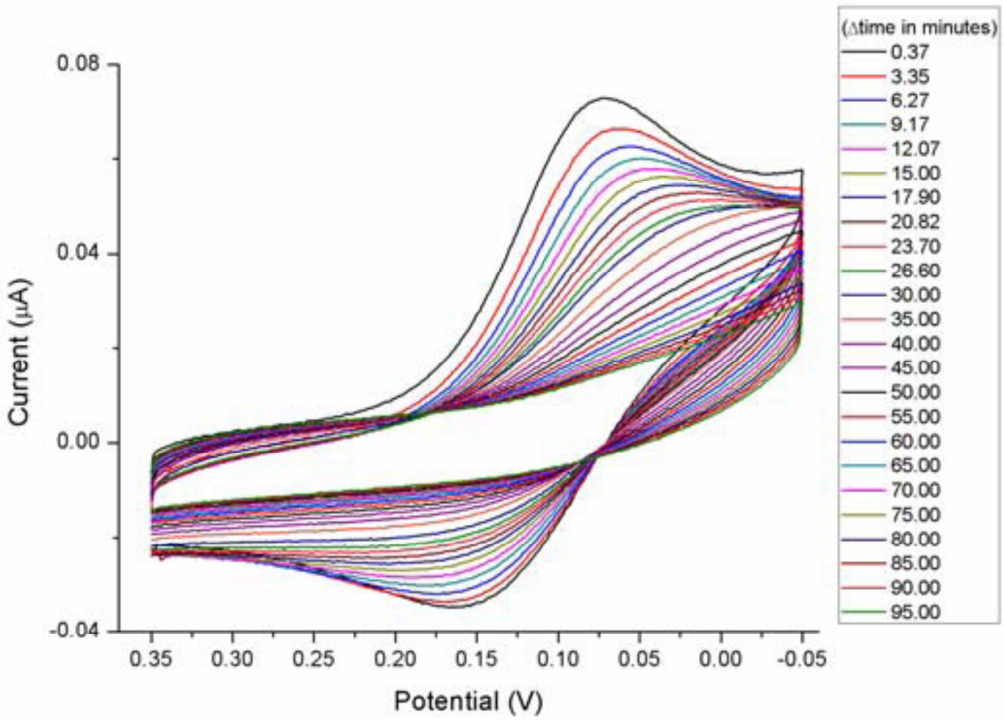
substrate binding site (4). Although plausible, this hypothesis was a distinct departure from the conventional view of peroxidase catalysis that electron transfer typically occurs from substrates positioned externally at the porphyrin edge of the heme. Binding of substrates in internal pockets in enzymes is, however, well established for cytochrome P450s. Recently it has been found that the internal site in DHP is an inhibitor binding site, rather than a substrate binding site. All 4-halophenols are capable of acting as inhibitors with the larger halogen atoms having the tightest binding (5). Consistent with this observation, it has been found that 2,4,6-trihalophenols, such as the native substrate 2,4,6-tribromophenol, are too large to gain entry into the distal pocket at ambient temperature and the evidence suggests that substrate oxidation takes place at an external binding site similar to other known peroxidases (6).

Understanding the redox properties of DHP is essential for understanding the functioning of the enzyme. For its dioxygen binding function, Fe(II) is the relevant redox state in DHP, as is the case with all globins. We have experimentally shown that Fe(II) DHP autooxidizes to Fe(III) DHP (unpublished data), which again is typical for a globin. For its enzymatic peroxidase function, DHP utilizes the high valent Fe(IV)=O oxyferryl redox state typical of peroxidases and cytochrome P-450's. This state can be reached from the Fe(III) resting state by reaction with hydrogen peroxide. The electrochemical research that we undertook has dealt only with the Fe(III)/Fe(II) redox couple of DHP. The objectives of this research were to 1) develop the diffusional and non-diffusional formats of DHP voltammetry, 2) to characterize the Fe(III)/Fe(II) redox thermodynamics, 3) to investigate the effect of substrate binding on redox properties, and 4) to undertake mutational studies aimed at elucidating the role of a peroxidase structural motif known as the aspartate-histidine-iron (Asp-His-Fe) triad. Descriptions of each of these areas are provided in the following sections.

