ABSTRACT

THOMPSON, MATTHEW KYLE. Distal Histidine Flexibility as the Key to Reactivity in Dehaloperoxidase-Hemoglobin from *Amphitrite ornata*. (Under the direction of Dr. Stefan Franzen).

Benthic coastal ecosystems can be a hazardous place. Many marine organisms release toxic chemicals into the water to eliminate their enemies. One marine organism, *Amphitrite ornata*, is able to withstand the chemical warfare because it poses a natural enzymatic antidote, dehaloperoxidase-hemoglobin (DHP).

The enzymes dehaloperoxidase-hemoglobin A and B (DHP A & B) are unique hemoglobins that function as peroxidases, capable of converting 2,4,6-trihalophenols into the corresponding 2,6-dihalogenated quinones. Similar to many myoglobins, the distal histidine (H55) of DHP A is observed in two conformations, open and closed. The conformations regulate oxygen binding and release during transport and storage in typical hemoglobins and myoglobins. However, in DHP A, the two conformations play an even greater role in enzymatic function.

DHP A possesses a unique internal binding cavity in the distal pocket above the heme large enough to accommodate 4-halophenol molecules present in the benthic ecosystems. Internal binding of the 4-halophenol molecules pushes the distal histidine to the open conformation and inhibits peroxidase function of the enzyme. Binding of 2,4,6-trihalophenol molecules, however, produces the opposite effect. 2,4,6-trihalophenols bind to DHP in such a way the forces the distal histidine into the closed position and prepares the enzyme for activation by $H_2O_2$. Activation of DHP by $H_2O_2$ requires that the distal histidine be in the closed position in order to serve as the acid/base catalyst for peroxidase chemistry. Upon activation, a tyrosyl radical (Y34) used to oxidize the toxic chemical warfare agents is
formed on DHP to a yield of nearly 100%, an order of magnitude greater than typical globins. Additionally, in the absence of substrate molecules, the open and closed states of the distal histidine provide alternative free radical decay pathways of the activated form of DHP leading to protein cross-linking and inactivation. This may protect *A. ornata* from unwanted deleterious oxidation chemistry further exemplifying the distal histidine’s flexible role in enzymatic activity.
Distal Histidine Flexibility as the Key to Reactivity in Dehaloperoxidase-Hemoglobin from *Amphitrite ornata*

by

Matthew Kyle Thompson

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APPROVED BY:

________________________________________________________________________
Stefan Franzen
Committee Chair

________________________________________________________________________
David A. Shultz

________________________________________________________________________
Reza A. Ghiladi

________________________________________________________________________
Tatyana Smirnova

________________________________________________________________________
Steven A. Lommel
DEDICATION

To my parents, Gary and Dorothy, my brother Marshal, and my wonderful wife, Tacita.
BIOGRAPHY

Matthew Kyle Thompson was born on May 17th, 1978 to Gary and Dorothy Thompson. He has one older brother, Marshal D. Thompson. He graduated from Bardstown High School in 1996 and then went on to complete bachelor’s degrees in chemistry and geology, graduating from the Eastern Kentucky University Honors Program in 2004. Matthew chose to pursue a PhD in chemistry at North Carolina State University in 2005 and successfully defended his dissertation in 2011. He will continue his education as a postdoctoral associate at Vanderbilt University.
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# TABLE OF CONTENTS

LIST OF TABLES.......................................................................................................................... viii

LIST OF FIGURES.......................................................................................................................... x

LIST OF SCHEMES........................................................................................................................ xxiv

Chapter 1  **Dehaloperoxidase-hemoglobin: An Overview of Current Research..**  1

- Introduction................................................................................................................................. 2
- Structure and Function............................................................................................................... 4
- Spectroscopic Characterization of DHP and the Unusual Flexibility of the Distal Histidine................................................................. 8
- Two-Site Competitive Inhibition in Dehaloperoxidase-hemoglobin............................................... 13
- Compound ES and Free Radical Decay in DHP Depend on the Conformation of the Distal Histidine............................................................... 17
- Current Catalytic Reaction Mechanism of Dehaloperoxidase-hemoglobin........................................ 20
- References................................................................................................................................. 22

Chapter 2  **Dehaloperoxidase-hemoglobin from *Amphitrite ornata* is primarily a monomer in solution.............................................................................**  26

- Abstract........................................................................................................................................ 27
- Introduction................................................................................................................................. 28
- Methods........................................................................................................................................ 30
  
  - Dehaloperoxidase expression and purification................................................................. 30
  - Dehaloperoxidase sample preparation.................................................................................. 30
  - Gel permeation chromatography......................................................................................... 31
  - SDS Polyacrylamide gel electrophoresis............................................................................. 31
  - Small angle X-ray scattering data collection and analysis....................................... 31
- Results......................................................................................................................................... 34
- Discussion................................................................................................................................... 45
- Conclusion................................................................................................................................. 27
- References................................................................................................................................. 49
LIST OF TABLES

CHAPTER 2

Table 2.1 Structural parameters determined from P(r) analysis (GNOM) of the SAXS data on His<sub>6</sub>-DHP ± 4-iodo-phenol (4-IP) substrate and DHP minus the His<sub>6</sub>-tag ................................................................. 41

CHAPTER 3

Table 3.1 Crystallographic Data Collection and Refinement Statistics ................... 62
Table 3.2 Michaelis-Menten Fit Parameters .................................................................. 73

CHAPTER 4

Table 4.1 TRSSA input parameters for the simulation of the tyrosyl radical EPR signals in Figure 4.6 ................................................................. 100
Table 4.2 The optimized rate constants of the reactions included in the kinetic model .......................................................................................................................... 104

APPENDIX A

Table A.1 RR frequencies (in cm<sup>-1</sup>) in the high wavenumber region of the various ferric DHP species, their normal mode assignments and depolarization ratios (ρ) (in brackets) compared to those of Mb .......................................................... 132

APPENDIX C

Table C.1 Kinetic parameters from the curve fitting to the phenomenological Michaelis-Menten equation .......................................................... 174
Table C.2 Calculated Gibb’s Free energies .................................................................. 177
APPENDIX E

Table E.1  Experimental Cl KIEs determined for different chlorophenols degradation catalyzed by HRP and DHP at 277 K.............................................. 213

Table E.2  Calculated kinetic isotope effects and saddle point characteristics for dehalogenation of TCP in anionic and radical forms in water at 300 K... 213

Table E.3  Relative energies (kcal/mol) of maxima located during C-Cl elongation in a water environment with respect to the initial Meisenheimer complex........................................................................................................... 218

Table E.4  Calculated kinetic isotope effects and saddle point characteristics for dehalogenation of 4-CP in radical form catalyzed by HRP at 300 K...... 221

APPENDIX G

Table G.1  The TRSSA generated parameters used to simulate spectrum B in Fig. 4.6 (principal tyrosyl radical)........................................................................................................... 264

Table G.2  The TRSSA generated EPR simulation parameters used to simulate spectrum C in Fig. 4.6 (pH 5 tyrosyl radical)......................................................... 265

Table G.3  Six reaction components participating in the reactions...................... 270

Table G.4  Ten reactions considered in the model.............................................. 271

Table G.5  Ten reactions considered in the model (continued)........................... 273

Table G.6  Corrections to rate constants............................................................ 277

Table G.7  Rate optimization target parameters................................................. 280

Table G.8  Optimized rate constants................................................................. 281
LIST OF FIGURES

CHAPTER 1

Figure 1.1 *Amphitrite ornata*........................................................................................................ 3

Figure 1.2 Common brominated aromatics found in *A. ornata* environment.............. 3

Figure 1.3 Structural comparison of Sperm Whale Myoglobin (PDB 1A6G), Dehaloperoxidase-hemoglobin (PDB 2QFK), Cytochrome c Peroxidase (PDB 1ZBY), and Horseradish Peroxidase (PDB 2ATJ)......................... 5

Figure 1.4 Structural comparison of the active sites of Cytochrome c peroxidase (PDB 1ZBY) and Dehaloperoxidase-hemoglobin (PDB 1EW6). In CcP, the distal arginine is observed in two conformations, whereas in DHP, the distal histidine is observed in two conformations. DHP lacks most of the typical peroxidase machinery found in CcP......................... 7

Figure 1.5 Typical DHP enzymatic assay. When activated with H₂O₂, DHP catalyzes the oxidation of 2,4,6-trichlorophenol (2,4,6-TCP) to 2,6-dichlorobenzoquinone (2,6-DCQ).................................................................................. 8

Figure 1.6 Correlation of room temperature resonance Raman spectrum and X-ray crystal structure (PDB 1EW6). The RR spectrum shows the presence of two heme species, 5cHS and 6cHS, that are attributable to the open and closed conformation of the distal histidine, respectively................. 9

Figure 1.7 Correlation of cryogenic temperature resonance Raman spectrum and X-ray crystal structure. The RR spectrum shows the predominately 6cHS heme, and the crystal structure is composed of the closed conformation of the distal histidine with H₂O bound to the heme iron..... 11

Figure 1.8 Correlation of deoxy DHP resonance Raman spectrum and X-ray crystal structure. The RR spectrum shows the predominately 5cHS heme, and the crystal structure is composed of the open conformation of the distal histidine with no O₂ bound to the heme iron. The low frequency RR spectrum displays the \( \nu(Fe-Im) \) mode which is only observed in the 5cHS deoxy form of globins and peroxidases.............. 12

Figure 1.9 Inhibition of DHP by 4-bromophenol (4-BP). In the presence of 4-BP, no 2,6-DCQ product is formed.......................................................... 14
Figure 1.10  Resonance Raman spectrum and X-ray crystal structure of DHP in the presence of 4-BP. The X-ray crystal of inhibitor-bound DHP displays an open distal histidine (H55) with no H$_2$O bound to the heme Fe, and the RR spectrum shows a predominately 5cHS heme.............................................. 15

Figure 1.11  Resonance Raman spectrum DHP in the presence of 2,4,6-TCP. The RR spectrum shows a predominately 6cHS heme species. The corresponding structure shows a depiction of a tri-halogenated phenol forcing the distal histidine into the closed position............................................ 16

Figure 1.12  EPR spectra of ferric DHP at different pH. The EPR spectra of ferric DHP at different pH can be deconvoluted into two species, and they are correlated to the two conformations of the distal histidine. The populations of the two species are stoichiometric with pH................................. 18

Figure 1.13  RFQ-EPR data showing pH-dependent tyrosine radical lineshapes. At pH 7, the free radical yield is nearly 93%, atypical of globins reacting with H$_2$O$_2$.................................................................................................. 19

Figure 1.14  Proposed radical pathways for productive and non-productive substrate oxidation. When the distal 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...................................................... 20

Figure 1.15  Proposed catalytic cycle of dehaloperoxidase-hemoglobin............................ 21

CHAPTER 2

Figure 2.1  Gel permeation chromatogram of the elution of an initial mixture GFP (MW ~ 27 kDa) and DHP (MW ~ 15.5 kDa)................................................................. 34

Figure 2.2  SDS-page of fractions collected from gel chromatography. The first and last lanes of each gel are protein standards. The remaining lanes are loaded with fractions 2, 4, 6, 8, etc. up to fraction 40 collected for the gel permeation chromatography. The boxed lanes represent fractions 16-20 for peak 1 and fractions 30-34 for peak 2 of the chromatogram in Figure 2.1 that were collected and analyzed by electronic absorption spectroscopy......................................................... 35

Figure 2.3  Electronic absorption spectra of DHP (solid line) and GFP (dashed line) after separation on the Sephacryl S-100 size exclusion column.
The spectra are of the concentrated fractions corresponding to the peak maxima of the chromatogram.

Figure 2.4 Single protein chromatograms of HHMb (dashed line) with MW = 17 kDa and DHP (solid line) with MW = 15.5 kDa.

Figure 2.5 Small-angle X-ray scattering Intensity I(Q) versus momentum transfer, Q, for (A) the His6-DHP concentration series at 3.0 mM (●), 2.0 mM (■), 1.5 mM (▲), 1.0 mM (◇) and 0.3 mM (▼) and for (B) the DHP minus His6-tag concentration series at 2.6 (○), 0.62 (□) and 0.31 mM (△). The solid lines represent final fits of the DAMMAVER models to these data. The χ2 fits of these models to the measured scattering data are 1.4 (A) and 2.2 (B).

Figure 2.6 P(r) profiles, calculated using PrView, from 3.0 mM His6-DHP (●) and 2.6 mM DHP (minus his6) (○). The dotted and the dashed lines represent the interatomic distance distribution for a monomer and a dimer, respectively, determined from the DHP crystal structure (pdb no. 2QFN). The solid red line represents P(r) profile expected from a 90:10 mixture of monomer and dimer calculated using the I(q) determined from the crystal structures. P(r) were normalized to unity.

Figure 2.7 Shown on the left is the average of 15 DAMMIN-derived model fits to the 3 mM His6-DHP SAXS data (orange balls) overlaid (using SUPCOMB) onto the structure (ribbon representation) of the dimer of DHP (pdb no. 2QFN). For orientation, the hemes are shown in ball-n-stick. Displayed on the right are four of the 15 individual DAMMIN models (NSD values for all varied from 0.483 – 0.626).

CHAPTER 3

Figure 3.1 Reaction scheme emphasizing the conformation of the distal histidine, H55, in response to binding of substrate, 2,4,6-TXP, and inhibitor, 4-XP. (a) Active enzyme: DHP with TXP substrate bound external to the heme pocket. The protein is 6cHS (aquo) with the distal H55 in a closed position. (b) When an inhibitor, 4-XP, binds in the internal pocket of DHP, H2O is displaced (5cHS) and the distal H55 is pushed to the open position. The resulting conformation leads to inactivation of the enzyme. (c) Addition of H2O2 leads to the formation of compound ES (37), the high-valent iron-oxo protein (Tyr) radical intermediate, which can lead to formation of the product 2,6-DXQ by
two-electron oxidation. Compound ES cannot be formed in the inhibitor-bound state since the Fe$^{III}$ site is blocked by 4-XP binding in the distal cavity. (d) In the resting state of DHP the distal histidine exists in two conformations known as open (red) and closed (blue). H$_2$O is bound to the heme iron only in the closed conformation.............

Figure 3.2 X-ray crystal structures of DHP at 100 K. (a) The metaquo form shows a hydrogen-bond interaction between the distal histidine (H55) and the water molecule coordinated to the ferric heme iron (PDB 2QFK) (closed conformation). (b) The deoxy form has a pentacoordinate heme iron. Two rotamers of H55 are observed in which H55 appears exclusively in a solvent exposed conformation (PDB 3DR9). This His conformation corresponds to the open form observed in Mb at pH 4.5 (15). (c) The three structures of 4-IP (3LB1; green), 4-BP (3LB2; light blue) and 4-CP (3LB3; grey) bound in the distal pocket of DHP are superimposed. 4-FP (3LB4) is not shown in the figure (see text)..........

Figure 3.3 Determination of internal binding affinity. (a) RR core size marker band region for WT-DHP (black), DHP with phenol (yellow), DHP with 4-FP (purple), DHP with 4-CP (blue), DHP with 4-BP (green), and DHP with 4-IP (red). The $\nu_2$, $\nu_3$ and $\nu_{10}$ frequencies are consistent with the increase of a 5cHS iron upon addition of the inhibitors. The final concentration of 4-IP was 1 mM (maximum solubility), and the final concentration of 4-BP, 4-CP, 4-FP, and phenol was 8 mM; protein concentration was 100 µM. Excitation wavelength was 406 nm; 1.7 cm$^{-1}$ resolution; laser power at the sample 60mW, and 300 s acquisition times. (b) Binding isotherms for 4-IP (red), 4-BP (green), 4-CP (blue), 4-FP (purple), and phenol (yellow). The isotherms clearly establish that the affinity of DHP for 4-XP inhibitors follows the pattern 4-IP > 4-BP > 4-CP > 4-FP, > phenol at pH 6..........................

Figure 3.4 Evidence of external binding. Resonance Raman core size marker band region for WT-DHP (a), DHP with 2,4,6-TBP (b), DHP with 2,4,6-TCP (c), and DHP with 2,4,6-TFP (d). The $\nu_2$, $\nu_3$, and $\nu_{10}$ frequencies shift to predominately 6cHS upon addition of the TXP substrates. The 5cHS population observed upon addition of 2,4,6-TFP may indicate that 2,4,6-TFP does enter the distal pocket to some degree, in agreement with cryogenic experiments (16, 35, 36). The final concentration of 2,4,6-TBP was 200 µM, and the final concentrations of 2,4,6-TCP and 2,4,6-TFP were 4 mM in 150 mM potassium phosphate buffer, pH 6. Excitation wavelength was 406 nm; 1.7 cm$^{-1}$ resolution; laser power at the sample 60mW, and 300 s acquisition times........................................
Figure 3.5 Kinetic assays showing inhibition by internal bound 4-XPs. (a - e) Time-dependent UV-Vis spectra from 0 seconds (red) to 120 seconds (purple). In the absence of 4-XP (a), the TCP substrate (312 nm) is converted to the DCQ product (273 nm). In the presence of 4-IP, 4-BP, and 4-CP (b - d), little product is formed with no significant decrease in the substrate band. In the presence of 4-FP (e), some turnover is observed, in agreement with the lower affinity of 4-FP to bind internally. (f) Inhibition of compound ES formation ($\lambda_{\text{max}}$ (Soret) = 420 nm) in ferric DHP at pH 7 due to increasing concentrations of 4-BP.

Figure 3.6 Michaelis-Menten analysis of inhibition. Initial reaction velocity versus substrate (2,4,6-TCP) concentration. Assays conducted of WT-DHP without the presence of inhibitor, WT-DHP with 125 uM 4-BP, WT-DHP with 250 uM 4-BP, and WT-DHP with 500 uM 4-BP. The Michaelis-Menten fit parameters are given in Table 2.

CHAPTER 4

Figure 4.1 EPR spectra of ferric DHP at different pH values and the result of their deconvolution into two individual HS ferric heme EPR signals, rhombic type 1 (R1HS) and ‘nearly axial’ (NAHS). The spectra were obtained at 10 K from the samples made by freeze-quenching 80 uM DHP at the indicated pH values mixed with equal volumes of buffer to give a final concentration of 40 uM. All spectra are plotted at similar amplitudes for comparative viewing. The instrumental conditions were as follows: microwave frequency $\nu_{\text{MW}} = 9.471$ GHz, microwave power $P = 3.18$ mW, modulation frequency $\nu_{m} = 100$ kHz, modulation amplitude $A_{m} = 5$ G, time constant $\tau = 82$ ms, scan rate $v = 22.6$ G s$^{-1}$, number of scans per spectrum $N_{S} = 1$. The principal g-factors of the signals R1HS and NAHS are indicated.

Figure 4.2 Relative concentration (left axis) of the two HS ferric heme forms in DHP, ‘rhombic type 1’ (R1HS) and ‘nearly axial’ (NAHS), at three different pH values. The error bars indicate maximal deviation from the average determined on the basis of three independent measurements. The concentration units are normalized to give the total concentration equal to unity. Also shown are the values of the rate constants $k_1$ (Compound ES formation, right axis) that have been obtained from the kinetic model described in the Kinetic Model section.
Figure 4.3  EPR spectra of 40 µM DHP reacted with 150 µM H_{2}O_{2} and freeze-quenched 0.5 s after mixing (all concentrations are final, pH 7). The control sample was obtained by mixing equal volumes of 80 µM DHP and pH 7 buffer and freeze-quenching 50 ms after mixing. Inset shows the g=6 area in greater detail with the H_{2}O_{2} treated spectrum magnified by a factor of 10. The spectra were recorded at 10 K at the same instrumental conditions as specified in the Fig. 4.1 legend......................