Voltammetry of DHP.

Although DHP is structurally a globin, our examination of its molecular surface from available x-ray crystal structures revealed a key similarity to cytochrome c (cyt c), namely a positively charged lysine patch near its heme edge. The archetypal lysine patch of cyt c is known to be essential for electrostatic binding and ET reactions with other biological redox partners as well as negatively charged electrode surfaces. Accordingly, an underlying tenet of this proposal was that cyt c, for which diffusional and non-diffusional voltammetry have been well worked out, could serve as a structural model for guiding the development of interfacial DHP electrochemistry. Although this tenet has been borne out, developing the voltammetry of DHP has presented numerous challenges that are still being worked out. Although we routinely obtain voltammetric responses for DHP, they are considerably less reversible and less stable than those typical of cytochrome c. One major reason for this inferior electrochemical behavior relates to two functional and structural aspects where DHP differs from cyt c. First, the primary function of globins is not electron transfer, as is the case for cytochromes. Thus the intrinsic electron transfer kinetics of DHP are expected to be much slower than for cyt c. Second, the lysine patch on DHP (4-5 lysines) is less extensive than that for cytochrome c (8-9 lysines), and the overall electrostatics are not as favorable. The net charge on DHP is +1 whereas the net charge on cyt c is +9. In addition to the fact that DHP is simply not as well suited for interfacial electron transfer, we encountered issues with sample purity that appear to be related to the instability of the voltammetric responses. Figure 1 shows typical diffusional cyclic voltammetry obtained for 15 uM DHP under aerobic conditions at a HSC_8OH/HSC_7COOH (3:1) mixed SAM modified gold electrode in 4 mM phosphate buffer, pH 6, scanned at 5 mV/s. As can be seen the initial

voltammogram is quasi-reversible but undergoes a time-dependent change to considerably more irreversibly behavior. As a result of this voltammetric instability, we have been forced to examine the thermodynamic redox properties of DHP using mediated electron transfer electrochemistry techniques (see below).



A

Figure 1

Figure 2 shows a typical non-diffusional CV response obtained for DHP under aerobic conditions immediately following adsorption to a (1:1) $HSC_{11}OH$ / $HSC_{10}COOH$ mixed self-assembled monolayer on an evaporated gold film electrode at a scan rate of 0.1 V/s in 6.0 mM potassium phosphate buffer, pH 6.00. The peak area is consistent with approximately one-third of a monolayer of electroactive DHP.