Figure 4.4  Kinetic dependences of total ferric heme state concentration in the course of the reaction of 40 µM ferric DHP with 120 µM H_{2}O_{2}, at pH 5, 6 and 7 (both concentrations are final). The ferric heme concentrations (symbols) were determined by EPR spectroscopy in the samples prepared by the RFQ method as described in the text. The lines are calculated from the kinetic model described in section Kinetic Modeling................................................................. 96

Figure 4.5  Kinetic dependences of total free radical concentration in the three pH series referred to in Fig. 4.4. The EPR spectra of the free radicals detected in these series are given in Fig. G.6. Symbols represent the experimental results; the lines are generated by the same kinetic model used to plot the curves in Fig. 4.4 (see section Kinetic Modeling for details).................................................................................................................. 96

Figure 4.6  Lineshapes of the free radical EPR spectra of the freeze-quenched samples of 40 µM DHP treated with 120 µM H_{2}O_{2}. A, pH 5, 50 ms after mixing. B, pH 7, 300 ms after mixing, multiplied by a factor of 0.06. The factor 0.06 was chosen empirically to construct a pure lineshape of the signal C=A-B. Both the principal radical (B) and pH 5 radical (C) EPR signals were simulated as neutral tyrosyl radical spectra (dotted lines). The parameters of the simulation (Tabel G.1 and Tabel G.2) were found by TRSSA............................................................... 99

Figure 4.7  Effect of hydrogen peroxide molar excess over heme on the extent of heme-protein cross-link formation in 80 µM DHP (curves, left axis). The concentration of the heme to protein cross-links was determined by HPLC, the dependences at three different pH values are presented. Three crosses are related to the right axis and represent the rate constant k_{2} of Compound ES to Compound RH conversion at the three pH values as found from the kinetic model described in section Kinetic Modeling.............................................................................................................. 101

Figure 4.8  Selected residues in the heme environment of DHP. Tyr34, the site suggested to host the principal radical, is in van der Waals interaction
with the heme porphyrin. Arg33 is in close proximity and might play a role in substrate binding. The pH 5 radical is proposed to be located on Tyr38 which can make a strong hydrogen bond with the distal His55. The PDB structure file 2QFK was used to generate the figure.

Figure 4.9 Conversion of the protonated oxoferryl heme to a ‘ferric heme + protein radical’ state, when the distal His55 is in the closed conformation. The Tyr38• radical is formed (the pH 5 radical) which might be in an equilibrium with its protonated form, the cation radical.

Figure 4.10 The optimized values of the second order (A) and first order (B) rate constants used in the kinetic model (see 3.2.5) are plotted as functions of pH. For comparative viewing, $k_3$ and $k_4$ dependences (B) are multiplied by the factors of 25 and 250, respectively, to bring the pH 5 data point to a similar position on the graph. Rate constants $k_6-k_{10}$ are set in the model to be proportional to $k_5$ and therefore they have the shape of the pH dependences identical to that of the $k_5$ dependence, and are not shown.

APPENDIX A

Figure A.1 Absorption spectra (panel A) and RR spectra (panel B) of $(\text{Fe}^{3+})\text{DHP}$ at pH 6 (a), $(\text{Fe}^{3+})\text{DHP-F}$ at pH 5 (b), $(\text{Fe}^{3+})\text{DHP-OH}$ at pH 9.6 (c) in 0.15 M potassium phosphate. Experimental conditions panel B: (a) 406.7 nm excitation wavelength, 5 mW laser power at the sample, average of six spectra with 300 s integration time, 1.3 cm$^{-1}$ spectral resolution; (b) 406.7 nm excitation wavelength, 5 mW laser power at the sample, average of twelve spectra with 300 s integration time, 1.3 cm$^{-1}$ spectral resolution; the asterisk indicates the reduced form (1354 cm$^{-1}$); (c) 413.1 nm excitation wavelength, 6 mW laser power at the sample, average of three spectra with 600 s integration time, 1.2 cm$^{-1}$ spectral resolution. The intensities are normalized to that of the $\nu_4$ band. Spectra have been shifted along the ordinate axis to allow better visualization.

Figure A.2 RR spectra of DHP (centre), DHP-4IP (1:30 molar ratio) (right), and DHP-TFP (1:320 molar ratio) (left) at 298 K (top) and 12 K (bottom) at pH 6. Experimental conditions: $\lambda_{exc}$: 406.7 nm, spectral resolution 1.3 cm$^{-1}$ (298 K) and 5 cm$^{-1}$ (12 K), power at the sample 5 mW (298 K) and 8 mW (12 K). DHP: average of six spectra with 300 s integration time (298 K), collection interval 8 s/0.5 cm$^{-1}$ (12 K). 4IP: average of six spectra with 300 s integration time (298 K), collection interval 6
Curve-fitting of the RR spectra of (Fe$^{3+}$)DHP at pH 6 (A), (Fe$^{3+}$)DHP-F at pH 5 (B), and (Fe$^{3+}$)DHP-OH at pH 9.6 (C) in 0.15 M potassium phosphate for parallel (∥) and perpendicular (⊥) polarized light, taken with 406.7 nm (A and B) and 413.1 nm (C) excitation wavelength.

Low frequency region RR spectra of (Fe$^{3+}$)DHP-OH. Experimental conditions: excitation wavelength 413.1 nm, 6 mW laser power at the sample, 1.2 cm$^{-1}$ spectral resolution. H$_2$O at pH 9.6 (average of three spectra with 900 s integration time), D$_2$O at pD 10.0 (average of twelve spectra with 900 s integration time) and H$_2^{18}$O at pH 9.6 (average of eighteen spectra with 900 s integration time). The intensities are normalized to that of $v_7$ (not shown). Spectra have been shifted along the ordinate axis to allow better visualization.

X-band EPR spectra showing the low field $g_\perp$ region of DHP (a), DHP-TFP 1:10 (b) and 1:320 molar ratio (c) and DHP-4IP (1:30 molar ratio) (d) at pH 6 in 0.15 M phosphate/glycerol 30 % (v/v). The spectra were recorded at 5 K, 1 mW microwave power, and 10 G modulation amplitude. Spectra have been shifted along the ordinate axis to allow better visualization.

Comparison between the heme binding pockets of (Fe$^{3+}$)Mb (PDB code 1A6K), (Fe$^{3+}$)DHP (PDB code 2QFK (subunit A)), and (Fe$^{3+}$)CCP (PDB code 1ZBY), showing the key residues and water
molecules in each case........................................................................................................ 145

APPENDIX B

Figure B.1   UV-Vis absorption spectra of (Fe³⁺) DHP (solid line) and 4IP-(Fe³⁺) DHP (dashed line) at pH 6 in 0.15 M potassium phosphate.......................... 159

Figure B.2   RR spectra of TFP-(Fe³⁺)DHP (40:1 molar ratio) at 12 (bottom) and 298 K (top) at pH 6 in 150 mM phosphate and 30% (v/v) of glycerol. Experimental conditions: λ_{exc}: 406.7 nm, spectral resolution 1.3 cm⁻¹ (298 K) and 5 cm⁻¹ (12 K), power at the sample 5 mW (298 K) and 8 mW (12 K), average of four spectra with 300 s integration time (298 K), collection interval 8 s/0.5 cm⁻¹ (12 K). Spectra have been shifted along the ordinate axis to allow better visualization........................................ 160

APPENDIX C

Figure C.1   Structures of DHP A with substrate and inhibitor bound to the enzyme. A.) Metaquo form with inhibitor bound (3LB1 is shown, but 3LB2, 3BL3, and 3LB4 are similar in structure); H55 open. B.) Deoxy form of DHP (3DR9);H55 open. 1C.) Metaquo form of DHP (2QFK); H55 closed D.) Metaquo form with substrate at proposed external substrate binding site; H55 closed........................................................................................................ 164

Figure C.2   Possible routes from phenol to quinone subsequent electron transfer to the heme oxoferryl intermediates of the DHP A enzyme. These structures were used for DFT calculations described in the text............. 165

Figure C.3   Single wavelength kinetics at 273 nm of the oxidation reaction of TCP by DHP as a function of time and product DCQ concentration obtained using a UV-VIS spectrometer. Assay conditions were [Ferric DHP] ~2.4μM, [TCP] ~ 150μM, [H₂O₂] ~ 1.2 mM, pH 7 KB buffer........................................ 173

Figure C.4   Single wavelength kinetics of oxidation of TCP by DHP as a function of substrate TCP concentration and enzymatic reaction initial velocity. The assay conditions were Ferric DHP ~2.1 μM, [TCP] ~ 150μM, [H₂O₂] ~ 0.1-1.9 mM, pH 7 KB buffer.......................................................... 173

Figure C.5   Plot of ln(k_{cat}) vs. 1/T with a fit to a line according to the Arrhenius Equation ln k_{cat} = -E_a/RT+ ln A. k_{cat} was obtained from the fit of the
initial velocity data to Eqn. 1................................................................. 175

Figure C.6  The Linear fit of ln(k_cat/K_M) vs. 1/T according to Arrhenius Equation (Table C.1). k_cat/K_M is sometimes called the efficiency of the enzyme... 176

Figure C.7  Energy level diagram based on DFT calculations.......................... 182

APPENDIX E

Figure E.1  The example of UV-Vis monitoring of the substrate conversion catalyzed by DHP. Panel A shows the spectrum of TCP disappearance and panel B presents the dependence of the absorbance at 313 nm on the degree of substrate conversion. The reactions were conducted in 100 mM potassium phosphate buffer (pH 7.0) at 4°C.............................. 204

Figure E.2  Two possible external binding sites of DHP: panel A – near the heme edge, panel B – dimer interface................................................................. 211

Figure E.3  Overlaid structures of reactants shown in magenta and a Meisenheimer complex (Mc) in yellow. Numbers indicate O-H bond lengths in Å in an attacking water molecule................................................................. 215

Figure E.4  Atom numbering in TCP molecule................................................... 216

Figure E.5  Snapshots of the intermediate structures obtained during elongating C-Cl bond in water. Panel A: three major structures related to the first observed maximum on the energy profile, panel B: three major structures related to the second observed maximum on the energy profile; in both cases, the starting point is shown in magenta, the maximum in orange and the first point right after it in green........................................ 218

Figure E.6  Interaction energy between bound 4-chlorophenol and neighboring residues in the active site of horseradish peroxidase. Red and blue bars denote substrate-aminoacid interaction energy for chlorine- and oxygen-facing the heme iron orientation, respectively.......................... 220

Figure E.7  The geometries of the enzyme-substrate (ES) and the enzyme-Meisenheimer complex (EMc) formed during the attack of water molecule on the cationic form of 4-CP in the active site of HRP. The numbers indicate the key geometrical parameters................................................. 223

Figure E.8  The comparison of interaction energies between bound 4-chlorophenol
(S) and formed Meisenheimer complex (Mc) and the neighboring residues in the active site of horseradish peroxidase. Red and blue bars denote substrate- and Meisenheimer complex-aminoacid interaction energy, respectively........... 225

Figure E.9 Overlaid structures obtained on C-Cl elongation pathway for MCP catalyzed by HRP. EMc complex is shown in green, the maximum on the energy profile in orange and the final – product structure in magenta.................. 226

Figure E.10 The estimated binding energies for MCP and TCP bound in the different sites of DHP.......................................................... 227

Figure E.11 Relative energies (kcal/mol) of Mc complex formed in water, DHP and HRP with respect to the corresponding substrate (in cation form) state.. 229

APPENDIX F

Figure F.1 Electronic absorption spectra of (black) WT-DHP and DHP with (yellow) phenol, (purple) 4-FP, (blue) 4-CP, (green) 4-BP, and (red) 4-IP. The Soret maximum undergoes a systematic blue shift as the substrate halogen is changed and follows the halogen series. The blue shift and a lowered extinction coefficient of the Soret are consistent with a shift from 6cHS to 5cHS heme. For the UV-vis spectroscopic data presented here, DHP concentration was 8 µM and the final concentration of 4-XP was 1 mM (maximum solubility) for 4-IP and 8 mM for 4-BP, 4-CP, 4-FP, and phenol in 150 mM potassium phosphate buffer, pH 6.......................................................... 240

Figure F.2 SVD component analysis. a) Column eigenvectors of SVD showing the grand mean (U1) of the data set and first difference eigenvector (U2) plotted versus wavenumber. The first difference spectrum indicates which peaks shift during the substrate titration. b) Row eigenvectors of SVD displaying well-defined curves corresponding to each of the two column eigenvectors. The VT2 row vector corresponds to the intensity changes and peak shifts of U2 and is used to establish the apparent dissociation constant.................................................. 241

Figure F.3 Electronic absorption spectra of WT-DHP (black), DHP with 2,4,6-TBP (green), DHP with 2,4,6-TCP (blue), and DHP with 2,4,6-TFP (red). The Soret band narrows and the CT1 band blue shifts upon the...
addition of 2,4,6-TBP and 2,4,6-TCP, consistent with conversion to aquo-6cHS heme. The blue shift of the Soret band upon addition of 2,4,6-TFP can be attributed to the 5cHS population observed in the Raman spectrum. The final concentration of 2,4,6-TBP was 200 µM, and the final concentrations of 2,4,6-TCP and 2,4,6-TFP were 4 mM in 150 mM potassium phosphate buffer, pH 6.

Figure F.4  Resonance Raman spectrum DHP with 3 mM 2,4,6-TCP only (red), DHP with 3 mM 2,4,6-TCP followed by the addition of 5 mM 4-BP (lower black), DHP with 5 mM 4-BP followed by the addition of 3 mM 2,4,6-TCP (upper black), and DHP with 5 mM 4-BP only (blue). The two black spectra, obtained by adding either the substrate or the inhibitor first to DHP, are identical, indicating that the substrate and the inhibitor are in equilibrium with the enzyme. Spectra were obtained in 150 mM potassium phosphate buffer, pH 6. Excitation wavelength was 406 nm; 1.7 cm⁻¹ resolution; laser power at the sample 60mW, and 300 s acquisition times.

Figure F.5  Time-dependent UV-vis spectra from 0 seconds (red) to 120 seconds (purple). Assay conditions were 2.4 µM DHP, 250 µM 4-FP, and 240 µM H₂O₂ in 100 mM potassium phosphate buffer at pH 7. Unlike 4-IP, 4-BP, and 4-CP, 4-FP has a low affinity to bind internally in DHP and is clearly oxidized to 1,4-benzoquinone.

Figure F.6  Time-dependent UV-vis spectra from 0 seconds (blue) to 120 seconds (teal). Assay conditions were 2.4 µM DHP, 240 µM H₂O₂, and 120 µM 2,4,6-TBP, pH 7 without (a) and with (b) addition of 240 µM 4-BP. (a) In the absence of 4-BP, the substrate (316 nm) is converted to product (291 nm). (b) In the presence of 4-BP, little product is formed with no significant decrease in the substrate band.

Figure F.7  (a) X-ray crystal structure of the heme active site showing hydrophobic amino acid residues surrounding the distal cavity (PDB 3FNW) (b) The location of the substrate halogen is analogous to the Xe₄ binding site in sperm whale met-Mb.

Figure F.8  X-ray crystal structure of the DHP monomer (PDB 2QFK). Blue residues are the distal pocket residues affected by internal binding of the inhibitor (see Figure F.7). Green residues are the dimer interface residues affected by substrate binding. Highlighted residues are based on ¹H-¹⁵N HSQC experiments of Davis et al.

Figure F.9  (a and b) Time-dependent UV-vis spectra from 0 seconds (red) to 120 seconds (purple). Assay conditions were 2.4 µM DHP, 250 µM 2,4,6-
TFP, and 240 μM H₂O₂ in 100 mM potassium phosphate buffer at pH 7 (a) and 2.4 μM DHP, 250 μM 2,4,6-TFP, 250 μM 2,4,6-TCP, and 240 μM H₂O₂ in 100 mM potassium phosphate buffer at pH 7 (b). Time traces of product formation (c)........................................................................................................ 248

APPENDIX G

Figure G.1 The freeze-quench attachment to the Update Instrument apparatus. The insets show the top view (with the chamber lid up), the bottom view (detached from the base) and the chamber lid........................................................... 251

Figure G.2 The EPR signal intensity of a ‘point’ sample as function of the sample vertical position in the resonator SP9703 (Bruker)................................. 253

Figure G.3 Correction factors for EPR samples shorter that the active zone of the resonator SP9703, which has been found to be 32 mm......................... 254

Figure G.4 An experimentally obtained kinetic dependence of free radical concentration before (top) and after (bottom) applying the procedure of EPR signal intensity correction for variable size of EPR samples........ 255

Figure G.5 Free radical EPR spectra of DHP reacting with H₂O₂ as recorded in six different samples (freeze-quenched). A – spectra as measured; B – the same spectra after each one was shifted to the left or to the right in accordance with the microwave frequency used to record each spectrum................................................................. 256

Figure G.6 Free radical EPR spectra of the reaction mixtures of 40 μM DHP + 120 μM H₂O₂ (final concentrations) at three different pH values freeze-quenched at the time indicated. DHP was freshly re-oxidised and cleaned of ferricianide for each of the pH value series. One cryotube of stock H₂O₂ solution (see section 2.3) was used for a session of making not more than 3-4 samples. All the spectra are presented normalised to the same intensity to highlight the lineshape change over the reaction time as well as differences in the shape at different pH values. The spectra were recorded at 10 K, the microwave frequency νₘₖ was slightly different for different EPR samples and was within the range of 9.469-9.473 GHz. All spectra were shifted along the magnetic field axis to correspond to some standard frequency (9.472169 GHz) as described in SI.Methods.3. Other instrumental conditions were; microwave power P = 0.05 mW, modulation frequency νₘ = 100 kHz,
modulation amplitude $A_m = 3$ G, time constant $\tau = 82$ ms, scan rate $v = 0.596$ G s$^{-1}$, number of scans per spectrum $NS = 1$...

Figure G.7 The $g=6$ region of the EPR signals of the two rhombic HS ferric heme forms of DHP. Similar spectra were obtained for slow-freeze and RFQ samples. Presented here is the result of spectral deconvolution of the slow-freeze samples, i.e. when a reaction mixture was placed in an EPR tube, and the tube was immersed in methanol kept on dry ice. The dotted line is the rhombic form (type 1 high spin form or $R1HS$) detected in the resting DHP (control). The continuous line is the rhombic form detected in the samples treated with $H_2O_2$ (type 2 high spin form or $R2HS$).

Figure G.8 HPLC of dehaloperoxidase before and after reaction with hydrogen peroxide measured at 280 nm (blue line) and 400 nm (red line). (A) HPLC chromatogram of dehaloperoxidase ($80$ µM). Acidic solvents used for HPLC analysis causes the separate elution of the heme moiety (peak i) and the apoprotein (peak ii). (B) Heme and protein oxidation products from the reaction between dehaloperoxidase ($80$ µM) and $H_2O_2$ ($160$ µM) at pH 5. Here peaks i and ii are decreased due to oxidative modifications that include heme to protein cross-linking (peak iii) and other oxidatively modified heme not covalently bound to the protein (peaks iv).

Figure G.9 Optical properties of heme to protein cross-linked species (blue line), unmodified heme (red line), oxidatively modified free hemes (green and purple lines). Spectra were taken from the chromatogram in Figure G.8, normalized with respect to the Soret peak and offset for clarity. Both unmodified and heme to protein cross-linked hemes have a typical ‘heme b’ spectra, corresponding to iron-protoporphyrin-IX, whereas the oxidatively modified hemes have a 720 nm band, characteristic of a ‘heme d’ chlorine heme spectra where one of the pyrrol rings is converted to a pyrroline.

Figure G.10 Two rotational conformations of the phenoxy ring in a tyrosyl radical with $\theta=45^\circ$ and $\theta=75^\circ$ (the sum of these complementing angles is $120^\circ$) have the same values of the hyperfine splitting constant for the two methylene protons.

Figure G.11 Two rotational conformations of the phenoxy ring in a tyrosyl radical with $\theta= -52^\circ$ and $\theta= -8^\circ$ (the sum of these complementing angles is $-60^\circ$) have the same values of the hyperfine splitting constant for the two methylene protons.
LIST OF SCHEMES

CHAPTER 1

Scheme 1.1  Reaction catalyzed by Dehaloperoxidase-hemoglobin............................ 6

CHAPTER 4

Scheme 4.1  Representative sequence of DHP activation, radical formation, and decay........................................................................................................... 104

Scheme 4.2  Equilibrium of oxoferryl heme and its protonated state......................... 109

APPENDIX C

Scheme C.1  Rate scheme for peroxidase catalysis...................................................... 170

APPENDIX E

Scheme E.1  Selected examples of enzymatic dehalogenation: (a) hydrolytic, (b) reductive, and (c) oxidative................................................................. 196

Scheme E.2  The catalytic cycle of DHP with 2,4,6-trichlorophenol (TCP) as a substrate.................................................................................................................. 200

Scheme E.3  The catalytic cycle of HRP with TCP as a substrate............................ 201
CHAPTER 1

Dehaloperoxidase-hemoglobin: An Overview of Current Research
Introduction

Dehaloperoxidase–hemoglobin (DHP) was first discovered in 1977 as the coelomic hemoglobin of the marine organism *Amphitrite ornata* (1). The original characterization was of a monomeric protein (mw ~12,200 Da) that possessed a high affinity for oxygen ($P_{50} = 2.8$ torr) typical of globins. Later, in 1996, DHP was rediscovered in studies of extracts from *A. ornata* tested for their ability to oxidize 2,4,6-tribromophenol (2,4,6-TBP) in the presence of $\text{H}_2\text{O}_2$ (2). Structural and functional characterization led to the recognition that the two proteins are the same. In fact, according to the Structure Classification of Proteins (SCOP) database, dehalo-“peroxidase” is best described as a globin.

*A. ornata* is classified as a terebellid polychate. It has a red-colored ringed body that is buried in the mud flats common in benthic coastal ecosystems (Figure 1.1). The red color is attributable to the oxygen-bound form of DHP, the most abundant protein in the organism. Two genes encode for DHP in *A. ornata*, DHP A and DHP B (3). 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* 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 hemoglobin (Hb) (3.6 megadaltons) known as the erythrocrucorin is located in the tentacles. (4) It remains unclear how DHP is related to this giant, extracellular erythrocrucorin.
Many organisms in benthic ecosystems produce brominated compounds as repellents to protect against predators (Figure 1.2). 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. While A. ornata is not directly involved in the predator-prey relationship, it is able to tolerate the toxic conditions, as a second function of DHP is the oxidation of 2,4,6-TBP to 2,6-dichlorobenzoquinone (2,6-DBQ) to reduce the concentration of the toxic phenols. Thus DHP is a bifunctional enzyme that stores oxygen similar to myoglobin but also acts as a peroxidase in the presence of peroxide and halogenated phenols.

![Figure 1.1. Amphitrite ornata.](image)

**Figure 1.1.** Amphitrite ornata.

![Figure 1.2. Common brominated aromatics found in A. ornata environment.](image)

**Figure 1.2.** Common brominated aromatics found in A. ornata environment.

[4-Bromophenol] [2,4-Dibromophenol] [2,4,6-Tribromophenol] [2,3,4-Tribromopyrrol]
This dissertation provides a summary and explanation of the current mechanism of action for DHP peroxidase activity that centers on the unusual flexibility of the distal histidine. The explanation presented herein is based on six published works, each of which are presented in the remaining chapters and appendices.

**Structure and Function**

DHP is a heme-containing peroxidase with a typical 3/3 α-helical globin fold. When crystallized, DHP is a dimer composed of two identical subunits having a total molecular weight of approximately 31 kDa (5, 6). Each subunit consists of eight α-helices (a-h) and an iron protoporphyrin IX prosthetic group. The single subunit structure is nearly isomorphous to myoglobin but is truncated to 137 amino acids rather than 153. Figure 1.3 provides comparison of DHP to sperm whale myoglobin (SWMb), Cytochrome c peroxidase (CcP), and horseradish peroxidase (HRP).
Figure 1.3. Structural comparison of Sperm Whale Myoglobin (PDB 1A6G) (7), Dehaloperoxidase-hemoglobin (PDB 2QFK) (8), Cytochrome c Peroxidase (PDB 1ZBY) (9), and Horseradish Peroxidase (PDB 2ATJ) (10).

The function of DHP is to catalyze the H₂O₂ dependent conversion of mono-, di-, and trihalogenated phenols into the corresponding quinone via the reaction given in Scheme 1. While the primary substrate is 2,4,6-tribromophenol, DHP is capable of removing all types of halogens from halophenols (2, 11). The enzyme likely uses a novel mechanism for
peroxidase activity that depends on the flexibility of the distal histidine given the hydrophobic nature of DHP’s distal heme cavity (Figure 1.4).

**Scheme 1.1.** Reaction catalyzed by Dehaloperoxidase-hemoglobin.

![Scheme 1.1](image)

The catalytic process of DHP involves a high valent iron-oxo, \( \text{Fe(IV)=O} \), intermediate formed upon addition of \( \text{H}_2\text{O}_2 \) and subsequent heterolytic cleavage of the O-O bond similar to HRP and \( \text{CcP} \), known as the Poulos-Kraut mechanism (12, 13). Figure 1.4 compares the active sites of \( \text{CcP} \) and DHP. In \( \text{CcP} \), the proximal side of the heme consists of a negatively charged aspartate residue hydrogen bonded to the \( \text{N}_\delta \text{H} \) of the proximal histidine. This gives rise to the Asp-His-Fe catalytic triad of typical peroxidases. This interaction, producing a negative charge build up on the heme Fe and initiating cleavage of the O-O bond of bound \( \text{H}_2\text{O}_2 \), is known as the “push” effect in peroxidase chemistry. On the distal side of peroxidases, the distal histidine and arginine residues work in a concerted effort to provide the “pull” effect of peroxidase chemistry. The histidine works as the acid/base catalyst that shuttles a proton from \( \text{O}_\alpha \) to \( \text{O}_\beta \) of \( \text{H}_2\text{O}_2 \) while the arginine stabilizes the developing negative charge on \( \text{O}_\beta \). The high resolution x-ray crystal structure of \( \text{CcP} \) shows the distal arginine in two conformations (9), while the x-ray structure of DHP clearly shows the distal histidine in
two conformations (6, 14). With only hydrophobic amino acid residues surrounding the distal cavity of DHP, it is likely that the distal histidine of DHP must work alone to activate bound H$_2$O$_2$. Nevertheless, while DHP lacks most structural features of typical peroxidases, it still maintains peroxidase activity (Figure 1.5).

**Figure 1.4.** Structural comparison of the active sites of Cytochrome c peroxidase (PDB 1ZBY) (9) and Dehaloperoxidase-hemoglobin (PDB 1EW6) (6). In CcP, the distal arginine is observed in two conformations, whereas in DHP, the distal histidine is observed in two conformations. DHP lacks most of the typical peroxidase machinery found in CcP.
Figure 1.5. Typical DHP enzymatic assay. When activated with \( \text{H}_2\text{O}_2 \), DHP catalyzes the oxidation of 2,4,6-trichlorophenol (2,4,6-TCP) to 2,6-dichlorobenzoquinone (2,6-DCQ).

Spectroscopic Characterization of DHP and the Unusual Flexibility of the Distal Histidine

The complete electronic absorption and resonance Raman spectroscopic characterization of DHP A is given in Appendix A. The material was published in *Biochemistry Journal* in 2010 (15) and provided the first spectroscopic evidence that there are two heme species present in the wild-type enzyme under physiological conditions. Comparison of the RR spectra to the available x-ray crystal structures of DHP revealed that the two heme species can be attributed to two conformations of the distal histidine, His55 (Figure 1.6).
Figure 1.6. Correlation of room temperature resonance Raman spectrum (15) and X-ray crystal structure (PDB 1EW6) (6). The RR spectrum shows the presence of two heme species, 5cHS and 6cHS, that are attributable to the open and closed conformation of the distal histidine, respectively.

The distal histidine is the most important determinant of reactivity in globins and peroxidases. In globins, the distal histidine stabilizes the capture and release of diatomic oxygen, whereas in peroxidases, the distal histidine serves as the acid/base catalyst for peroxidase chemistry. A combination of resonance Raman (15, 16), EPR (17-20) and X-ray crystallographic (8, 14, 21, 22) studies suggest 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 and the ‘closed’ or internal conformation at pH 7. In the closed conformation, H55 hydrogen-bonds to, and stabilizes, a heme-bound water or oxygen
molecul (8). Figure 1.6 compares the room temperature resonance Raman spectrum to the room temperature x-ray crystal structure. In the crystal structure, the distal histidine is observed in both conformations, and 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, the distal H55 is observed in the internal (closed) conformation. When the heme Fe is 5-coordinate, water is absent, and the distal H55 is in the external (open) conformation. The room temperature resonance Raman spectrum confirms the existence of both a 5-coordinate high-spin and 6-coordinate high-spin heme species.

Further support for this correlation can be found by comparing the low temperature RR spectrum and crystal structure (8, 15). At cryogenic temperature, the crystal structure of DHP A shows the distal histidine only in the closed position with water bound to the heme iron, and the 12 K RR spectrum shows predominately a 6-coordinate high-spin heme (Figure 1.7). Moreover, the crystal structure of the deoxy form of DHP A shows the distal histidine in the open conformation (14), and the RR spectrum of deoxy DHP is representative of an Fe$^{2+}$ 5-coordinate high-spin heme (Figure 1.8) (23).
Figure 1.7. Correlation of cryogenic temperature resonance Raman spectrum (15) and X-ray crystal structure (8). The RR spectrum shows the predominately 6cHS heme, and the crystal structure is composed of the closed conformation of the distal histidine with H₂O bound to the heme iron.
Figure 1.8. Correlation of deoxy DHP resonance Raman spectrum (23) and X-ray crystal structure (14). The RR spectrum shows the predominately 5cHS heme, and the crystal structure is composed of the open conformation of the distal histidine with no O\(_2\) bound to the heme iron. The low frequency RR spectrum displays the \(\nu(\text{Fe-Im})\) mode which is only observed in the 5cHS deoxy form of globins and peroxidases.

The same distal histidine conformations involved in dehaloperoxidase function regulate oxygen binding and release during transport and storage by hemoglobins and myoglobins. 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 only observed in an X-ray structure at pH 4.5 (24), when it is expected to be protonated thereby disrupting hydrogen bonding to the iron-bound ligand. However, in DHP,
the open form is formed far more readily and is observed in the X-ray crystal structures and RR spectra at pH 6.0 (6, 14, 15, 25).

Because of the quaternary dimeric form of DHP observed in the crystal structure, it is reasonable to assume that allosteric interactions between the monomer may give rise to the different conformations of the distal histidine, i.e. the histidines of each subunit may communicate and alternate in an open/closed fashion to yield the observed spectroscopic data. Such allosteric communication is often observed in hemoglobin cooperativity where the quaternary structure of hemoglobin changes in response to oxygen tension, and the binding constant of each heme iron in the αβ heterodimer is modulated by the ligation state of the others. This, however, is not the case for DHP. Chapter 2 of this dissertation provides evidence that, although dimeric in the crystal form, DHP is a monomer in solution under physiological conditions (26). Therefore, the dynamic action of the distal histidine observed in DHP is of a single subunit of the enzyme.

Two-Site Competitive Inhibition in Dehaloperoxidase-hemoglobin

The first x-ray crystal structure of DHP showed a 4-iodophenol (4-IP) molecule bound inside the protein, above the heme, but not ligated to the iron (6). From that initial structure, a reasonable hypothesis was formed that the internal binding site is the substrate binding and active site. We have systematically investigated that hypothesis, and it has now been replaced with extensive data showing that the internal binding site is an inhibitor site. The inhibition of DHP is significant because the ratio of 4-bromophenol (4-BP) to 2,4,6-tribromophenol (2,4,6-TBP) is approximately 2:1 in the coastal estuary environment where
A. ornata is found (27, 28), and at that ratio, 2:1, the peroxidase function of DHP is almost completely inhibited (Figure 1.9). Chapter 3 of this dissertation provides a detailed spectroscopic and crystallographic analysis of the two-site mechanism of inhibition and a Michaelis-Menten kinetic analysis that clearly establishes the competitive nature (16). Furthermore, Appendix C provides a detailed kinetic analysis of DHP and strong support that DHP does in fact follow Michaelis-Menten kinetic behavior (29).

**Figure 1.9.** Inhibition of DHP by 4-bromophenol (4-BP). In the presence of 4-BP, no 2,6-DCQ product is formed.

Figure 1.10 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. The x-ray crystal structure of bound 4-BP shows the distal histidine in the open conformation, and the corresponding RR spectrum is of a 5-coordinate high-spin species. Substrate binding, however, produces the opposite effect (Figure 1.11). While the actual location of the
external substrate binding site remains unknown, the effect is clear. The resonance Raman spectrum of DHP in the presence of 2,4,6-trichlorophenol shows a 6-coordinate high-spin species. Given the flexibility of the distal histidine and the fact that 2,4,6-TXP molecules have never been observed to bind internally under physiological conditions, binding of the substrate molecule must cause an allosteric effect that pushes the distal histidine into the closed position, priming the enzyme for peroxidase activity.

Figure 1.10. Resonance Raman spectrum (15) and X-ray crystal structure of DHP in the presence of 4-BP (16). The X-ray crystal of inhibitor-bound DHP displays an open distal histidine (H55) with no H2O bound to the heme Fe, and the RR spectrum shows a predominately 5cHS heme.
Figure 1.11. Resonance Raman spectrum DHP in the presence of 2,4,6-TCP (16). The RR spectrum shows a predominately 6cHS heme species. The corresponding structure shows a depiction of a tri-halogenated phenol forcing the distal histidine into the closed position.

Based on the structural and spectroscopic data presented in Chapter 3 of this dissertation, this unique push-of-war mechanism of the distal histidine involves two independent but concerted competitive binding events. The first arises from the steric interactions of 4-XP binding in the distal pocket preventing ligation of H$_2$O$_2$ to the heme iron. The second is allosteric communication by movement of the distal histidine resulting in two-site competitive binding between the substrate and inhibitor pair. Thus, inhibition by 4-BP appears to involve three simultaneous effects: 1) H$_2$O$_2$ coordination to the heme iron is impeded, 2) displacement of the distal H55, which is the acid-base catalyst in the peroxidase
mechanism (30, 31), out of the cavity, and, subsequent 3) rearrangement of the external binding site by the solvent-exposed (open) distal H55 conformation.

**Compound ES and Free Radical Decay in DHP Depend on the Conformation of the Distal Histidine**

The formation of radical intermediates in peroxidases was first observed in cytochrome c peroxidase (CcP), where a tryptophan radical (Try191, Figure 1.4) is an on-pathway electron transfer intermediate leading to oxidation bound cytochrome c (32). Feducia et al were the first to show that DHP exhibits a similar behavior, but with the protein radical on a tyrosine rather than a tryptophan (18). They named the activated tyrosyl radical form of DHP compound ES by analogy to CcP. Considering the on-pathway nature of the tryptophan radical in CcP, it was reasonable to consider that the tyrosine radical associated with Compound ES in DHP might lead to elucidation of the external substrate binding site. Chapter 4 of this work provides a detailed rapid free-quench analysis of the DHP Compound ES free radical signals, including simulations of the lineshapes in order to establish which of the five tyrosines in DHP holds the free radical (20).

The open and closed conformations of the distal histidine were again confirmed by rapid freeze-quench EPR spectroscopy. The EPR lineshape of wild-type ferric DHP varies with pH (Figure 1.12). The spectra were deconvoluted into two lineshapes and assigned to the open and closed states of the distal histidine by comparison to a previous EPR signal of ferric DHP in the presence of 4-BP. In the presence of 4-BP, the distal histidine is open, and the EPR lineshape is nearly identical to that of the “Open H55” shown in Figure 1.12.
Furthermore, the relative ratio of the two EPR signals is stoichiometric with pH, favoring the open conformation at low pH and the closed conformation at high pH, in line with the previous x-ray crystallographic and spectroscopic data.

![EPR spectra of ferric DHP at different pH](image)

**Figure 1.12.** EPR spectra of ferric DHP at different pH (20). The EPR spectra of ferric DHP at different pH can be deconvoluted into two species, and they are correlated to the two conformations of the distal histidine. The populations of the two species are stoichiometric with pH.

The conformation of the distal histidine plays an integral role in the free radical formation of Compound ES. Because the distal histidine is required for acid/base catalysis and activation of the bound H$_2$O$_2$, it must be in the closed conformation. This is further evidenced by the relative free radical yield at different pH as shown in Figure 1.13. At pH 7, the free radical yield is ~ 93% compared to only ~ 25% at pH 5 (20). Thus the enzyme is tuned to be most productive at physiological pH, 7.4.
Figure 1.13. RFQ-EPR data showing pH-dependent tyrosine radical lineshapes (20). At pH 7, the free radical yield is nearly 93%, atypical of globins reacting with H$_2$O$_2$.

As can be seen in Figure 1.13, 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 oxoferryl 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 oxoferryl. 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$_2$O$_2$. At this low pH, the oxoferryl can be protonated leading to the formation of a hydroxyl radical in the interior of DHP. The tyrosyl radical then decays via two alternative pathways that also depend on the conformation of the distal H55 (Figure 1.14). When H55 is “closed” (minor population at pH 5), the hydroxyl radical is transferred to Y38, and a “protein biradical” is formed, whereas when H55 is “open,” a protein-heme crosslink forms in DHP (20).
Figure 1.14. Proposed radical pathways for productive and non-productive substrate oxidation (20). When the distal 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.

Current Catalytic Reaction Mechanism of Dehaloperoxidase-hemoglobin

This new understanding of the flexible distal histidine’s involvement in activation, inhibition, radical formation, and radical decay in dehaloperoxidase-hemoglobin has significantly contributed to the proposed overall catalytic reaction mechanism given in Figure 1.15. Shown is the complete reaction cycle for DHP based on all of the currently available and/or published data. The remaining sections of this dissertation describe, in
detail, the highlighted sections of the reaction cycle and how each is influenced by the flexibility of the distal histidine.

Figure 1.15. Proposed catalytic cycle of dehaloperoxidase-hemoglobin.
References


CHAPTER 2

Dehaloperoxidase-hemoglobin from *Amphitrite ornata* is primarily a monomer in solution
Abstract

The crystal structures of the dehaloperoxidase-hemoglobin from *A. ornata* (DHP A) each report a crystallographic dimer in the unit cell. Yet, the largest dimer interface observed is 450 Å², an area significantly smaller than the typical value of 1200-2000 Å² and in contrast to the extensive interface region of other known dimeric hemoglobins. To examine the oligomerization state of DHP A in solution, we used gel permeation by fast protein liquid chromatography (FPLC) and small angle X-ray scattering (SAXS). Gel permeation experiments demonstrate that DHP A elutes as a monomer (15.5 kDa) and can be separated from green fluorescent protein (GFP), which has a molar mass of 27 kDa, near the 31 kDa expected for the DHP A dimer. By SAXS, we found that DHP A is primarily monomeric in solution, but with a detectable level of dimer (~10%), under all conditions studied up to a protein concentration of 3.0 mM. These concentrations are likely 10–100-fold lower than the *K₅* for dimer formation. Additionally, there was no significant effect either on the overall conformation of DHP A or its monomer-dimer equilibrium upon addition of the DHP A inhibitor, 4-iodophenol.
Introduction

The dehaloperoxidase-hemoglobin from *A. ornata* (DHP A) has been discovered twice. For many years, DHP A was known simply as the coelomic hemoglobin (Hb) of *A. ornata*\(^1\). However, in 1996 DHP A was discovered for a second time as an enzyme in *A. ornata* capable of oxidizing 2,4,6-tribromophenol (2,4,6-TBP). Based on functional characterization of DHP A, it was determined to be a dehaloperoxidase\(^2\). DHP A was identified from extracts of *A. ornata* based on its ability to oxidize brominated phenols, which are prevalent naturally occurring pollutants in coastal waters\(^3\). There are two Hb genes\(^4\) known as *dhpA* and *dhpB*. While DHP A has been studied in all published work until recently\(^5\), DHP B was shown to be a dehaloperoxidase with a turnover rate three times greater than DHP A.