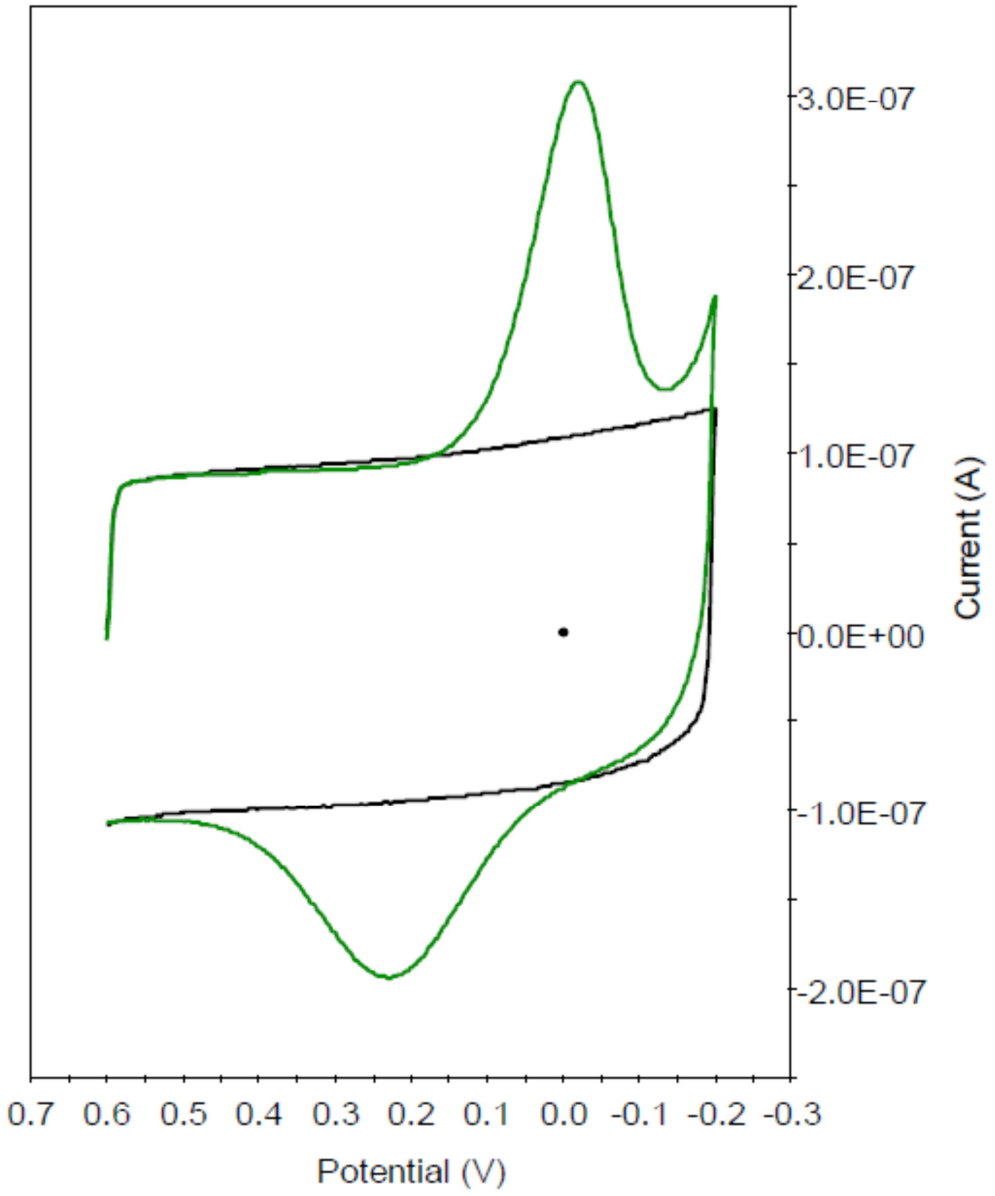


Figure 2

Although the initial CV response following adsorption is well formed, the signal is gradually lost over a period of minutes to an hour (data not shown), ostensibly due to desorption and/or surface denaturation. Efforts are continuing to optimize the interfacial electrochemistry of DHP.

Fe(III)/Fe(II) Redox Thermodynamics.

Anaerobic thin-layer spectroelectrochemistry was used to measure the formal reduction potential (E°') of the Fe(III)/Fe(II) redox couple of DHP over the pH 5-7 range. In this work, electron transfer mediators were employed to couple the DHP heme to the electrode surface. Typical results are shown here for a pH of 7.

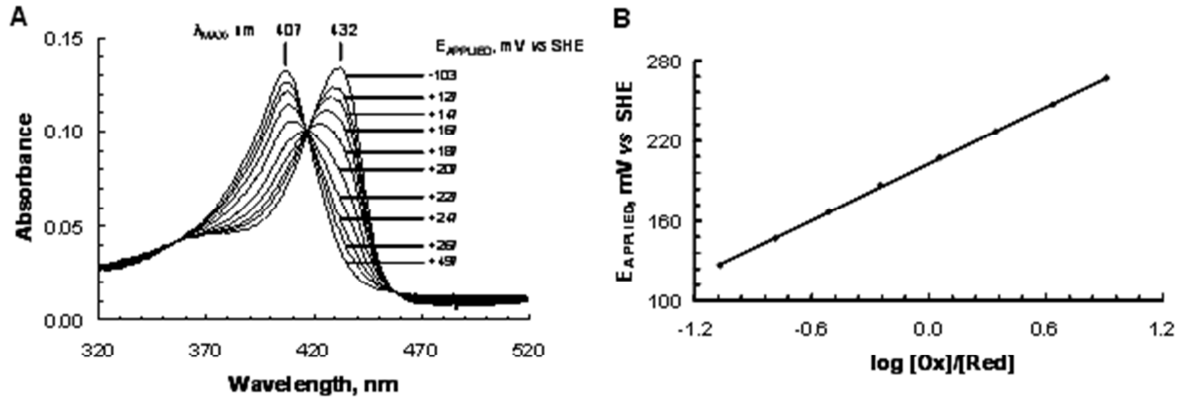
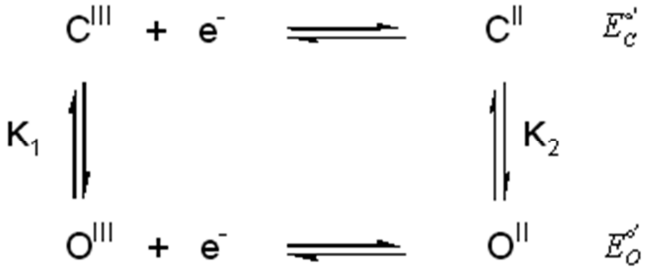