Despite the functional characterization of DHP A, it was recognized in the late 1990s that its structure is best described as a globin, which is its classification in the Structure Classification of Proteins (SCOP) database. The original X-ray crystal structures of DHP A discuss the globin structure but make no mention of the fact that DHP A was previously known as the Hb of *A. ornata*\(^6-8\). It is still not known how the two DHP genes relate to the two known Hbs in *A. ornata*\(^1\)— the monomer coelomic Hb and the giant extracellular Hb. However, the intracellular Hb was determined to be a monomer with molecular weight 15.5 kD\(^1\). The extracellular hemoglobin or erythrocruorin has a molecular weight of approximately 3,000 kD\(^9\). DHP A was rediscovered in 1996 in studies of extracts from *A. ornata* tested for their ability to oxidize 2,4,6-tribromophenol in the presence of H\(_2\)O\(_2\).\(^2\) DHP A was named dehaloperoxidase in that work, and it was described as a dimer\(^2\), with no mention of a previous characterization of the same sequence as a monomeric coelomic Hb. It
was pointed out first in 2001 that the dehaloperoxidase and the hemoglobin of *A. ornata* have the same sequence, and given that DHP A is a globin, it is clear that the two proteins are one and the same. The complete description of the two functions depends, to a significant extent, on whether there are protein-protein interactions that lead to dimerization, as is often observed in Hbs.

The X-ray crystal structure served as the basis for the characterization of DHP A as a dimer subsequent to its rediscovery in 1996. The crystallographic dimer in the X-ray crystal structures consists of a weakly bound interface. The dimer interface consists of only three amino acid residues, Arg122, Asn126 and Asp72. These three amino acids form two salt bridges and one hydrogen bond with a dimer surface area of ~210 Å². Submissions of 10 X-ray crystal structures have been subjected to analysis by the program PISA, which locates all possible crystallographic dimers and reports the contact surface area. PISA analysis reveals that the observed crystallographic dimer has the smallest surface area of any of the 5 other dimer interfaces identified. The largest interface in DHP A identified by PISA has a surface area of 475 Å², and consists of residues Arg10, Asp12, Glu57, Asn61, and Asp68. The surface areas identified in DHP A are similar in DHP B and DHP A/B mixed crystals. These values are all significantly smaller than the typical value of 1200-2000 Å² observed in protein-protein interaction databases. The relatively weak binding between molecules of DHP A also stands in contrast to the extensive interface region of dimeric hemoglobins such as that of *Scapharca*. To resolve the issue of the oligomerization state of DHP A in solution, we conducted quantitative gel permeation studies using fast protein liquid chromatography (FPLC) and small angle X-ray scattering (SAXS) experiments. SAXS is a
useful technique for the determination of overall molecular size and shape of a protein in solution.\textsuperscript{17} The SAXS profile represents the average scattering from all conformational states weighted by the number of molecules in each of those conformational states, and thus it is an excellent method for the detection of protein oligomerization in solution. We also tested if oligomerization of DHP A would be affected by the presence of an inhibitor, 4-iodophenol, which binds in the internal binding pocket of the enzyme. Such effects could be analogous to the allosteric effectors in hemoglobins, which could affect the binding of diatomic molecules as well as the stability of any dimer. Herein, we report that DHP A is primarily monomeric in solution but with a detectable level of dimer (~10\%) under all conditions studied up to a protein concentration of 3.0 mM, including those in the presence of up to a 20-fold molar excess of 4-iodophenol.

**Methods**

**Dehaloperoxidase expression and purification**- DHP was expressed and purified as previously described for both the his-tagged\textsuperscript{18} and non-his-tagged\textsuperscript{11} forms.

**Dehaloperoxidase sample preparation**- A stock solution of his-tagged DHP (His\textsubscript{6}-DHP) at a concentration of 3.0 mM in potassium phosphate buffer (pH 7.0) was diluted with a matched buffer to make a His\textsubscript{6}-DHP concentration series at 100\% (3.0 mM), 75\% (2.0 mM), 50\% (1.5 mM), 25\% (1.0 mM) and 10\% (0.30 mM). A stock solution of non- His\textsubscript{6}-DHP at a concentration of 40 mg/ml (2.6 mM) in 20 mM cacodylate buffer pH 6.5 was used to make a DHP concentration series at 100\% (2.6 mM), 25\% (0.62 mM) and 12.5 \% (0.31 mM). The concentration of DHP stock was determined using the extinction coefficient for the Soret
band in a 1 cm cuvette\textsuperscript{18}. All dilutions were made gravimetrically. A series of His\textsubscript{6}-DHP were prepared at 0.1, 0.5, 1.4 mM and 1.4 mM His\textsubscript{6}-DHP with a molar ratio of added 4-iodophenol to DHP of 19, 3.3, 0.8 and 0, respectively.

**Gel Permeation Chromatography**- A mixture of 20 \( \mu \)M DHP and 10 \( \mu \)M green fluorescent protein (GFP) in 50 mM sodium phosphate buffer containing 150 mM NaCl, pH 7 and 5\% glycerol was loaded onto a GE Healthcare HiPrep 26/60 Sephacryl S-100 high resolution size exclusion separation column using an AKTAprime FPLC pump/analysis system from Amersham Biosciences. The flow rate was set to 1 mL/min and 1 mL fractions were collected starting at approximately 143 mL while the absorbance was monitored at 280 nm.

**SDS Polyacrylamide Gel Electrophoresis**- 10 \( \mu \)L of each fraction collected from the GE Healthcare HiPrep 26/60 Sephacryl S-100 high resolution column were mixed with an equal volume of Laemmli sample buffer from Bio-Rad. The resulting mixtures were loaded onto a Bio-Rad 15\% Tris-HCl polyacrylamide ready-gel and run for approximately 1 hour at 150 mV. The gels were stained with Bio-safe Coomassie stain and imaged on a LI-COR Biosciences Odyssey infrared imaging system.

**Small Angle X-Ray Scattering data collection and analysis**- The small-angle scattering intensities of all samples and their corresponding matched buffer were measured at Brookhaven National Lab beamline X21. His\textsubscript{6}-DHP data were measured at one visit in September 2007. The DHP minus his-tag data were measured on a separate visit in March 2008. The Intensity at zero angle, \( I_0 \), and the radius of gyration (Rg), [the root-mean-square distance of each atom from the center-of-mass] were determined using both a Guinier
approximation of the low Q-range data (where $Q \times R_g \leq 1.3$) as well as the first and second moments of the probability distribution of interatomic vectors, $P(r)$, calculated as an inverse Fourier transform of the scattering data using the program packages GNOM$^{19}$ as well as PrView. ([http://danse.chem.utk.edu/prview.html](http://danse.chem.utk.edu/prview.html)).

*Guinier Analysis.* SAXS from globular proteins will exhibit a linear relationship at low Q according to the Guinier approximation: 

$$I(Q) = I_0 \cdot e^{(-R_g^2 Q^2/3)}$$

A plot of the natural log of intensities as a function of squared angular momentum, $Q^2$, will produce a straight line at low $Q$, permitted $Q \times R_g \leq 1.3$, with slope equal to $R_g^2/3$. $I_0$, being the Y-intercept of this plot, is also extrapolated from these data. Values of $R_g$ and $I_0$ determined by Guinier analysis (data not shown) were all within error of those determined by $P(r)$ analysis (shown in Table 2.1).

*P(r) Analysis.* Values for $I_0$ and $R_g$ were also calculated from the $0^{th}$ and $2^{nd}$ moment of the $P(r)$ profile, respectively. GNOM$^{19}$ determines the probability of finding scattering centers within the protein separated by a distance of $r$, in angstroms, via an inverse Fourier transform of the scattering data over the entire Q-range measured ($0.016$ – $0.402$ Å$^{-1}$). This process requires defining a $D_{\text{max}}$, or maximum vector distance between scattering centers (atoms) and evaluating the resultant behavior of the $P(r)$ transform$^{20}$. For high quality data with accurate background subtractions, such as presented herein, $D_{\text{max}}$ can be chosen with reasonable reliability. The $P(r)$ profiles shown in Figure 2.6 were generated using the program package, PrView. ([http://danse.chem.utk.edu/prview.html](http://danse.chem.utk.edu/prview.html)) The indirect method that the PrView software is based on is that of P. Moore$^{21}$. It is a more robust method for selecting the ‘soft’ $D_{\text{max}}$ parameter that best fits the data.
**Oligomer Analysis of SAXS data.** The $I_0$ is the intensity of the scattered radiation through zero angle and is related to the number of scattering particles per unit volume and the square of the particle volume thus, it is directly proportional to the square of the molecular weight of the protein. A lysozyme standard (14.2 kDa) run in the same sample cell on the same day was determined to exhibit an $I_0/c$ value of 1.7 for the His$_6$-DHP +/- inhibitor experiment, where $c$ is the concentration in mg/ml. The DHP samples with the His$_6$-tag removed were measured on a separate trip to Brookhaven and the $I_0/c$ value of the lysozyme standard determined for those experiments was 35.1. Using these values and the known molecular weights of the DHP monomer (15.5 kDa) and the dimer (31 kDa), the expected $I_0/c$ was calculated to be 1.86 ($=1.7 \times 15.5/14.2$) for 100% His$_6$-DHP monomer and 3.71 ($=1.7 \times 31/14.2$) for 100% His$_6$-DHP dimer, where $c$ is measured in mg/ml. For the second set of experiments, the expected $I_0/c$ is 40.4 for 100% DHP monomer and is 80.8 for 100% DHP dimer. The measure $I_0/c$ values for each sample were compared to these expected $I_0/c$ values to estimate the monomer-dimer ratio. The volume fractions of oligomers were also checked using the overall structural parameters as determined by OLIGOMER\textsuperscript{22}.

**Modeling of SAXS data.** Three-dimensional scattering shapes that best fit the SAXS data over a Q-range of $0.016 – 0.402 \text{ Å}^{-1}$ for the 3 mM His$_6$-DHP and the 2.6 mM DHP data were generated using the program DAMMIN\textsuperscript{23}. For His$_6$-DHP, 15 models were generated (average NSD of 0.593 ± 0.010) and 12 models were generated for DHP (average NSD of 0.589 ± 0.021). The models were overlaid and averaged using DAMAVER software\textsuperscript{24}. The averaged model of His$_6$-DHP was superimposed and combined with the high-resolution
crystal structure of DHP (pdb no. 2QFN) using SUPCOMB20 so as to minimize the normalized spatial discrepancy (NSD)\textsuperscript{25}.

**Results**

Results of the gel permeation chromatography on a mixture of GFP and DHP A are shown in Figure 1.1. The chromatogram consists of two peaks separated by 12 mL. The first peak eluted from the Sephacryl S-100 column at approximately 159.5 mL and the second at approximately 171.5 mL, which correspond to molar masses between 35 kDa (β-lactoglobulin @ ~141.5 mL) and 12.4 kDa (cytochrome C @ ~192 mL) according to the manufacturer’s test specifications of the column.

![Gel permeation chromatogram of the elution of an initial mixture GFP (MW ~ 27 kDa) and DHP (MW ~ 15.5 kDa).](image)

**Figure 2.1.** Gel permeation chromatogram of the elution of an initial mixture GFP (MW ~ 27 kDa) and DHP (MW ~ 15.5 kDa).

Fractions collected from the S-100 size exclusion column were analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 2.1). The first and last lanes of the SDS-page gels were loaded with Bio Rad Precision Plus protein standard. Protein standards representing 25
kDa and 15 kDa molecular weights are highlighted. The remaining lanes were loaded with fractions 2, 4, 6, 8, etc. up to fraction 40. Two distinct rows of bands appear in the SDS-page gels. One row is just larger than 25 kDa and the other is just under 15 kDa.

**Figure 2.2.** SDS-page of fractions collected from gel chromatography. The first and last lanes of each gel are protein standards. The remaining lanes are loaded with fractions 2, 4, 6, 8, etc. up to fraction 40 collected for the gel permeation chromatography. The boxed lanes represent fractions 16-20 for peak 1 and fractions 30-34 for peak 2 of the chromatogram in Figure 2.1 that were collected and analyzed by electronic absorption spectroscopy.

Fractions corresponding to the peaks of the chromatogram (16-20 for peak 1 at 159.5 mL and 30-34 for peak 2 at 171.5 mL) were collected, concentrated, and analyzed by electronic absorption spectroscopy (Figure 2.3). The electronic absorption spectrum of the ~ 15 kDa protein is characteristic of ferric DHP with the Soret band at 406 nm, Q₁ and Q₀ bands at 505 and 529 nm, respectively, and the charge transfer (CT₁) band at 637 nm. The electronic absorption spectrum of the larger (~ 27 kDa) protein is clearly GFP with two excitation maxima at 395 nm and 470-490 nm. The band at 577 nm in the spectrum of DHP can be attributed to the presence of a small amount of the ferrous form of the protein (Soret at 418 nm, and β and α bands at 542 and 577 nm, respectively).
Figure 2.3. Electronic absorption spectra of DHP (solid line) and GFP (dashed line) after separation on the Sephacryl S-100 size exclusion column. The spectra are of the concentrated fractions corresponding to the peak maxima of the chromatogram.

Additionally, comparison of the chromatograms of DHP and horse heart myoglobin (HHMb) with MW = 17 kDa shows that even the slightly larger HHMb elutes at a lower volume than DHP (Figure 2.4). Note that the elution volume of DHP in Figure 2.4 is different than that in Figure 2.1 only because the smaller volume HiPrep 16/60 Sephacryl S-100 column (120 mL total volume for the 16/60 rather than 320 mL total volume for the 26/60) was used for the individual protein chromatograms. Regardless, the elution volume constant, $K_{EV}$, given as the elution volume of the protein divided by the total column volume,
is approximately equal for DHP in either chromatogram. $K_{EV} = 171.5 \text{ mL/320 mL} = 0.536$ for the 26/60 column and $K_{EV} = 62.8 \text{ mL/120 mL} = 0.523$ for the 16/60 column.

**Figure 2.4.** Single protein chromatograms of HHMb (dashed line) with MW = 17 kDa and DHP (solid line) with MW = 15.5 kDa.

The measured SAXS data on a concentration series of His$_6$-DHP and of DHP minus the His$_6$-tag samples are shown in Figure 2.5A and B, respectively. The data on the native DHP sequence are very similar to those on His$_6$-DHP indicating that the presence of a
Figure 2.5. Small-angle X-ray scattering Intensity $I(Q)$ versus momentum transfer, $Q$ for (A) the His$_6$-DHP concentration series at 3.0 mM (●), 2.0 mM (■), 1.5 mM (▲), 1.0 mM (♦) and 0.3 mM (▼) and for (B) the DHP minus His$_6$-tag concentration series at 2.6 (○), 0.62 (□) and 0.31 mM (Δ). The solid lines represent final fits of the DAMMAVER models to
these data. The $\chi^2$ fits of these models to the measured scattering data are 1.4 (A) and 2.2 (B).

The hexahistidine tag did not alter the conformational state of the protein. The interatomic vector distribution functions [P(r)] determined from the 3.0 mM His$_6$-DHP and the 2.6 mM DHP data are shown in Figure 2.6. The first, major peak in each P(r) is representative of a globularly-folded structure of maximum dimension approximately 40 Å. It overlays with the P(r) calculated from the crystal structure of the monomer quite well (dotted line, Figure 2.6). This peak, however, is followed by a tail that extends the interatomic vector distance probability out to a maximum of ~85 Å (± 10 Å) indicating that there may also be some dimer (dashed line, Figure 2.6) that is contributing to the measured scattering in these samples.

**Figure 2.6.** P(r) profiles, calculated using PrView, from 3.0 mM His$_6$-DHP (●) and 2.6 mM DHP (minus his$_6$) (○). The dotted and the dashed lines represent the interatomic distance distribution for a monomer and a dimer, respectively, determined from the DHP crystal structure (pdb no. 2QFN). The solid red line represents P(r) profile expected from a 90:10 mixture of monomer and dimer calculated using the I(q) determined from the crystal structures. P(r) were normalized to unity.
The Rg and I₀ can be calculated from the second and zeroth moments of the P(r), respectively. Rg is defined as the root mean square distance of all elemental volumes from their center of mass, weighted by their scattering densities. The forward scatter (I₀) values measured for all samples were each well below that expected for a dimer, but above that expected for a monomer (Table 2.1). We calculated the expected I₀/c for a mixture of monomer to dimer from 0 – 100%. The percent dimerization in each sample was determined by the intersection of the measured and expected I₀/c values. Within error, all samples consistently present at ~20% dimer at all conditions tested, including those in the presence of 4-iodophenol. In order to check our I₀-based estimate of the fraction of DHP dimer in solution, we fit the measured SAXS data to linear combinations of the SAXS curves (form factors) computed from the crystal structure of the dimer (pdb no. 2QFN) and only chain A from that structure (monomer) using OLIGOMER®. This approach resulted in fits that estimate the volume fraction of dimer to be slightly lower (~15% for His₆-DHP and <10% for DHP) than that from the I₀-based estimate of 20% (Table 2.1). It is likely that equilibrium exists between monomer and dimer, but that, over the concentration range of these experiments (0.3 – 3 mM DHP), we are well below the K_d for the dimer. At 10-fold lower concentration than the K_d, one would expect 10% dimer and at 100-fold lower, 4% dimer. Using either the I₀-based or the structure-factor based analysis, we do not detect a concentration-dependent trend in the volume fraction of dimer thus, it is likely that we are either working at the detection limit of this analysis or there is a more complicated mixture of minor oligomeric forms contributing to the measured scattering than simply monomer and dimer. In any case, all samples including those with inhibitor present exhibit similar
scattering profiles and calculated dimer/monomer ratios (within error) indicating that the presence of bound 4-iodophenol does not alter the ratio of monomer to dimer in solution significantly. In all of the samples studied,

**Table 2.1.** Structural parameters determined from P(r) analysis (GNOM) of the SAXS data on His$_6$-DHP ± 4-iodo-phenol (4-IP) substrate and DHP minus the His$_6$-tag.

| Concentration (mM) | Rg ± σ (Å) | I$_0$ ± σ (relative) | Measured I$_0$/c | Estimated$^a$ % dimer | Calculated$^b$ % dimer | $\chi^2$ |   |
|-------------------|------------|----------------------|------------------|------------------------|------------------------|--------|
| **His$_6$-DHP 4-IP** |           |                      |                  |                        |                        |        |
| 3.0               | 20.50 ± 0.06 | 122 ± 0.13           | 2.20             | 19                     | 13.7                   | 7.17   |
| 2.0               | 19.69 ± 0.06 | 86.83 ± 0.08         | 2.36             | 22                     | 16.4                   | 9.56   |
| 1.5               | 19.32 ± 0.05 | 74.46 ± 0.06         | 2.37             | 23                     | 17.6                   | 6.3    |
| 1.0               | 18.78 ± 0.07 | 117.77 ± 0.05        | 2.27             | 22                     | 18.0                   | 5.09   |
| 0.3               | 18.42 ± 0.14 | 131.14 ± 0.04        | 2.30             | 24                     | 14.2                   | 1.12   |
| 0.1               | 16.88 ± 0.11 | 14.19 ± 0.02         | 2.27             | 23                     | 6.9                    | 1.24   |
| 0.5               | 17.78 ± 0.10 | 22.38 ± 0.05         | 2.35             | 26                     | 14.9                   | 2.04   |
| 1.4               | 19.84 ± 0.09 | 59.63 ± 0.08         | 2.30             | 24                     | 16.2                   | 5.18   |
| **DHP**           |           |                      |                  |                        |                        |        |
| 2.6               | 18.13 ± 0.01 | 767.1 ± 0.22         | 43.0             | 7                      | 3.6                    | 23.95  |
| 0.62              | 17.29 ± 0.03 | 177.4 ± 0.13         | 50.5             | 24                     | 7.4                    | 6.10   |
| 0.31              | 17.25 ± 0.05 | 83.75 ± 0.10         | 50.6             | 26                     | 8.1                    | 2.91   |

$^a$ Estimated from I$_0$ analysis as described in Materials and Methods. Based on the lysozyme standards, the I$_0$/c expected for a 100% monomer would be 1.86 (40.4 for the DHP experiments) and for a 100% dimer the expected I$_0$/c value would be 3.71 (80.8 for the DHP experiments).

$^b$ Calculated from fits to crystal structure form factors of pdb no. 2QFN using OLIGOMER$^{22}$ analysis. Calculated Rg values from the monomer and dimer crystal structures were 15.77 and 22.94 Å, respectively.
up to the highest concentration of 3.0 mM, the radius of gyration ranged from 16.9 Å to 20.5 Å consistent with assignment of between 80 – 95 % of the sample as monomer and 20 – 5% as dimer DHP or approximately 10% dimer. Additionally, if the volume fraction of dimer (V_d) in solution is ~0.1, there would be a 30% increase in the measured intensity of the monomer-dimer mixture over that of the monomer only solution (I_0 being proportional to the [Mwt]^2). From the I_0 analysis, we saw about a 20% increase in the measured intensity over that expected for a monomer, consistently across all concentrations of DHP. Therefore, it is likely that we are measuring a mixture closer to that with a V_d around 10%. Within error, this value is confirmed by our results from analysis of our SAXS data using OLIGOMER.

The Rg of the crystal structure of one DHP monomer (extracted from pdb no. 2QFN) is 15.77 Å and of the DHP dimer is 22.94 Å. The Rg values determined from our SAXS data on each of the DHP samples were between 17 and 20 Å (see Table 2.1). SAXS data is measured for the time-averaged ensemble of molecules in solution. The Rg calculated from our SAXS analysis of His_6-DHP is larger than that for a monomer but smaller than that expected for a dimer. This result is consistent with what one would expect for a mixture of mostly monomer, with some contribution from the longer vectors between the two subunits of the less populated dimer. We calculated the scattering profile for a mixture of 90:10 monomer:dimer using the intensity profiles determined from the crystal structure of the dimer and the monomer. The resultant P(r) is plotted (red line) on Figure 2.6 and overlays quite well with our measured scattering data for the DHP samples.

A model of the three-dimensional scattering shape (orange balls) that fit the 3.0 mM His_6-DHP SAXS data with a \( \chi^2 \) of 1.429 is shown on the left of Figure 2.7. This shape was
averaged from 15 individual fits (four of which are displayed on the right of Figure 2.7), that were all very similar to one another (average NSD of 0.593 ± 0.010). This figure also displays an overlay of the averaged model fit with the crystal structure (ribbon representation) of the dimer of DHP (pdb. no. 2QFN). This SAXS-based model reflects that the majority of the measured scattering intensity comes from intra-subunit vectors for the monomer in solution with a minor contribution to the scattering from the long inter-subunit vectors of the less populous dimer. It is interesting to note that the SAXS-based model reflects vector distances from both the intrasubunit scattering as well as the longer vectors from the heme region of the second subunit within the small dimer population. It is likely that the longer scattering vectors can be attributed to scattering from the Fe centers within the heme, as these atoms have a higher scattering density and thus will contribute more strongly to the measured scattering data. Also of note, the overlay of the DHP dimer crystal structure to the SAXS-based model would suggest that the dimer that is present in solution is likely in the same conformation as that of the dimer that was crystallized. It is possible then that crystal contacts formed upon crystallization of DHP stabilize the dimer form, relative to the monomer shifting that equilibrium and resulting in crystals of the dimer only.
Figure 2.7. Shown on the left is the average of 15 DAMMIN-derived model fits to the 3 mM His$_6$-DHP SAXS data (orange balls) overlaid (using SUPCOMB) onto the structure (ribbon representation) of the dimer of DHP (pdb. no. 2QFN). For orientation, the hemes are shown in ball-n-stick. Displayed on the right are four of the 15 individual DAMMIN models (NSD values for all varied from 0.483 – 0.626).
Discussion

DHP was originally reported as a dimer with a MW = 30,790 Da based on gel permeation chromatography\(^2\). However, in that initial report the only data actually shown consisted of an SDS-PAGE gel in which the DHP monomer can clearly be distinguished with a MW = 15,500 Da. Our gel permeation chromatogram in Figure 2.1 clearly shows the separations of GFP (MW ~ 27 kDa) and DHP (MW ~ 15.5 kDa). GFP was selected as a suitable comparison protein because dimeric DHP would be expected to elute from the Sephacryl column before GFP whereas monomeric DHP would elute after. Analysis of the eluted fractions indicates that DHP elutes after GFP and has a molecular weight around 15 kDa as expected for the monomeric form.

The X-ray crystal structure was also reported in preliminary data published in 1996\(^8\). DHP crystallizes as a dimer in those original X-ray structures (1EWA and 1EW6) and in most, but not all, of the crystal forms observed to date\(^5\)\(\text{-}7\)\(\text{-}10\)\(\text{-}13\). A review of the published DHP X-ray structures\(^5\)\(\text{-}10\)\(\text{-}13\) using the program PISA, recently implemented as a tool in the Protein Data Bank, reveals that the surface areas of the dimer interfaces in DHP A and B are much smaller than expected for a physiological dimer. Although the fact that DHP A has been stated to be a dimer in nearly every publication to date\(^13\)\(\text{-}18\)\(\text{-}26\)\(\text{-}34\), the present report is the first significant study of DHP A protein-protein interactions in solution. The experiments in this report were designed to test the assumption that DHP A is, in fact, a dimer. Contrary to the initial interpretation of the X-ray crystal structure data, the SAXS data on either recombinant His\(_6\)-DHP or DHP minus the his-tag indicate that, in solution at the concentration range from 0.3
to 3 mM, there is an equilibrium between the monomeric and dimeric forms that is approximately 90% monomeric.

It is known from the X-ray crystal structure that a range of para-halophenols bind in the distal pocket of DHP A\textsuperscript{13}. Based on the systematic trends in those structures, phenol binding was not expected to change the radius of gyration of a monomer to a measurable extent. Our interpretation of the Rg value for DHP A is that it is slightly larger than that for a true monomer indicating a small equilibrium population of the dimer. If this interpretation is correct, the extent of dimer formation is increased slightly by the binding of a halogenated phenol in the internal binding pocket\textsuperscript{33,35-36}. However, this is a very small effect (change of ~6% at maximum) and could be further complicated by the fact that the concentration of DHP varies 10-fold for these samples.

Multimeric hemoglobins exist in solution in equilibrium with their monomeric subunits. For example, the binding constant of the subunits in human hemoglobin is 5.6 μM\textsuperscript{37}. Since DHP A has a dimeric form in the X-ray crystal structure, the real issue is the concentration at which the dimer is formed in solution. For example, Scapharca hemoglobin is also observed in dimer and monomer forms\textsuperscript{16}. However, in the Scapharca case the dimer forms at very low concentration ~10\textsuperscript{-8} M. The SAXS data in Figure 2.5 show a relatively consistent presence of a dimer component, but the concentration range is orders of magnitude higher than observed for Scapharca. Moreover, for the separation chromatogram presented in Figure 2.1, very small amounts of the two proteins were used to avoid overloading the column and ensure proper separation. The extinction coefficient of the non-His\textsubscript{6}-DHP was calculated using the online Peptide Calculator from Northwestern University to be ε\textsubscript{280} \approx 12,210 cm\textsuperscript{-1}M\textsuperscript{-1}.
Given that the maximum 280 absorbance of DHP in the chromatogram is 5.89 mAu, we can approximate the concentration of DHP at the time of elution to be $4.8 \times 10^{-7}$ M. At this low concentration of DHP, no dimeric form is observed to elute before or during the GFP elution peak. Therefore, the monomer/dimer equilibrium for DHP exists somewhere in the range 0.48 µM to 0.3 mM. Nevertheless, we can conclude that DHP A protein-protein interactions in solution are sufficiently weak that we may consider DHP A to be primarily a monomer at all concentrations used for any experimental work to date.

**Conclusion**

The conclusion that DHP A is primarily monomeric in solution is confirmed by quantitative gel permeation chromatography and SAXS. Our SAXS data on either recombinant His$_6$-DHP or DHP minus the his-tag, indicate that, in solution at the concentration range from 0.3 to 3 mM, there exists an equilibrium between monomeric and dimeric forms that is ~90% monomer, indicating that the $K_d$ for dimer formation would be 10 to 100-fold higher than the concentration range of these experiments. All DHP samples, including those with 4-iodophenol present, exhibit similar scattering profiles and calculated dimer/monomer ratios, indicating that the presence of inhibitor does not significantly alter the ratio of monomer to dimer in solution. Although we have not yet conducted SAXS experiments on DHP B, we have co-crystallized DHP A and B and crystallized DHP B. DHP B behaves in a manner similar to DHP A, with a similar unit cell and lack of a well-defined dimer interface. DHP B differs from DHP A by only 5 amino acids, none of which are in the crucial interface regions. Clearly, there is interest in understanding the role of the small dimer...
component and the possible protein-protein interactions that may be involved in formation of the giant Hb of *A. ornata*. Based on the present work, we suggest that other proteins must be involved in the formation of the giant Hb structure. In conclusion, considering these SAXS and gel permeation results, we suggest that DHP A and B should henceforth be referred to as monomers.

Acknowledgements

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CHAPTER 3

Internal binding of halogenated phenols in dehaloperoxidase-hemoglobin

inhibits peroxidase function
Abstract

Dehaloperoxidase from the annelid, *Amphitrite ornata*, is a catalytically active hemoglobin-peroxidase that possesses a unique internal binding cavity in the distal pocket above the heme. The previously published crystal structure of dehaloperoxidase shows 4-iodophenol bound internally. This led to the proposal that the internal binding site is the active site for phenol oxidation. However, the native substrate for dehaloperoxidase is 2,4,6-tribromophenol, and all attempts to bind 2,4,6-tribromophenol in the internal site under physiological conditions have failed. Herein, we show that the binding of 4-halophenols in the internal pocket inhibits enzymatic function. Furthermore, we demonstrate that dehaloperoxidase has a unique two-site competitive binding mechanism in which the internal and external binding sites communicate through two conformations of the distal histidine of the enzyme resulting in non-classical competitive inhibition. The same distal histidine conformations involved in dehaloperoxidase function regulate oxygen binding and release during transport and storage by hemoglobins and myoglobins. The present work provides further support that there exists an external site on dehaloperoxidase for substrate oxidation as is typical for the peroxidase family of enzymes.
Introduction

The two dehaloperoxidase-hemoglobins from *Amphitrite ornate* (DHP A and DHP B), are the first characterized hemoglobins (Hbs) that have natural peroxidase functions (1-6). Hbs, including DHP, are readily identified by their characteristic 3/3 α-helical protein structure. While Hbs are primarily associated with O₂ storage and transport, the characteristic globin fold actually encodes a diversity of protein functions. In addition to allosteric regulation of oxygen uptake, globins minimize the autooxidation rate of the heme iron, discriminate against CO binding, and carry out other natural functions such as NO binding and oxidation to nitrate. DHP brings this functional diversity to a new level by combining the seemingly contradictory functions of reversible oxygen binding (globin) and hydrogen peroxide activation (peroxidase).

Although Hbs and heme peroxidases are structurally distinct and perform different functions, both have a common heme cofactor and iron binding site, with a histidine residue positioned on each side (Figure 3.1). The proximal histidine is coordinated to the heme iron and provides a charge relay that supports either the ferrous (Fe²⁺) or ferric (Fe³⁺) iron oxidation state (7, 8), dependant on the local environment of globins or peroxidases, respectively. In myoglobins (Mbs) and Hbs, the distal histidine stabilizes diatomic oxygen during uptake and transport, whereas in peroxidases, the distal histidine serves as the acid-base catalyst necessary for heterolytic O-O bond cleavage which constitutes the activation of bound hydrogen peroxide. Allostery in hemoglobin (Hb) is vital for the proper uptake of oxygen in the lungs and release of oxygen to respiring tissues (9). While allostery may modify a binding constant or facilitate communication between multimers in cooperative
Figure 3.1. Reaction scheme emphasizing the conformation of the distal histidine, H55, in response to binding of substrate, 2,4,6-TXP, and inhibitor, 4-XP.
(a) Active enzyme: DHP with TXP substrate bound external to the heme pocket. The protein is 6cHS (aquo) with the distal H55 in a closed position.
(b) When an inhibitor, 4-XP, binds in the internal pocket of DHP, H$_2$O is displaced (5cHS) and the distal H55 is pushed to the open position. The resulting conformation leads to inactivation of the enzyme.
(c) Addition of H$_2$O$_2$ leads to the formation of compound ES (37), the high-valent iron-oxo protein (Tyr) radical intermediate, which can lead to formation of the product 2,6-DXQ by two-electron oxidation. Compound ES cannot be formed in the inhibitor-bound state since the Fe$^{III}$ site is blocked by 4-XP binding in the distal cavity.
(d) In the resting state of DHP the distal histidine exists in two conformations known as open (red) and closed (blue). H$_2$O is bound to the heme iron only in the closed conformation.
proteins such as Hb, allosteric inhibition in heme peroxidases is an off-switch that can cause the enzyme to become completely inactive (10-12). The significance of both allostery and inhibition is more complex in a dual function protein like DHP since the regulation involves not only each individual function, but also the switch between functions.

The first X-ray crystal structures of DHP A (PDB 1EW6 and 1EWA), obtained at room temperature, showed two features that are unique in the Hb superfamily (1). First, the distal histidine was observed in two conformations at pH 6, identified as open and closed. The closed conformation is the commonly observed conformation shown in Figure 3.2a, in which the histidine is in the distal pocket and interacts with a ligand coordinated to the heme iron (13). In the open conformation (Figure 3.2b), the distal histidine (H55) has swung out to a solvent exposed position (14). Although the open conformation is known in sperm whale Mb, it is only observed below pH 4.5 when the distal histidine is protonated (15). We have previously shown by comparison of X-ray crystal structures, 3DR9 and 2QFK, at 100 K that the open and closed conformations in DHP A are correlated with the 5-coordinate and 6-coordinate forms of the heme iron, respectively (13, 14). Furthermore, the unique flexibility of the distal histidine has been shown to play an important role in heme-coordinated ligand stabilization (16). The second unprecedented observation in the initial X-ray crystal structure of DHP A was the presence of a substrate analog, 4-iodophenol, in a well-defined position in the distal pocket of the globin but not coordinated to the heme iron (1). This unusual mode of binding in a Hb led to the suggestion that the internal binding site is the substrate binding site (1). We have systematically investigated this hypothesis and found that the 4-halogenated phenols (4-XP) that bind internally are inhibitors, rather than substrates. The
Figure 3.2. X-ray crystal structures of DHP at 100 K.
(a) The metaquo form shows a hydrogen-bond interaction between the distal histidine (H55) and the water molecule coordinated to the ferric heme iron (PDB 2QFK) (closed conformation). (b) The deoxy form has a pentacoordinate heme iron. Two rotamers of H55 are observed in which H55 appears exclusively in a solvent exposed conformation (PDB 3DR9). This His conformer corresponds to the open form observed in Mb at pH 4.5 (15). (c) The three structures of 4-IP (3LB1; green), 4-BP (3LB2; light blue) and 4-CP (3LB3; grey) bound in the distal pocket of DHP are superimposed. 4-FP (3LB4) is not shown in the figure (see text).
active site for oxidation of substrates such as 2,4,6-tribromophenol (2,4,6-TBP), 2,4,6-trichlorophenol (2,4,6-TCP) and 2,4,6-trifluorophenol (2,4,6-TFP) is external (17). The possible location of an external substrate binding site on DHP A has recently been established via backbone NMR experiments (18). Given the extensive data available on DHP A relative to the more recently characterized DHP B, the remainder of this study will focus on DHP A, which will be referred to as DHP for brevity.

In the present work, we provide detailed evidence that internal binding of 4-XPs inhibits peroxidase function of DHP. The result is contradictory to the previous hypothesis that the distal pocket binding site for 4-XP is the substrate binding site for phenol oxidation. This work builds on a number of observations that support the existence of distinct binding sites for 4-BP and 2,4,6-TBP and demonstrates that the distinct sites are involved in inhibition of a competitive nature. Normally, competitive inhibition implies that the substrate and inhibitor compete for the same binding site. However, both structural and kinetic evidence presented below suggest that DHP exhibits a form of competitive inhibition, formally known as allosteric or non-classical competitive inhibition (19-22), in which the inhibitor binds remote to the active site and creates a conformational change in the enzyme that prevents substrate from binding. The X-ray crystallographic, resonance Raman (RR), and kinetic data presented here are consistent with competitive inhibition between the internal and external sites mediated by the distal histidine (H55). The proposed functional role for the distal histidine, as the switch that leads to peroxidase inhibition (open) and activation (closed), overlays the regulation of oxygen binding affinity by the same histidine.
in the open (low affinity) and closed (high affinity) conformations traditionally observed in globins (15, 23, 24).

**Methods**

**Materials.** Buffer salts were purchased from Fisher Scientific. All other reagents were purchased from Sigma-Aldrich and used without further purification.

**Crystallization, Data Collection and Processing.** Recombinant wild type protein was expressed in *E. coli*, purified, and characterized as previously described (13). In order to obtain crystals of DHP complexed with substrate analogs, the protein, at a concentration of 8 mg/ml dissolved in 10 mM Na cacodylate pH 6.5, was incubated on ice for 30 min with the para-halogenated phenols (1.5 mM 4-IP, 10 mM 4-BP or 10 mM 4-CP, respectively), and crystallized using the hanging drop vapor diffusion method with the reservoir solution containing unbuffered 0.2 M ammonium sulfate and 32 % PEG 4000 (w/v) as described previously (13, 14). The crystals were cryoprotected in a solution containing 0.2 M ammonium sulfate, 35% PEG 4000 (w/v), and 15% PEG 400 as the cryoprotectant. Data were collected at 100 K on the SER-CAT 22-ID beamline at the APS synchrotron facility using a wavelength of 1 Å for crystals derivatized with 4-chloro- and 4-fluorophenol, 0.91942 Å for crystals derivatized with 4-bromophenol, and 1.5 Å for crystals derivatized with 4-iodophenol. The latter two wavelengths were chosen to be able to collect two data sets in a single-wavelength anomalous dispersion mode in order to correctly orient the halogenated phenol in its electron density. The collected diffraction data sets were processed using HKL2000 program suite (25). The new crystals belong to the same space group, P2₁₂₁₂₁, as the ferric water-ligated (metaquo) form (PDB entry 2QFK), and the structures
were solved by molecular replacement using 2QFK coordinates as a starting model in the Phaser molecular replacement program (26). The structure determination and refinement calculations were performed using the CCP4 suite of programs (27, 28) whereas the visualization and manual model building were conducted using Coot model building software (29). Waters were placed with the Coot routine, Find Waters, using 2F_o-F_c contoured at 1 \( \sigma \) level and F_o-F_c maps at 3\( \sigma \) level. The occupancies were refined manually until no residual Fo-Fc density remained. Final models were obtained by iterative cycles of model building in Coot using 2F_o-F_c (contoured at 1 \( \sigma \) level) and F_o-F_c electron density maps (contoured at 3\( \sigma \) level) and positional and anisotropic B factor structure refinement using Refmac5 (30) in the CCP4 suite of programs (Collaborative Computational Project, 1994) and CNS. (31) Simulated annealing and composite omit maps were constructed with the CNS program. All the figures were prepared using PyMOL (DeLano, 2002). The refinement statistics of the four X-ray crystal structures (3LB1, 3LB2, 3LB3, and 3LB4) are given in Table 3.1.
### Table 3.1. Crystallographic Data Collection and Refinement Statistics

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<sup>a</sup> Crystal containing 4-halophenol in complex with wt DHP; the letter specifies the halogen substituent.<br><sup>b</sup> Values in parenthesis are the highest resolution shell.<br><sup>c</sup> R<sub>merge</sub> = (Σ<sub>i</sub>Σ<sub>j</sub>{}) x 100%, where I<sub>i</sub> is the ith measurement and <I(h)> is the weighted mean of all measurements of I(h).<br><sup>d</sup> R<sub>work</sub> = Σ|F<sub>o</sub> – F<sub>c</sub>|/ΣF<sub>o</sub> x 100%, where F<sub>o</sub> are observed and F<sub>c</sub> calculated structure factors, respectively; R<sub>free</sub> is R factor for the subset (5%) of reflections selected before, and not included in the refinement.<br><sup>e</sup> rmsd is root mean square deviation.<br><sup>f</sup> Calculated using PROCHECK.
Electronic Absorption Spectroscopy and Kinetic Assays. Recombinant his-tagged wild type protein was expressed in *E. coli* and purified as previously described (17, 18). Initial inhibition experiments were conducted in 100 mM potassium phosphate buffer at pH 7 using an Agilent 8453 UV-vis spectrometer equipped with a temperature control and Hewlett Packard UV-Visible Chemstation Software set to kinetics mode. The concentration of DHP in each sample was approximately 2.4 µM, and temperature was equilibrated to 20 °C. A 100-fold excess of H₂O₂ (240 µM) was added to the cuvette to initiate the assay. Electronic absorption spectra were taken every 2 seconds for 2 minutes, monitoring the 273 nm peak of the 2,6-dichloro-1,4-dibenzoquinone product (2,4-DCQ). The assays were repeated with the addition of 250 µM 4-bromophenol in each sample to demonstrate the inhibition effect.