Figure 3

The value of E°' determined at pH 7 (100 mM potassium phosphate buffer under ambient temperature) was +0.202 ± 0.006 V *vs* SHE. Remarkably, this gives DHP the most positive Fe(III)/Fe(II) reduction potential among known intracellular globins, falling approximately 150 mV and 50 mV above typical myoglobins and hemoglobins, respectively. What makes this finding so remarkable is the fact that DHP has a significant peroxidase activity. Unlike globins, whose fairly positive reduction potentials stabilize the Fe(II) state in order to effect dioxygen binding, peroxidases typically feature considerably more negative reduction potentials that stabilize the Fe(III) resting state. For example, horseradish peroxidase has a formal potential that falls 0.5 V more negative. We have proposed that the ability of DHP to maintain such a positive

potential appears to derive from an unusual redox-coupled conformational change involving the distal histidine (H55), which has previously been shown to correlate with the coordination state of the heme iron. In the internal (closed) conformation, H55 hydrogen bonds with and stabilizes iron-coordinated H_2O, whereas in the external (open) conformation, H55 hydrogen bonds to a heme propionate and results in a five-coordinate Fe. We have shown that the following thermodynamic cycle that links electron transfer to the conformational change is consistent with an Fe(II) state that strongly favors the open conformation.



In this cycle, C^{III} and C^{II} denote the Fe(III) and Fe(II) states in the closed (C) conformation, and O^{III} and O^{II} denote the Fe(III) and Fe(II) states in the open (O) conformation. We have also identified a second contribution to the driving force that appears to arise from destabilization of the ferric state due to repositioning of H55 relative to other globins.

Substrate Binding on DHP Redox Properties

At ambient temperature, 2,4,6-trihalophenol (TXP) substrate molecules are known to react at an exterior surface location that has not yet been identified (*6,7)*. However, there are other physiological halophenol derivatives, such as the monosubstituted para-halophenols (4-XP), which are known to bind internally and to inhibit the peroxidase function. If DHP is found

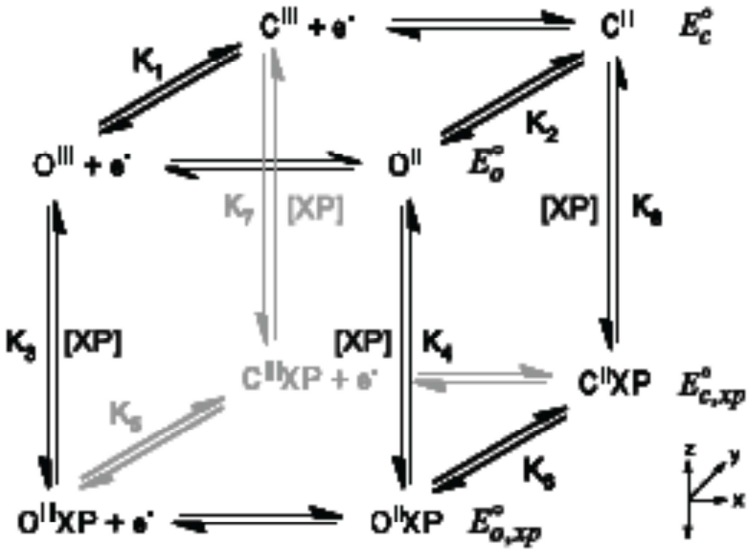
to follow the conventional peroxidase heme-edge electron transfer route for substrate oxidation, then the substrate binding site is expected to reside external to the active-site region. To complement existing data concerned with halophenol binding to DHP, we undertook a redox thermodynamic study focusing on the modulation in the Fe(III)/Fe(II) reduction potential that occurs upon exposure of DHP to various halophenol molecules. Anaerobic thin-layer spectroelectrochemistry was used to measure the formal reduction potential of DHP in the presence and absence of various halophenols including mono-, di-, and tri-halogenated. The data obtained are shown in the following table where the final column indicates the concentration ratio of halophenol (XP) to protein (P):