Inhibition assays for Michaelis-Menten analysis were conducted on a Cary 100 UV-vis equipped with a Cary temperature control system and an Applied Photophysics RX2000 Rapid Kinetics Spectrometer Accessory. The Applied Photophysics pre-mixing chambers were temperature controlled with a Fisher Scientific Isotemp 3006S set to 25 °C. Instruments were controlled by Cary WinUV software in kinetics mode set to monitor the 273 2,4-DCQ peak every 0.1 s for 60 seconds. Assay conditions were 2 µM DHP initiated with 2 mM H₂O₂ in 100 mM potassium phosphate buffer at pH 7. Eight assays were completed at each of the 2,4,6-TCP concentrations. The assays were then repeated at each of the 2,4,6-TCP concentrations with the presence of 125, 250, and 500 µM 4-BP. The initial velocity, V₀, of enzyme turnover was obtained for each concentration of the 2,4,6-TCP substrate and the 2,4,6-TCP substrate with inhibitor. The V₀ versus 2,4,6-TCP concentration
data were fit independently to the Michaelis-Menten equation using non-linear squares in IgorPro5.0.

**Resonance Raman Spectroscopy.** All protein samples used in resonance Raman experiments were purified as above and maintained in 150 mM potassium phosphate buffer, pH 6. The final protein concentration for all RR samples 100 µM. Para-halogenated substrate analogs were introduced to final concentrations of 8 mM for 4-BP, 4-CP, 4-FP, and phenol, and to 1 mM for 4-IP. The concentration of 4-IP is limited by its low solubility at 25°C. The samples were placed into 5 mm diameter glass NMR tubes and stored on ice until used.

Resonance Raman spectra were obtained by Soret band excitation using a Coherent Mira 900 titanium sapphire (Ti:sapphire) laser. The Ti:sapphire laser was pumped using a Coherent Verdi 10 frequency doubled diode pumped Nd:vanadate laser generating 10 W at 532 nm. The beam generated from the Ti:sapphire is tunable through approximately 700-1000 nm, and was sent through a Coherent 5-050 doubler to generate a normal working range of 400-430 nm for Soret band excitation. The beam was collimated and cylindrically focused to a vertical line of ~0.5 mm on the sample. Scattered light was collected with a Spex 1877 triple spectrometer (2400 grooves/mm final stage grating) equipped with an ISA SPEX liquid nitrogen-cooled CCD at ~1.7 cm⁻¹ resolution. Computer acquisition of the data was accomplished with SpectraMax 2.0 software. The spectra were calibrated using known peaks from indene, toluene, and carbon tetrachloride standards.

**Binding Isotherm Analysis.** A titration data set for each inhibitor binding to DHP was collected via a series of resonance Raman spectra. Each sample contained 100 µM DHP in
150 mM potassium phosphate buffer at pH 6, with concentrations of the inhibitor from 0 to 8 mM (0 to 1 mM for 4-IP only). Singular value decomposition was performed on the spectral data set using Igor Pro 5.0. The SVD analysis yields 1-dimensional column and row eigenvectors; columns corresponding to changes with respect to wavenumber and rows corresponding to changes with respect to concentration. The SVD row eigenvectors representing the intensity changes and the peak shifts of the Raman data set were fit using non-linear least squares to the single site binding equation

\[
\theta = \frac{[I]}{K_d + [I]}
\]

to determine apparent substrate dissociation constants, \(K_d\), where \(\theta\) is the fraction 5-coordinate high spin protein and \([I]\) is the concentration of inhibitor.

**Results**

*Crystallography.* Previously published X-ray crystal structures (13, 14) and spectroscopic data (16) strongly suggest a role for distal histidine flexibility in DHP. Figures 3.2a (closed) and 2b (open) are PDB structures 2QFK and 3DR9, respectively. As mentioned above, in the metaquo form, the distal His is stabilized in the closed conformation by hydrogen bonding to the heme-coordinated water molecule (Figures 3.2a), and the heme iron is 6-coordinate high spin. However, unlike other Hb structures (32-34), in the 5-coordinate deoxy form the His is observed in the open conformation (Figure 3.2b). Therefore the open and closed conformations in DHP are correlated with the 5-coordinate and 6-coordinate forms of the heme iron. Figure 3.2c shows an overlay of the new heme pocket structures of DHP co-crystallized with 4-IP (3LB1), 4-BP (3LB2), and 4-CP (3LB3) following established
protocols (13). The 4-XPs bind in a conformation close to that originally reported for 4-IP (1). The occupancy of the 4-IP, 4-BP, and 4-CP molecules is greater than 90% in all three structures. The structure of DHP with 4-FP (3LB4) is not shown due to its low occupancy (< 50%) and for clarity of the figure. Upon binding of these molecules in the internal site, the heme-coordinated water molecule is displaced and the histidine is pushed into the open conformation, thus the iron is 5-coordinate high spin (see also Figure 3.1 for schematic). The secondary structure of DHP A exhibits remarkably little change when 4-XPs bind in the distal pocket. The backbone root-mean-square deviations from the metaquo structure are around 0.4 Å or less, and the pairwise main chain differences between the complexed structures are on the order of 0.1 to 0.2 Å. On the other hand, superposition of the structures shows that, as the size of the para-halogen atom increases, the position of the 4-XP molecules bound in the distal pocket shifts slightly towards the heme-7-propionate and the solvent-exposed distal histidine.

**Binding of parahalogenated phenols.** While the X-ray crystal structures provide meaningful insight into DHP in the solid state, resonance Raman spectroscopy was used to investigate the solution state properties of halophenol binding. Figure 3.3a compares the RR spectra of wild type DHP (WT-DHP) with those obtained upon addition of phenol, and the 4-XP molecules (X = F, Cl, Br, I). The 5-coordinate high spin (5cHS) core size marker band frequencies \( v_3 \) at 1494 cm\(^{-1} \), \( v_2 \) at 1568 cm\(^{-1} \) and \( v_{10} \) at 1632 cm\(^{-1} \) systematically become more intense at the expense of the aquo 6-coordinate high spin (6cHS) heme state \( v_3 \) at 1481
Figure 3.3. Determination of internal binding affinity.
(a) RR core size marker band region for WT-DHP (black), DHP with phenol (yellow), DHP with 4-FP (purple), DHP with 4-CP (blue), DHP with 4-BP (green), and DHP with 4-IP (red). The $\nu_2$, $\nu_3$ and $\nu_{10}$ frequencies are consistent with the increase of a 5cHS iron upon addition of the inhibitors. The final concentration of 4-IP was 1 mM (maximum solubility), and the final concentration of 4-BP, 4-CP, 4-FP, and phenol was 8 mM; protein concentration was 100 µM. Excitation wavelength was 406 nm; 1.7 cm$^{-1}$ resolution; laser power at the sample 60mW, and 300 s acquisition times. (b) Binding isotherms for 4-IP (red), 4-BP (green), 4-CP (blue), 4-FP (purple), and phenol (yellow). The isotherms clearly establish that the affinity of DHP for 4-XP inhibitors follows the pattern 4-IP > 4-BP > 4-CP > 4-FP, > phenol at pH 6.
cm$^{-1}$, $v_2$ at 1562 cm$^{-1}$ and $v_{10}$ at 1611 cm$^{-1}$) for the series of 4-XP bound DHP relative to WT-DHP. Similar systematic changes are also observed in the corresponding electronic absorption spectra. The Soret maximum undergoes a systematic blue shift as the substrate halogen is changed and follows the halogen series (Figure F.1). Therefore, in agreement with the X-ray crystal structures, binding of 4-XP in the internal pocket is consistent with the loss of the 6cHS population and subsequent movement of the distal His to the open, solvent exposed, position. Figure 3.3b shows that 4-halophenols bind in the distal pocket with a binding affinity that follows the trend I > Br > Cl > F > H, with apparent dissociation constants of 0.536, 1.15, 1.78, 3.72, and 10.0 mM, respectively. We use the term, apparent dissociation constant, because the binding isotherms represent the fraction of enzyme converted to 5cHS, which does not necessarily reflect total binding to the enzyme. The relative binding affinity of 4-FP reflects its low occupancy in the crystal structure. The binding isotherms were determined using the change in relative intensities and the frequency shifts of the core size heme vibrational modes measured by RR spectroscopy and obtained from the data shown in Figure 3.3a by singular value decomposition (as shown in Figure F.2).

**Binding of trihalogenated phenols.** In contrast to the binding of 4-XPs, binding of 2,4,6-TXP substrates produces an increase of the 6cHS species. Figure 3.4 shows the change of the RR core size marker bands, indicating the formation of a predominately 6cHS heme ($v_3$ at 1481 cm$^{-1}$, $v_2$ at 1562 cm$^{-1}$ and $v_{10}$ at 1611 cm$^{-1}$) when 2,4,6-TBP and 2,4,6-TCP bind to DHP. While the binding of 2,4,6-TFP also produces predominately 6cHS heme, an appreciable
amount of 5cHS ($v_3$ at 1494 cm$^{-1}$, $v_2$ at 1568 cm$^{-1}$ and $v_{10}$ at 1632 cm$^{-1}$) is also formed, indicating that 2,4,6-TFP binds either externally or internally, unlike 2,4,6-TCP or 2,4,6-

![Diagram of Raman spectra showing vibrational modes $v_2$, $v_3$, $v_{10}$, and $v_{C=C}$.

**Figure 3.4. Evidence of external binding.**
Resonance Raman core size marker band region for WT-DHP (a), DHP with 2,4,6-TBP (b), DHP with 2,4,6-TCP (c), and DHP with 2,4,6-TFP (d). The $v_2$, $v_3$, and $v_{10}$ frequencies shift to predominately 6cHS upon addition of the TXP substrates. The 5cHS population observed upon addition of 2,4,6-TFP may indicate that 2,4,6-TFP does enter the distal pocket to some degree, in agreement with cryogenic experiments (16, 35, 36). The final concentration of 2,4,6-TBP was 200 µM, and the final concentrations of 2,4,6-TCP and 2,4,6-TFP were 4 mM in 150 mM potassium phosphate buffer, pH 6. Excitation wavelength was 406 nm; 1.7 cm$^{-1}$ resolution; laser power at the sample 60mW, and 300 s acquisition times.

TBP. This result is in agreement with previous studies of DHP showing that 2,4,6-TFP binds internally at cryogenic temperatures (16, 35, 36), but binds non-specifically to both sites at room temperature (16, 18). The corresponding electronic absorption spectra of 2,4,6-TXP binding (Figure F.3) change accordingly. More importantly, we can control the distal
histidine and force DHP to adopt either the 5cHS or 6cHS state by changing the concentrations of 4-BP and 2,4,6-TCP present in solution. Figure F.4 shows that the same ratio of 5cHS:6cHS heme is achieved by first adding DHP to 2,4,6-TCP and then adding 4-BP or by first adding DHP to 4-BP and then adding 2,4,6-TCP. The result demonstrates that 2,4,6-TCP and 4-BP compete for their respective binding locations until an equilibrium between the two states is attained. The X-ray crystal structures show the mutual exclusivity of the closed conformation of the distal histidine and the binding of 4-XP in the distal pocket. Since binding of 2,4,6-TCP forces the distal histidine into the closed position, it must also effectively remove 4-BP from the distal pocket. Thus, by correlation of the X-ray crystal structures and the RR data, we conclude that binding at the internal and external sites are mutually exclusive events.

**Enzyme inhibition by parahalogenated phenols.** Davis et al. confirmed the existence of two distinct binding sites for 2,4,6-TCP and 4-BP, and they showed that DHP has little activity toward 4-BP compared to 2,4,6-TCP (18). We have therefore tested the effect internal binding of 4-XP has on the turnover of 2,4,6-TCP. Figure 3.5 provides kinetic evidence that the oxidation of 2,4,6-TCP is in fact inhibited by internal binding of 4-halophenols. The substrate, 2,4,6-TCP, is readily converted to product, 2,6-dichloroquinone (2,6-DCQ), when DHP is activated with excess H_{2}O_{2} (Figure 3.5a). However, in the presence of even a 2:1 ratio of 4-XP:2,4,6-TCP, little turnover of 2,4,6-TCP to 2,6-DCQ is observed (Figures 3.5b-5e). In fact, the only appreciable turnover is observed in the presence of 4-FP, in agreement with the binding isotherms from Figure 3.2b that suggest only a small fraction of 4-FP binds internally. Since the 4-XP molecules bind internally and force the distal histidine to adopt
the open conformation, it is likely that they prevent the formation of compound ES (37), the H$_2$O$_2$ activated protein radical form of DHP analogous to compound I of cytochrome c peroxidase (38) (see Figure 3.1c). Indeed, Figure 3.5f shows that increasing the concentration of 4-BP systematically blocks the formation of compound ES.

Figure 3.5. Kinetic assays showing inhibition by internal bound 4-XPs. (a - e) Time-dependent UV-Vis spectra from 0 seconds (red) to 120 seconds (purple). In the absence of 4-XP (a), the TCP substrate (312 nm) is converted to the DCQ product (273 nm). In the presence of 4-IP, 4-BP, and 4-CP (b - d), little product is formed with no significant decrease in the substrate band. In the presence of 4-FP (e), some turnover is observed, in
agreement with the lower affinity of 4-FP to bind internally. (f) Inhibition of compound ES formation ($\lambda_{max}$ (Soret) = 420 nm) in ferric DHP at pH 7 due to increasing concentrations of 4-BP.

**Michaelis-Menten inhibition/kinetic analysis.** Since the unique internal binding of 4-XPs inhibits the peroxidase function of DHP at a remote external site, it is of interest to determine the type of inhibition that occurs between the two sites. The solubility of the native substrate, 2,4,6-TBP, is relatively low, ~200 µM, which limits its usefulness for kinetic studies. However, 2,4,6-TCP is an excellent substrate and has higher solubility than 2,4,6-TBP. Therefore, we have used 2,4,6-TCP rather than 2,4,6-TBP for the following kinetic studies in order to demonstrate enzymatic inhibition by 4-BP (Figure 3.6). The data obtained with and without 4-BP were fit independently to a Michaelis-Menten kinetic model. The fit parameters for the kinetic data are summarized in Table 3.2. $K_M$ increases as the concentration of inhibitor increases, but $V_{max}$ is essentially unchanged within the limits of the fitting errors. Ideally, one would use substrate concentrations several times greater than $K_M$, i.e. $[S]_{max} >> K_M$, but even using 2,4,6-TCP, substrate solubility remains the limiting factor. Thus, errors in the fit data arise as $K_M$ becomes greater than $[S]_{max}$. While we recognize this limitation, the relative effect of the inhibitor is clearly observed. Based on these data, the substrate/inhibitor pair exhibit competitive inhibition in DHP. Although results of this type cannot be demonstrated for 2,4,6-TBP because of its limited solubility, kinetic assays of 2,4,6-TBP with 4-BP under identical conditions to those used for 2,4,6-TCP with 4-BP yield the same complete inhibition effect (Figure F.5).
Figure 3.6. Michaelis-Menten analysis of inhibition.
Initial reaction velocity versus substrate (2,4,6-TCP) concentration. Assays conducted of WT-DHP without the presence of inhibitor, WT-DHP with 125 µM 4-BP, WT-DHP with 250 µM 4-BP, and WT-DHP with 500 µM 4-BP. The Michaelis-Menten fit parameters are given in Table 2.1.

Table 2.1. Michaelis-Menten fit parameters.

<table>
<thead>
<tr>
<th></th>
<th>$V_{\text{max}}$ (µM/s)</th>
<th>$K_M$ (mM)</th>
</tr>
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<tbody>
<tr>
<td>No Inhibitor</td>
<td>13.1 ± 1.32</td>
<td>1.32 ± 2.11</td>
</tr>
<tr>
<td>125 µM 4-BP</td>
<td>15.3 ± 2.94</td>
<td>2.53 ± 6.11</td>
</tr>
<tr>
<td>250 µM 4-BP</td>
<td>13.7 ± 4.12</td>
<td>2.80 ± 1.06</td>
</tr>
<tr>
<td>500 µM 4-BP</td>
<td>10.4 ± 4.58</td>
<td>3.08 ± 1.71</td>
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Discussion

The shifts in the relative position of 4-XPs bound in the distal pocket of DHP suggest that there are two driving forces that stabilize the molecule. First, the para-halogen atom fills a cavity in the protein. Cavities in Mb have been studied by determining Xe binding sites using X-ray crystallography (39). The cavity within DHP that is filled by the para-halogen is surrounded by hydrophobic residues (Figure F.6) and resembles the Xe4 binding site in
sperm whale Mb (39). The second factor is the interaction of the hydroxyl group with Y38, heme-7-propionate and, to a lesser extent, with H55 (Figure 3.2c). As the atomic radius of the halogen decreases, the hydroxyl group moves into closer contact with H55, Y38, and the heme propionate. Therefore, as shown in Figures 3.2 and 3.3, a single atom, the para halogen X in 4-XP, determines the binding affinity of the molecule in the distal pocket. Since the bound 4-XP displaces the water coordinated to the heme iron, it is also expected that it prevents the coordination of H₂O₂, and thereby inhibits peroxidase activity. The finding that increased concentrations of 4-BP systematically block the formation of the radical enzymatic intermediate (compound ES (37), see Figure 3.1) in ferric DHP at pH 7 supports this hypothesis. Moreover, in accord with this hypothesis, the H55V mutation effectively eliminates the enzymatic activity of DHP (40).

On the basis of these results, and in agreement with the known mechanism for the peroxidase family members, we propose that enzymatic oxidation of substrates such as 2,4,6-TXP (X = I, Br, Cl, F) occurs at an external site. Recent ¹H⁻¹⁵N HSQC experiments on ¹³C/¹⁵N labeled DHP clearly indicate different binding interactions between 4-BP inhibitor and 2,4,6-TCP substrate (18). 4-BP binding causes deviations in the internal binding pocket residues (F24, F35, F21, H55, and V59) (see Figure F.7) while 2,4,6-TCP binding affects the distal H55 and amino acids residing at the protein dimer interface (R122, G1, S129, and L76) (see Figure F.8) (18). Interestingly, the common amino acid affected by either binding event, as observed by NMR, is the distal H55. The combination of resonance Raman and NMR strongly supports the existence of an external substrate binding site. Consistent with these observations, every attempt to infuse 2,4,6-TXPs into the distal pocket in crystals of DHP
using identical conditions used for the 4-XP X-ray structures has resulted in no observable binding.

It appears from the RR data in Figure 3.4 that 2,4,6-TFP binds both externally and internally, which implies that it acts as substrate and inhibitor, respectively. It is not possible to directly measure whether 2,4,6-TFP inhibits itself, i.e. autoinhibition. However, 2,4,6-TFP is a poor substrate for DHP compared to 2,4,6-TCP or 2,4,6-TBP, which may be in part due to its propensity to bind as an inhibitor. Figure F.9 provides a kinetic summary of the inhibition of 2,4,6-TCP by 2,4,6-TFP. It is clear from the data that 2,4,6-TFP does in fact inhibit 2,4,6-TCP oxidation. The sigmoidal shape of 2,6-DCQ product formation suggests that 2,4,6-TFP is oxidized until its concentration is reduced such that it no longer acts as an inhibitor to 2,4,6-TCP oxidation. 2,4,6-TFP was used in a number of previous binding studies as a model for the native substrate because of its high solubility and similar substitution pattern (16, 35, 36).