	E^o (mV)	E^o Shift (mV)	pH	$[XP_t] / [P_t]$
no exposure	204 ± 5		7	
no exposure	218 ± 1		6	
4-chlorophenol	170 ± 6	-48	6	45
4-bromophenol	164 ± 3	-54	6	45
2,4-dichlorophenol	149 ± 2	-69	6	45
2,4-dibromophenol	159 ± 8	-59	6	10
2,4,6-trichlorophenol	193 ± 5	-25	6	10

It can be seen that a modulation of the Fe(III)/Fe(II) oxidation/reduction potential occurs upon exposure to any of the halophenols and that the shifts are always negative, indicating that binding is stronger to the Fe(III) form of DHP relative to the Fe(II) form. The modulation is more substantial for internally binding mono-substituted halophenols (4-XP) than for the externally binding tri-substituted halophenols (2,4,6-TXP). From these data, it also appears likely that di-substituted halophenols (2,4-DXP) are also able to bind into the heme pocket given the similarity of their reduction potential shifts to those of the 4-XP species. If this proves to be a valid conclusion, the implication would be that the binding cavity could be important to the overall

function of the enzyme for reasons not considered before since the di-substituted halophenols are active substrates of the enzyme (whereas the monosubstituted halophenols are known to be inhibitors).

As noted in the previous section, the DHP Fe(III)/Fe(II) electron transfer reaction is coupled to a conformational change involving the distal histidine (H55). In light of the impact of substrate (XP) binding described in the current section, we have developed a thermodynamic cubic scheme that accounts for these interactions:



The equilibrium constants for the DHP Fe(III) state are denoted using superscript "III" Roman numerals and are defined as follows:

$K_1 = [O^{III}]/[C^{III}]$

$K_5 = [O^{III}XP]/[C^{III}XP]$

$K_3 = [O^{III}][XP]/[O^{III}XP]$

$K_7 = [C^{III}][XP]/[C^{III}XP]$

The equilibrium constants for the DHP Fe(II) state are denoted by superscript "II"

Roman numerals and are defined similarly:

$K_2 = [O^{II}]/[C^{II}]$

$K_6 = [O^{II}XP]/[C^{II}XP]$

$K_4 = [O^{II}][XP]/[O^{II}XP]$

$K_8 = [C^{II}][XP]/[C^{II}XP]$

The Fe(III)/Fe(II) redox couples are indicated for the following conformational and binding states:

O^{III}/O^{II} ($E°_o$): open conformation

C^{III}/C^{II} ($E°_c$): closed conformation

$O^{III}XP/O^{II}XP$ ($E°_{c,xp}$): open conformation with halophenol bound

$C^{III}XP/C^{II}XP$ ($E°_{c,xp}$): closed conformation with halophenol bound

Mutation of DHP at the Methionine-86 Position

Compared to other intracellular globins (myoglobins, hemoglobins), DHP displays a high level of peroxidase reactivity for the O – O bond cleavage reaction of heme iron-bound H_2O_2:

$$DHP + H_2O_2 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} HDHP\text{-}O_2H \xrightarrow{k_2} Cpd\ ES + H_2O$$

The rate constant for the cleavage step has been determined to be $k_2 = 3.56 \times 10^4\ M^{-1}\ s^{-1}$ at pH 7 (8). In comparison, the values that have been reported for globins are on the order of $10^2\ M^{-1}s^{-1}$ and those for peroxidases on the order of $10^7\ M^{-1}s^{-1}$. When H_2O_2 binds to peroxidases in the Fe(III) state, the peroxide undergoes deprotonation by the distal histidine yielding the Compound 0 (Cpd 0). In DHP, this occurs by H55 and forms the putative DHP Cpd 0 (HDHP-O_2H). O – O bond cleavage results in the formation of Compound ES (Cpd ES), a species oxidized two

electrons beyond the Fe(III) state that features an Fe(IV)-oxo/amino acid radical structure. H_2O_2 activation in peroxidases has been mechanistically interpreted in terms of the so-called "push-pull" effect (*9*). The "push" effect refers to either proximal side ligands or structure that control the amount of electron density that is "pushed" onto the Fe-atom, and the "pull" effect results from distal side acid/base catalysis and/or stabilization of developing negative charge on the heme iron-bound H_2O_2. The principal "push" effect in peroxidases has been attributed to a structural motif known as the Asp-His-Fe triad, which features an internal anionic electron-rich amino acid (Asp) (10). The Asp-His-Fe triad is absent in globins, and it is not known whether its presence would significantly affect the O-O bond cleavage rate constant. Despite prior attempts reported in the literature, protein engineering studies had failed to realize a stable globin mutant featuring this triad. In our work, we have been able to for the first time successfully install an Asp-His-Fe triad into a globin protein, DHP, by creating the stable M86D mutant. However, due to a structural rearrangement that occurs resulting in the formation of a 6cLS species (we propose that it is a ferric hemochrome based on both UV-visible and rRaman spectroscopy), we were unable to determine whether a "push" effect can affect the rate of O – O bond cleavage in a globin because the new sixth ligand, H55, acts as a strong inhibitor to peroxide binding. Two other M86 mutants were also prepared and characterized, the M86E and the M86A versions. These proteins were crystallized and x-ray crystal structures were solved and submitted to the Protein Data Bank in collaboration with Vesna de Serrano and Stefan Franzen. The mutants were also characterized by UV-Visible spectroscopy, C-13 nmr spectroscopy, and electrochemistry, and assayed for enzymatic activity. The following figure shows the UV-visible spectra for DHP(M86A) (blue), DHP(M86E) (red) and DHP(M86D) (black):