The proposed external active site is consistent with pH-dependent studies of enzyme activity. The greatest activity of DHP for oxidation of 2,4,6-TCP was observed at pH 7.5 (41). Since at this pH, 2,4,6-TCP (pKa 6.4 (42)) is in the phenolate form, it is unlikely that it will be able to enter the distal pocket. On the other hand, the pKa of 4-BP is 9.3 (43) so the inhibitor will be protonated and hence neutral at pH 7.5. It is well known that buried charges are not stable in proteins. Attempts to place a buried charge in Mb by the mutation V68D resulted in ligation of the negatively charged carboxylate to the ferric heme iron, thus neutralizing the charge (44). Hence, neither the structural nor the functional observations of enzymatic activity are consistent with substrate binding in the distal pocket, but the same
considerations are consistent with the internal binding of 4-bromophenol as an inhibitor. In line with this reasoning, the pKa of 2,4,6-TFP is \(~7.2\) (45), the highest of all of the TXP’s studied. Thus 2,4,6-TFP will have the highest percentage of the phenol form at physiological pH, and therefore the greatest propensity to enter the distal cavity of the enzyme.

The correlation of the X-ray structural and resonance Raman data elucidates the key role played by the flexibility of the distal histidine. The X-ray crystal structure of Lebioda et al. (PDB 1EW6) shows that H55 is in equilibrium between the open and closed conformations at room temperature (1). Nicoletti et al. showed the $\nu_3$ band of the RR spectrum displays an approximately 40:60 ratio of 5cHS and 6cHS forms (16). Binding of 4-XP to DHP forces the distal H55 and H$_2$O out of the distal pocket (Figures 3.1b, 3.2c, and 3.3a). Accordingly, the RR spectra in the presence of 4-XP are typical of 5cHS heme, indicating that the water molecule has been expelled. Conversely, the RR spectroscopic data in Figure 3.4, and the UV-vis data in Figure F.3, show that binding of 2,4,6-TXP increases the population of aquo 6cHS heme. Thus, external binding of a 2,4,6-TXP substrate forces the distal H55 into the closed position, stabilizing the heme iron-coordinated water molecule (Figure 3.1a). Substrate binding, then, enforces the closed H55 conformation, which strengthens H$_2$O$_2$ coordination to the heme iron and positions H55 to serve as the acid-base catalyst in the peroxidase mechanism. Furthermore, we have demonstrated that the 5cHS:6cHS ratio can be driven to either extreme by varying the concentrations of inhibitor and substrate (Figure F.4) indicating mutual exclusivity between their respective binding sites.
The kinetic data presented in Figure 3.6 and Table 3.2 demonstrate the competitive nature of the inhibition that occurs between the substrate/inhibitor pair. Based on the structural and spectroscopic data presented herein, this unique mechanism involves two independent but concerted competitive binding events. The first arises from the steric interactions of 4-XP binding in the distal pocket preventing ligation of H₂O₂ to the heme iron. The second is allosteric communication by movement of the distal histidine resulting in two-site competitive binding between the substrate and inhibitor pair. Thus, inhibition by 4-XP appears to involve three simultaneous effects: 1) H₂O₂ coordination to the heme iron is impeded, 2) displacement of the distal H55, which is the acid-base catalyst in the peroxidase mechanism (46-48), out of the cavity, and, subsequent 3) rearrangement of the external binding site by the solvent-exposed (open) distal H55 conformation. Therefore, DHP exhibits non-classical two-site competitive inhibition in which the inhibitor and substrate have mutually exclusive binding interactions. When the substrate is bound, the distal histidine is in the internal, or active, conformation (6cHS), and the inhibitor cannot access the internal site (see Figure 3.1a). On the contrary, when the inhibitor is bound, the distal histidine is in the solvent-exposed, or inactive, conformation (5cHS), and the substrate cannot access the external site (see Figure 3.1b). The two-site competitive inhibition is driven by the same open/closed conformational change that has been studied for many years in Mb (15, 24).

**Concluding Remarks**

Since the X-ray crystal structure of DHP showing the substrate analog, 4-IP, bound in the distal pocket (PDB 1EWA) was the first observation of internal binding, it was
reasonable to consider the internal site may serve as the active site (1). However, structural, functional and spectroscopic studies have repeatedly contradicted that assumption (17, 18, 35, 36, 41, 49). The finding that 4-BP and 2,4,6-TBP act as an inhibitor and a substrate, respectively, for DHP is noteworthy since both molecules are present in benthic ecosystems (50). However, since 4-BP is not an oxidation product of 2,4,6-TBP, this inhibitor/substrate pair is not part of a feedback system for this enzyme. Organisms such as Notomastus lobatus (among many others) synthesize 4-BP and 2,4,6-TBP, but this does not appear to be the case for A. ornata (3, 51). Instead, DHP, which is the most abundant protein in A. ornata, oxidizes 2,4,6-TBP to 2,6-dibromo-1,4-benzoquinone (Figure 3.1). The substrate 2,4,6-TBP acts as both a repellent, protecting marine organisms from predators, and a potentially lethal toxin. Therefore, the degradation of 2,4,6-TBP must be a protective function that minimizes the concentration of the highly toxic molecule in A. ornata (50). Although the reason for the inhibition of 2,4,6-TBP oxidation by 4-BP is not known, it is clear that DHP is a finely tuned enzyme that has an unusual mechanism for inhibitor specificity.

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References


CHAPTER 4

Compound ES of dehaloperoxidase decays via two alternative pathways depending on
the conformation of the distal histidine
Abstract

Dehaloperoxidase (DHP) is a respiratory hemoglobin (Hb) that catalyzes the conversion of trihalophenols to dihaloquinones in the presence of hydrogen peroxide. Ferric heme states of the resting DHP and the free radical intermediates formed under H$_2$O$_2$ treatment were studied by low temperature EPR spectroscopy in the range of reaction time of 50 ms – 2 min at three different pH values. Two high spin ferric heme forms were identified in the resting enzyme and assigned to the open and closed conformations of the distal histidine, His55. Two free radicals were found in DHP activated by H$_2$O$_2$: the radical associated with Compound ES has been assigned to Tyr34, the other radical - to Tyr38. The Tyr34 radical is formed with a very high relative yield (almost 100% of heme), atypical of other globins. The HPLC analysis of the reaction products showed a pH dependent formation of covalent heme-to-protein cross-links. The stable DHP Compound RH formed under H$_2$O$_2$ in the absence of substrates is proposed to be a state with the ferric heme covalently cross-linked to Tyr34. A kinetic model of the experimental data suggests that formation of Compound RH or the Tyr38 radical are two alternative routes of Compound ES decay. Which route is taken depends on the conformation of His55: in the less populated closed conformation, the Tyr38 radical is formed, but in the major open conformation, Compound ES decays yielding Compound RH, a product of safe termination of the two oxidizing equivalents of H$_2$O$_2$ when no substrate is available.
Introduction

Dehaloperoxidase-hemoglobin (DHP), originally isolated from the terebellid polychaete *Amphitrite ornata*, is the first characterized hemoglobin known to have natural peroxidase function. When activated with hydrogen peroxide, DHP catalyzes the overall two-electron oxidation of 2,4,6-trihalophenol (2,4,6-TXP) to the corresponding 2,6-dihaloquinone (Scheme 1.1)\(^1\). *A. ornata* cohabits benthic ecosystems with organisms such as *Thelespus crispis* and *Notomastus lobatus* that secrete brominated aromatics as predatory defense mechanisms\(^2-4\). To survive the toxic build up, the coelomic oxygen binding hemoglobin in *A. ornata* has evolved to perform a dehaloperoxidase function that results in a decrease in the concentration of toxic 2,4,6-TXP. DHP has a typical globin fold though low amino acid sequence homology with other members of the globin family\(^5\). Two isoforms of DHP are found in *A. ornata* (DHP A and DHP B), both of which can bind oxygen reversibly\(^6\). The occurrence of globins in annelids is known to occur in two general contexts. First, a monomeric hemoglobin is observed in the coelom. Second, a giant hemoglobin is found in the tentacles and other compartments outside the coelom\(^7\). As a peroxidase, DHP acts two orders of magnitude faster than myoglobin (Mb)\(^8\) and only an order of magnitude slower than horseradish peroxidase (HRP)\(^9\). Thus DHP lies in a unique median, sharing structural homology with globins and peroxidase activity with HRP.

Six available X-ray structures of DHP\(^5,10-12\) demonstrate that the distal histidine, His55, may adopt an open (outside the distal pocket) or closed (inside the pocket) conformation. This high flexibility of the distal histidine has been implicated in the mechanism of switching between oxygen storing and peroxidase activity\(^11\). The first X-ray crystal structure of DHP
showed the substrate analogue 4-iodophenol bound in the distal pocket with His55 pushed into the open conformation. This led to the proposal that the distal pocket is the substrate binding site. However, structural, spectroscopic and kinetic data suggest the existence of an external binding site, while binding in the internal site, in fact, inhibits peroxidase function. Recently, Thompson et al. demonstrated that DHP exhibits non-classical competitive inhibition, in which the inhibitor and substrate pair communicate between the internal and external binding sites, respectively, through two conformations of the distal histidine.

Upon activation by H$_2$O$_2$, DHP is converted to the oxoferryl heme state with a free radical on the globin assigned to a tyrosine. This state of DHP was interpreted as Compound ES by analogy with cytochrome c peroxidase (CcP) and was implicated in substrate oxidation. In the absence of substrates, Compound ES evolves stoichiometrically into a new optically detectable stable species termed Compound RH (reversible heme intermediate) which can be reduced by external reductants to yield ferrous DHP which combines with oxygen to give Compound III (a ferrous-oxo state).

The electron paramagnetic resonance (EPR) spectra of the radical associated with Compound ES were shown to be different when the activation was conducted at pH 5 and pH 7. This raises the question of whether the structural properties of Compound ES change with pH or a parallel process is leading to formation of a different free radical on the enzyme. This paper is aimed at elucidation of the mechanism of free radical formation on DHP when the enzyme is treated with H$_2$O$_2$. Herein we show that two different tyrosyl radicals, Tyr34• and Tyr38•, are responsible for the pH dependence of the free radical EPR spectrum, but only
the Tyr34• radical is associated with Compound ES. The other radical is formed during one of the two alternative routes of Compound ES decay.

**Experimental**

**Materials.** Buffer salts were purchased from Fisher Scientific. All other reagents were purchased from Sigma-Aldrich and used without further purification. DHP A$^6$ was studied in this paper.

**6XHis-tagged DHP A growth and purification.** Recombinant his-tagged wild type DHP protein was expressed in *E. coli* and purified as previously described$^{14}$. All experiments in this report were conducted in 100 mM potassium phosphate buffer.

**Hydrogen peroxide solutions.** The H$_2$O$_2$ solution was prepared from a 30% reagent grade H$_2$O$_2$ solution from Fisher Chemicals to a final concentration of 15 mM for the stock solution. Aliquots of 15 mM H$_2$O$_2$ stock solution in 100 mM potassium phosphate buffer at pH 7 were stored in cryotubes at liquid nitrogen temperature. The cryotubes were thawed and diluted in 100 mM potassium phosphate buffer shortly before use. One cryotube was used for making no more than 3-4 freeze-quenched samples, which normally took no longer than 1 hour. This protocol was followed in order to maintain the peroxide concentration constant during several hours sample preparation sessions.

**EPR sample preparation.** Wilmad SQ EPR tubes (Wilmad Glass, Buena, NJ) were used for EPR samples. To minimize the effect that slightly different size of tubes might have on the quantitative results, only tubes with outer diameter 4.05 ± 0.07 mm and inner diameter 3.12 ± 0.04 mm (mean ± range) were selected for use. When a set of samples was prepared
by freezing the same protein solution in the selected Wilmad SQ tubes, the random error in
the EPR signal intensities was determined to be very low (1\textendash{}3\%).

The rapid freeze quenching (RFQ) of the EPR samples was performed by a combined use
of an Update Instrument (Madison, Wisconsin) mixing machine and a home built apparatus
for freezing the ejected mixtures on the surface of a rapidly rotating aluminium disk kept at
liquid nitrogen temperature (see Supporting Information, SI.G.Methods.1). The freezing
time achievable by this apparatus was calibrated by the method described elsewhere \cite{19}
based on azide binding to horse metmyoglobin (metMb), where the rate constant of the binding was
determined optically by an Applied Photophysics SX18MV diode array spectrophotometer.

DHP and H$_2$O$_2$ solutions were kept at 4°C in the Update Instrument freeze-quenching
apparatus and were mixed in the mixing chamber kept just outside the ice bath (at ca. 15°C).
The samples corresponding to the time points in the range of 50 \textendash{} 300 ms were made using
aging hoses of variable length. For longer reaction times, a delay was used between two
pushes of the ram of the freeze-quenching apparatus. The sample frozen on the cold disk
was transferred to a funnel attached to an EPR tube and packed with a Teflon tipped stainless
steel rod. The packing factor was found to be persistently close to 50\% (±4\%). The freeze-
quenched samples, when packed, were of variable length, sometimes shorter than the active
zone of the resonators. This affected the intensity of the EPR signals. To make a correction
for this effect, we used the sample length specific coefficients obtained as described in
SI.G.Methods.2.

**EPR spectra measurement and processing.** All EPR spectra were measured on a Bruker
EMX EPR spectrometer (X-band) at a modulation frequency of 100 kHz. Accurate g-values
were obtained using the built-in microwave frequency counter and a 2,2-diphenyl-1-picrylhydrazyl powder standard, the g-value for which is \( g = 2.0037 \pm 0.0002 \) \(^{20}\). A spherical high quality Bruker resonator SP9703 and an Oxford Instruments liquid helium system were used to measure the low temperature EPR spectra. The EPR spectra of the blank samples (frozen water) were subtracted from the EPR spectra of the enzyme samples to eliminate the base line caused by the resonator’s walls, quartz insert, or quartz EPR tube.

The freeze-quenched control samples were obtained by mixing a DHP solution with buffer. The \( g_{\parallel} = 2 \) component of the high spin (HS) ferric heme signal, obtained from the DHP control samples, has been subtracted from the EPR spectra of the protein radicals formed under peroxide treatment using individual coefficients of subtraction for each spectrum as described elsewhere \(^{21}\). Thus, the free radical spectra presented in this paper are not contaminated with the HS ferric heme DHP EPR signal.

To measure intensities of overlapping EPR signals in a composite EPR spectrum, the procedure of spectral subtraction with a variable coefficient was used \(^{22,23}\). Different EPR samples are measured at slightly different microwave frequencies because of the variation in the physical characteristics of the EPR tubes. Therefore, the same EPR signal in two different EPR samples can appear at slightly different values of the magnetic field. To increase the accuracy of the spectral subtraction, this error has been corrected by shifting each experimental spectrum to the left or to the right along the magnetic field in accordance with the difference between the frequency used to record an individual spectrum and a frequency value chosen as a standard for the whole array of experimental spectra. Thus, a good alignment of all EPR spectra on the magnetic field axis has been achieved. Fig. G.5
(SI.G.Methods.3) illustrates the effect of the spectral shifting procedure on a set of spectra obtained from different samples. It should be noted that the spectra position correction based on the microwave frequency value will not have an accurate outcome (e.g. as that shown in Fig. G.5) if the magnetic field is drifting. It normally takes several hours for the EMX spectrometer to achieve stable magnetic field characteristics. Therefore all our EPR experiments were performed after the spectrometer had a considerable warming up time (2-3 days).

**EPR spectra simulation.** Tyrosyl radical EPR spectra were simulated using SimPow6. The Tyrosyl Radical Spectra Simulation Algorithm (TRSSA) was used to generate the EPR spectral simulation parameters on an input of only two parameters, the phenoxyl ring rotation angle and the spin density on atom C1 of the radical.

**Assignment of tyrosyl radicals to specific tyrosine residues.** The Phenol Ring Rotation Angle Database has been used to identify the tyrosine residues in DHP with the same, or similar, rotational conformation of the phenol group (the ring rotation angle) as the conformation determined from the simulation of the tyrosyl radical EPR spectrum.

**Kinetic modeling.** Time dependences of the concentration of total ferric heme state and total free radical, at three different pH values, were modelled using Microsoft Office Excel 2007 while the Excel Solver tool was used to optimize the rate constants by finding the minimum of the average distance between experimental and calculated values. Three average distance parameters attributable to different pH value series were added together to a single value which has been minimized during global optimization.
**Reverse-phase HPLC.** The reverse-phase HPLC method for the identification of heme-protein cross-links was adapted from that of Osawa and Korzekwa. DHP (80 µM) was reacted with H₂O₂ (0-1600 µM) in 15 mM sodium acetate buffer (pH 5.0) or sodium phosphate buffer (pH 6.0 or 7.0). The reaction was allowed to stand for at least 2 h prior to HPLC analysis. Samples were analyzed on an Agilent HP1100 HPLC equipped with a diode array spectrophotometer, and fitted with a Zorbax StableBond 300 C3 column (250 mm × 4.6 mm) with a 12 mm × 4.6 mm guard column. Solvents were (A) water containing 0.1% trifluoroacetic acid (TFA) and (B) acetonitrile containing 0.1% TFA. The gradient was initially 35% solvent B, stable for 10 min, increasing to 37% solvent B over 5 min. This was increased to 40% solvent B over 1 min and then to 43% solvent B over 10 min. The flow rate was 1 mL min⁻¹, and the temperature was 25°C. The concentration of heme to protein cross-linking was determined by integration of the heme to protein cross-linked HPLC peak at 400 nm and comparing the area to a standard.

**Results**

**Heme states in the resting (ferric) enzyme.** The EPR spectra of rapidly freeze-quenched DHP in the resting (ferric) state at three different pH values are shown in Fig. 4.1. While no low spin ferric heme forms are seen in the spectra, the EPR signal of the HS form in the g=6 region shows a slightly different lineshape at pH 5, 6 and 7. The differences were used to deconvolute the signal into two components: a rhombic component, with the g-values g₁ and g₂ well separated (R1HS, rhombic type 1 high spin), and a nearly axial component, i.e. when the difference between g₁ and g₂ is small (NAHS, nearly axial high spin). The R1HS EPR
The signal \((g_1 = 6.15, g_2 = 5.51, g_3 = 1.996)\) was obtained as a difference spectrum, \(\text{R1HS} = \text{pH 5'} - 0.38 \times \text{pH 7'}\). The NAHS signal \((g_1 = 5.97, g_2 = 5.62, \text{ and } g_3 = 1.997)\) was constructed as \(\text{NAHS} = \text{pH 7'} - 0.69 \times \text{pH 5'}\). The coefficients of the subtractions were determined empirically to yield the best pure lineshapes of the individual EPR signals.

**Figure 4.1.** EPR spectra of ferric DHP at different pH values and the result of their deconvolution into two individual HS ferric heme EPR signals, rhombic type 1 (R1HS) and ‘nearly axial’ (NAHS). The spectra were obtained at 10 K from the samples made by freeze-quenching 80 \(\mu\)M DHP at the indicated pH values mixed with equal volumes of buffer to give a final concentration of 40 \(\mu\)M. All spectra are plotted at similar amplitudes for comparative viewing. The instrumental conditions were as follows: microwave frequency \(\nu_{\text{MW}} = 9.471 \text{ GHz}\), microwave power \(P = 3.18 \text{ mW}\), modulation frequency \(\nu_{\text{m}} = 100 \text{ kHz}\), modulation amplitude \(A_{\text{m}} = 5 \text{ G}\), time constant \(\tau = 82 \text{ ms}\), scan rate \(v = 22.6 \text{ G s}^{-1}\), number of scans per spectrum \(N_S = 1\). The principal g-factors of the signals R1HS and NAHS are indicated.

The relative concentrations of the heme states responsible for the two signals, R1HS and NAHS, were estimated by measuring the relative intensities of the signals in the DHP spectra at pH 5, 6 and 7 and expressing them in common units when the values of second integrals of
R1HS and NAHS were taken into account. The result is presented in Fig. 4.2, which shows a stoichiometric transition of R1HS into NAHS as pH increases.

**Figure 4.2.** Relative concentration *(left axis)* of the two HS ferric heme forms in DHP, ‘rhombic type 1’ (R1HS) and ‘nearly axial’ (NAHS), at three different pH values. The error bars indicate maximal deviation from the average determined on the basis of three independent measurements. The concentration units are normalized to give the total concentration equal to unity. Also shown are the values of the rate constants $k_1$ (Compound ES formation, *right axis*) that have been obtained from the kinetic model described in the Kinetic Model section.