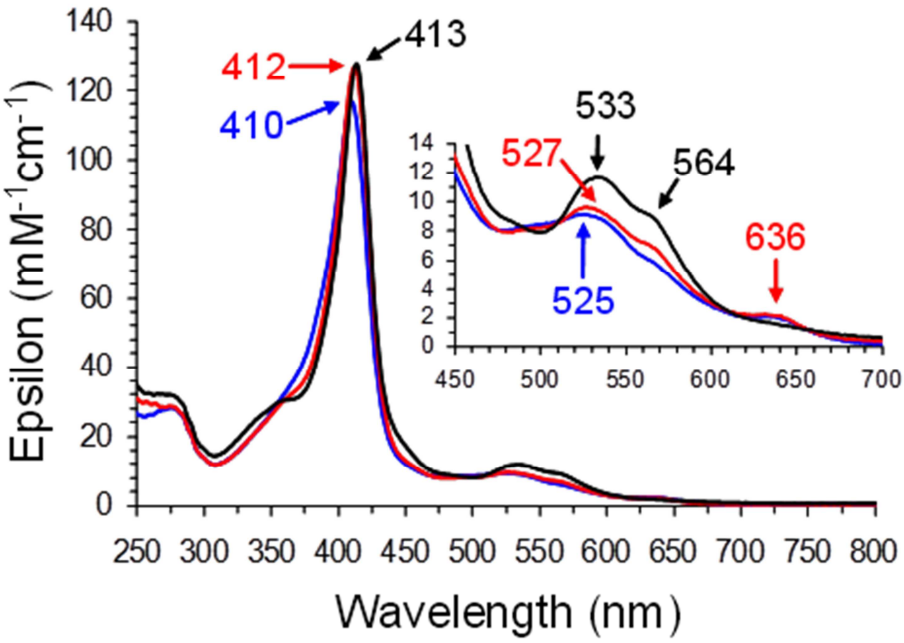
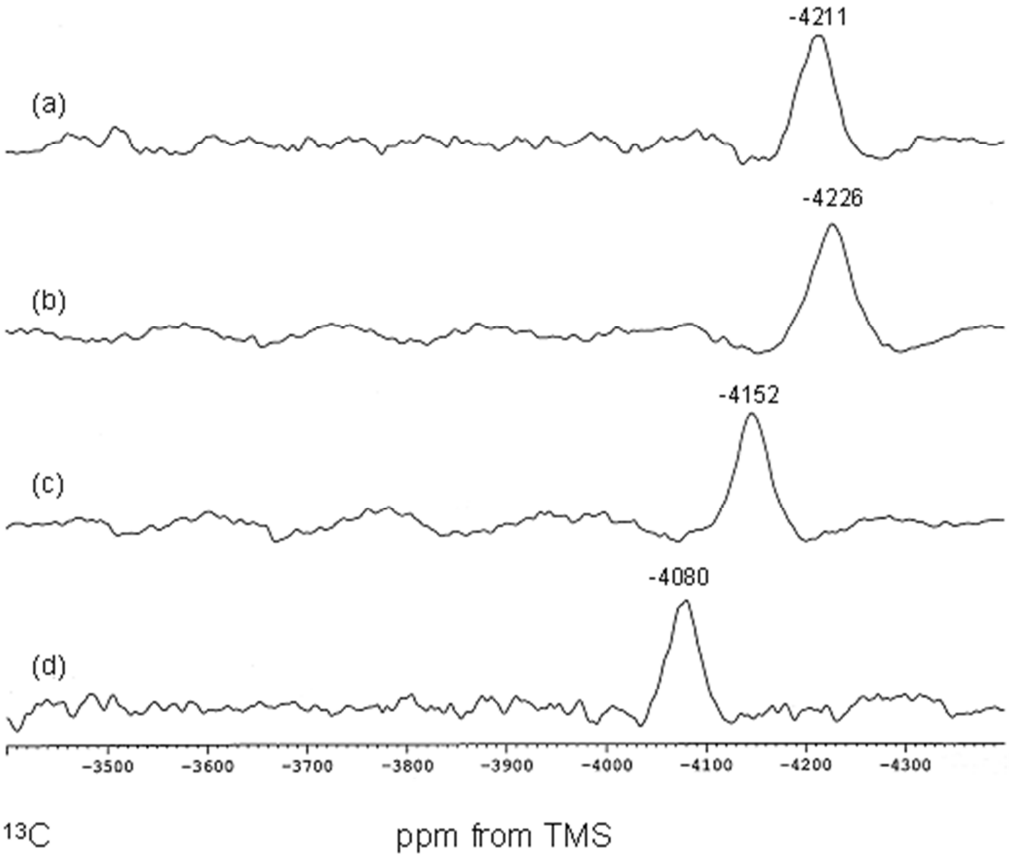


Figure 4

^{1}H and ^{13}C NMR spectra of the $^{13}C^{15}N$-labeled cyanide-bound ferric forms of wt-DHP, DHP (M86 mutants), horse myoglobin and horseradish peroxidase were collected at pH 7.0 and 25 °C. The next figure shows the ^{13}C nmr spectra for the DHP species: (a) wt-DHPA, (b) DHP(M86A), (c) DHP(M86E) and (d) DHP(M86D)). It was hoped that these spectra would allow us to evaluate the "push" effect by evaluating the amount of electron density on the heme iron but this was not possible due to the structural rearrangement that occurred in the M86D mutant giving rise to the 6cLS species. Nonetheless, we were able to correlate the electron density deduced from the nmr spectra with the reduction potentials of these mutants determined by thin layer spectroelectrochemistry. The higher electron densities on the M86E and M86E mutants were consistent with the more negative reduction potentials that were obtained, namely, 112 and 76 mV vs SCE, respectively.

B


-4211
(a)
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(b)
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(c)
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(d)
-3500
-3600
-3700
-3800
-3900
-4000
-4100
-4200
-4300
13C
ppm from TMS

Figure 5

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