**DHP reacting with H$_2$O$_2$.** When hydrogen peroxide is added to DHP, the paramagnetic ferric heme state is transformed to the EPR silent oxoferryl form leading to a decrease of the $g$=6 signal intensity in the EPR spectrum. At the same time, a protein bound free radical is formed resulting in the appearance of a $g$=2.004 EPR signal (Fig. 4.3). This state of DHP with a protein bound free radical was first observed by Feducia et al. and interpreted as Compound ES$^{17}$. 

94
**Figure 4.3.** EPR spectra of 40 μM DHP reacted with 150 μM H₂O₂ and freeze-quenched 0.5 s after mixing (all concentrations are final, pH 7). The control sample was obtained by mixing equal volumes of 80 μM DHP and pH 7 buffer and freeze-quenching 50 ms after mixing. Inset shows the g=6 area in greater detail with the H₂O₂ treated spectrum magnified by a factor of 10. The spectra were recorded at 10 K at the same instrumental conditions as specified in the Fig. 4.1 legend.

**Concentration of the ferric heme and the free radicals as a function of reaction time.**

Kinetic dependences of the ferric heme state and the free radical concentrations were studied in DHP reacting with H₂O₂ at three different pH values (Fig. 4.4 and Fig. 4.5).
Figure 4.4. Kinetic dependences of total ferric heme state concentration in the course of the reaction of 40 μM ferric DHP with 120 μM H₂O₂, at pH 5, 6 and 7 (both concentrations are final). The ferric heme concentrations (symbols) were determined by EPR spectroscopy in the samples prepared by the RFQ method as described in the text. The lines are calculated from the kinetic model described in section 3.2.5.

Figure 4.5. Kinetic dependences of total free radical concentration in the three pH series referred to in Fig. 4.4. The EPR spectra of the free radicals detected in these series are given in Fig. G.6. Symbols represent the experimental results; the lines are generated by the same kinetic model used to plot the curves in Fig. 4.4 (see section 3.2.5 for details).
The concentrations of the ferric heme state were measured from the intensities of the HS ferric heme EPR signal, and, in the absence of detectable low spin forms, were assumed to reflect the total ferric heme concentration. The average intensity of the HS ferric heme EPR signals in the control samples (freeze-quenched on mixing with buffer containing no peroxide) were thus normalized to the initial ferric enzyme concentration determined optically (40 μM after 2-fold dilution). The small changes in the proportion of R1HS and NAHS were ignored in the measurements.

The total free radical concentrations were determined in common units of the second integral of the EPR spectra (Fig. G.6, SI.G.Results.1), measured at low microwave power, after the g=2 component of the HS ferric heme state (measured in the control, i.e. when no H₂O₂ was added) was subtracted as described elsewhere. The subtraction coefficients for each spectrum were found from the kinetics of the HS forms shown in Fig. 4.4. The integral values were then corrected for variable sample size and normalized to absolute concentration values using a Cu²⁺ concentration standard.

While reporting the ‘total free radical concentration’ in Fig. 4.5, we should note that the radical composition was not uniform in the series studied. The lineshape of the free radical EPR signals was different in the different pH series. The lineshape also changed during the reaction time. Generally, the resolution of the hyperfine structure in the free radical EPR spectrum becomes poorer as time progresses (Fig. G.6). In different pH series, the initial lineshape persisted for different lengths of reaction time, i.e. for 0.5 s at pH 5, for 3 s at pH 6 and for 6 s at pH 7.
The experimental kinetic dependences of the ferric heme and free radical at pH 5, 6 and 7 have been fitted by the dependences computed from a kinetic model (Fig. 4.4 and Fig. 4.5). The model is described in section Kinetic Modeling.

**High spin ferric heme after H₂O₂ addition.** Although a significant decrease in the concentration of the HS ferric heme forms is seen during the reaction of DHP with H₂O₂, the relative proportion of the rhombic (R1HS) and nearly axial (NAHS) ferric heme forms after the addition of H₂O₂ is similar to the relative proportion before the addition of H₂O₂. This indicates that the two forms might be in a fast equilibrium. Two small but noticeable effects should be noted though. First, the relative concentration of the axial form is slightly higher in the samples of DHP reacting with H₂O₂ than in the resting (control) samples. Second, when the ferric heme starts to recover from the EPR invisible oxoferryl form (e.g. 1 min after mixing, pH 7, Fig. 4.4), a weak signal of a new rhombic HS ferric heme (type 2, R2HS) can be seen in the spectrum. It shows a slightly greater degree of rhombicity than R1HS and is characterized by the g-factors $g_1 = 6.39$ and $g_2 = 5.42$ (Fig. G.7).

**Identification of two different hydrogen bonded tyrosyl radicals.** Fig. S6 demonstrates that the lineshapes of the free radical EPR spectra of DHP treated with H₂O₂ are different at different pH values. The difference is particularly evident when the pH 5 and pH 7 series are compared. We used the procedure of spectral subtraction with a variable coefficient to identify two different free radical EPR signals (Fig. 4.6). One is present at all
Figure 4.6. Lineshapes of the free radical EPR spectra of the freeze-quenched samples of 40 μM DHP treated with 120 μM H₂O₂. A, pH 5, 50 ms after mixing. B, pH 7, 300 ms after mixing, multiplied by a factor of 0.06. The factor 0.06 was chosen empirically to construct a pure lineshape of the signal C=A-B. Both the principal radical (B) and pH 5 radical (C) EPR signals were simulated as neutral tyrosyl radical spectra (dotted lines). The parameters of the simulation (Table S1 and Table S2) were found by TRSSA 25.

pH values studied and is particularly strong in the pH 7 spectra. We will refer to this EPR signal as that originating from the principal free radical. The other signal is generally of a much weaker intensity and is observable at pH 5 (henceforth referred to as the pH 5 radical signal).

The two EPR signals have been simulated as tyrosyl radical EPR spectra (Fig. 4.6). The simulation parameters are given in Table G.1 and Table G.2. Each of these two sets of 30 parameters has been found by TRSSA 22,25,28 when only two input parameters of the algorithm were varied. The optimal values of these two parameters for the two radicals are specified in Table G.1.

The values of the spin density on C1 for the two radicals are both in the upper part of the range of values (0.35-0.42) in other protein bound tyrosyl radicals 25. This means that both
Table 4.1. TRSSA\(^a\) input parameters for the simulation of the tyrosyl radical EPR signals in Figure 4.6

<table>
<thead>
<tr>
<th></th>
<th>McConnell spin density on atom C1</th>
<th>Phenoxy ring rotation angle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Principal radical (B)</td>
<td>0.400</td>
<td>45 (or 75)</td>
</tr>
<tr>
<td>pH 5 radical (C)</td>
<td>0.420</td>
<td>-8 (or -52)</td>
</tr>
</tbody>
</table>

\(^a\)Tyrosyl Radical Spectra Simulation Algorithm

\(^b\)There are always two values of rotation angle \(\theta\) that give identical sets of simulation parameters \(^{25,29}\), see (SI.G.Results.2) for details.

Tyrosyl radicals are hydrogen bonded. In fact, the value of 0.42 for the pH 5 radical is at the top of the range and indicates that the H-bond engaging this radical must be strong.

The principal tyrosyl radical, found in all three pH series, is characterized by the phenoxy ring rotation angle of 45° or 75°. This angle in the pH 5 tyrosyl radical is either -8° or -52°. By using the Phenol Ring Rotation Angle Database \(^{26}\), an assignment of the radicals has been performed. The most likely site of the principal radical is either Tyr28 or Tyr34, and the most likely site of the pH 5 radical is Tyr38. For details of the analysis leading to this conclusion, see SI.Results.2.

**Formation of heme to protein cross-links.** Reverse-phase HPLC was employed to study the oxidation of the heme prosthetic group in the reaction of DHP with H\(_2\)O\(_2\) (Fig. G.8). The chromatograms obtained and the optical properties of the eluted fractions (Fig. G.9) allowed identification of a DHP form in which the heme is covalently cross-linked to the globin. The H\(_2\)O\(_2\) concentration profile of the cross-link formation is pH dependent as shown in Fig. 4.7.
Figure 4.7. Effect of hydrogen peroxide molar excess over heme on the extent of heme-protein cross-link formation in 80 µM DHP (curves, left axis). The concentration of the heme to protein cross-links was determined by HPLC, the dependences at three different pH values are presented. Three crosses are related to the right axis and represent the rate constant $k_2$ of Compound ES to Compound RH conversion at the three pH values as found from the kinetic model described in section 3.2.5.
Kinetic model. The time dependences of the concentrations of total ferric heme and total free radical have been modelled (Fig. 4.4 and Fig. 4.5) by calculating a kinetic evolution of ferric DHP reacting with $\text{H}_2\text{O}_2$. A set of 10 chemical reactions involving 6 reaction components were postulated:

1. $\text{Fe(III)} + \text{H}_2\text{O}_2 \rightarrow ^\bullet \text{Fe(IV)}$

2. $^\bullet \text{Fe(IV)} \rightarrow \text{Fe(III)}$

3. $^\bullet \text{Fe(IV)} \rightarrow ^\bullet \text{Fe(III)}$

4. $\text{Fe(IV)} \rightarrow ^\bullet \text{Fe(III)}$

5. $^\bullet \text{Fe(IV)} + ^\bullet \text{Fe(IV)} \rightarrow \text{Fe(IV)} + \text{Fe(IV)}$

6. $^\bullet \text{Fe(IV)} + ^\bullet \text{Fe(III)} \rightarrow \text{Fe(IV)} + \text{Fe(III)}$

7. $^\bullet \text{Fe(IV)} + ^\bullet \text{Fe(III)} \rightarrow \text{Fe(IV)} + ^\bullet \text{Fe(III)}$

8. $^\bullet \text{Fe(III)} + ^\bullet \text{Fe(III)} \rightarrow \text{Fe(III)} + \text{Fe(III)}$

9. $^\bullet \text{Fe(III)} + ^\bullet \text{Fe(III)} \rightarrow \text{Fe(III)} + ^\bullet \text{Fe(III)}$

10. $^\bullet \text{Fe(III)} + ^\bullet \text{Fe(III)} \rightarrow \text{Fe(III)} + \text{Fe(III)}$

The rationales behind postulating these reactions, together with a number of assumptions made, are given in SI.G.Results.3. Briefly, reaction (1) describes formation of Compound ES. The latter decays via two alternative reactions (2) and (3). Reaction (4) is a decay of the ferryl heme state into the ‘ferric+radical’ state. Reactions (5) to (10) are six radical recombination reactions that can take place between three types of radical species in the model – a radical on DHP in the ferric heme state •Fe(III), a radical when DHP is in the ferryl state •Fe(IV), and a bi-radical DHP when the heme is ferric •Fe(III)•. For a time point of the evolution, the derivatives by time of the concentrations (rates) for each component were formulated via the rate constants and the components’ concentrations. The concentrations dependences on time (t) were then obtained by stepwise integration of the derivatives over the time range of choice. The graphs showing the time dependences of the total free radical concentration and of the total ferric heme state were combined as follows:

\[
\text{[Total free radical]}(t) = •\text{Fe(III)}(t) + 2 •\text{Fe(III)•}(t) + •\text{Fe(IV)}(t)
\]

\[
\text{[Total ferric heme]}(t) = \text{Fe(III)}(t) + •\text{Fe(III)}(t) + •\text{Fe(III)•}(t)
\]

The Excel file *Kinetic_model.xls* allows visual monitoring of all constructed kinetic dependences on one screen. A change in any one of the 32 parameters (ten rate constants × three pH values + [DHP]₀ + [H₂O₂]₀) instantly produces change(s) in the calculated kinetic dependences. The initial DHP and H₂O₂ concentrations were not varied during the fitting process. The fitting procedure was based on minimizing the ‘target’ parameters, the average absolute difference between experimental and calculated concentration values, defined for each pH series (TₚH₅, TₚH₆ and TₚH₇) and for all three sets of data when optimizing the rate constants globally (T=TₚH₅+TₚH₆+TₚH₇) (see details in SI.Results.3.). The constraints
imposed on the rate constants have allowed diminishing of the 30D variable space down to 13 variables (see SI. Results. 3.). Table G.2 presents the optimized values of the 30 rate constants that were used to plot the continuous lines in Fig. 4.4 and Fig. 4.5. This set of the rate constants produces the T parameter equal to 12.728 μM which is, in average, 2.1 μM per one of the 6 experimental kinetic dependences modelled. We consider this a good accuracy of fitting for the case of total protein concentration of 40 μM.

**Table 4.2.** The optimized rate constants of the reactions included in the kinetic model

<table>
<thead>
<tr>
<th>pH</th>
<th>$k_1$, M$^{-1}$s$^{-1}$</th>
<th>$k_2$, s$^{-1}$</th>
<th>$k_3$, s$^{-1}$</th>
<th>$k_4$, M$^{-1}$s$^{-1}$</th>
<th>$k_5$, M$^{-1}$s$^{-1}$</th>
<th>$k_6$, M$^{-1}$s$^{-1}$</th>
<th>$k_7$, M$^{-1}$s$^{-1}$</th>
<th>$k_8$, M$^{-1}$s$^{-1}$</th>
<th>$k_9$, M$^{-1}$s$^{-1}$</th>
<th>$k_{10}$, M$^{-1}$s$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>2.53×10$^4$</td>
<td>2.291</td>
<td>0.093</td>
<td>0.009</td>
<td>14.52×10$^4$</td>
<td>19.36×10$^4$</td>
<td>38.72×10$^4$</td>
<td>25.81×10$^4$</td>
<td>51.63×10$^4$</td>
<td>103.3×10$^4$</td>
</tr>
<tr>
<td>6</td>
<td>3.78×10$^4$</td>
<td>0.642</td>
<td>0.017</td>
<td>0.002</td>
<td>5.11×10$^4$</td>
<td>6.81×10$^4$</td>
<td>13.63×10$^4$</td>
<td>9.08×10$^4$</td>
<td>18.17×10$^4$</td>
<td>36.33×10$^4$</td>
</tr>
<tr>
<td>7</td>
<td>9.47×10$^4$</td>
<td>0.444</td>
<td>0.005</td>
<td>0.001</td>
<td>2.81×10$^4$</td>
<td>3.75×10$^4$</td>
<td>7.50×10$^4$</td>
<td>5.00×10$^4$</td>
<td>10.01×10$^4$</td>
<td>20.01×10$^4$</td>
</tr>
</tbody>
</table>

**Discussion**

The enzyme activation can be represented as a sequence of transformations (Scheme 2) when the intermediates are the species detectable by optical and/or EPR spectroscopy.

**Scheme 4.1.** Representative sequence of DHP activation, radical formation, and decay.

![Scheme 4.1](image)

We now consider in detail the individual intermediates presented in Scheme 2 from the perspective of their spectroscopic properties.

**Resting DHP – relation between the two HS forms and the His55 in open and closed conformations.** We have found two HS ferric heme forms in the resting enzyme – a ‘nearly
axial’ signal and a rhombic one (NAHS and R1HS, Fig. 4.1). These two forms are in a pH dependent equilibrium, with R1HS being the acidic and NAHS – the alkaline component (Fig. 4.2). The axial HS ferric heme state in the globins, such as Mb and Hb, has a water molecule coordinated by the distal histidine \(^\text{30-32}\). Therefore, we suggest that NAHS is a 6-coordinate HS ferric heme state (6cHS) of DHP when the distal His55 is in the closed conformation coordinating a water molecule. Recently, Nicoletti et al. showed that binding of 4-iodophenol to DHP produces a rhombic type EPR lineshape with g-values \(g_1 = 6.22\), \(g_2 = 5.50\), and \(g_3 = 1.99\) \(^\text{33}\) similar to the resting state R1HS shown in Fig. 4.1 and Fig. G.7. Binding of 4-iodophenol in the distal heme side results in the distal His55 being pushed out of the pocket to the open conformation \(^\text{5}\). Therefore, we can assign R1HS to a 5-coordinate HS ferric heme state (5cHS) of DHP in the open His55 conformation.

The forms R1HS (5cHS, open) and NAHS (6cHS, closed) are in a fast pH-dependent equilibrium in the resting state. The correlation of the His55 position and heme coordination has been observed by X-ray crystallography, nuclear magnetic resonance and resonance Raman spectroscopies \(^\text{10,11,14,15,33}\). The equilibrium is shifted towards R1HS as the pH is lowered (Fig. 4.2) because of increasing protonation of the distal His55 which leads to the disruption of the hydrogen bonding to the water molecule in the sixth position of the heme iron. This displaces the distal His55 towards the open conformation thus producing a 5cHS heme state. Another way to rationalize the effect of pH on the ‘open’ / ‘closed’ equilibrium is to think that one excessive positive charge on the histidine (at lower pH) makes it harder for the residue to approach the positively charged heme iron.
The equilibrium between the R1HS and NAHS ferric heme states seems to be slightly affected after the reaction starts: the decrease of R1HS on H$_2$O$_2$ addition is marginally greater than the decrease of NAHS. In fact, the EPR spectra of the RFQ samples sometimes showed a complete disappearance of the R1HS form just 50 ms after mixing, leaving just a small intensity axial HS ferric heme EPR signal (not shown). We suggest that the reaction with H$_2$O$_2$ starts when the distal His55 is in the closed conformation. It is known that if a heme protein lacks a histidine in the distal side of the heme, the reaction can still be initiated, but Compound 0 (a transient complex of H$_2$O$_2$ and the ferric heme) is formed as the first intermediate $^{21,34}$. Since no Compound 0 has been observed in DHP upon reaction with H$_2$O$_2$, it appears feasible that peroxide reacts with the heme iron when His55 is in the closed conformation. It is also known that the open, 5cHS, form of DHP is inactive and does not convert to compound ES $^{16}$.

Once the oxoferryl heme state starts to be reduced in the course of the reaction, a new rhombic ferric species, R2HS, is formed with a slightly stronger rhombic distortion of the heme than in R1HS (Fig. G.7). This is likely to be an open His55 conformation of DHP, similar to R1HS but modified due to a peroxide-induced change in the heme geometry.

**Compound I, Compound ES and the substrate oxidation site.** Compound I is a term commonly used to describe the two electron oxidized form of a heme protein, in which one oxidizing equivalent originates from the ferric iron, resulting in the oxoferryl heme state, and the other comes from the $\pi$-system of the porphyrin yielding a $\pi$-cation radical.

Compound I has not been reported in DHP. Instead, the oxidation state of DHP formed shortly after mixing with H$_2$O$_2$ has been interpreted as Compound ES $^{17}$ by analogy with CcP.
18, in which the heme is oxidized to the oxoferryl state, and a free radical is formed on an amino acid. $\text{H}_2\text{O}_2$ reacting with respiratory heme proteins, such as Mbs and Hbs, also produces an oxoferryl heme state and a protein bound radical. However, an essential difference with Compound ES in CeP is in the amount of the free radical produced. The apparent yield of the protein bound free radical in the globins is never greater than a few percent 22. We show now that in DHP the free radical concentration is nearly quantitative with respect to the enzyme concentration (a ~93% yield at 150 ms, pH 7, Fig. 4.5). This fact makes the ‘oxoferryl + radical’ oxidation state of DHP more like classical Compound ES in CeP rather than the ‘oxoferryl + radical’ state in the globins.

We have assigned the Compound ES radical (the ‘principal radical’) to either Tyr28 or Tyr34 on the basis of its EPR spectrum simulation (3.2.3.). These two residues are both solvent exposed and provide possible sites for electron transfer from the halogenated substrate. We favor the assignment of the radical to Tyr34. One reason for this is that it is closer to the heme and it is in direct contact (by the van der Waals spheres) with the heme porphyrin (Fig. 4.8). More importantly, Tyr34 in DHP A is a conserved tyrosine residue in a number of globins. Structural homologues of this residue – Tyr103 in horse Mb, sperm whale Mb and in human Mb and Tyr42 in the $\alpha$-subunit of human Hb – all are reportedly the sites of free radical formation when these globins react with $\text{H}_2\text{O}_2$ 22,35-38.

The principal radical in DHP A Compound ES is found at a very high relative yield and therefore is very likely to be related to the mechanism of substrate oxidation. It is feasible to
Figure 4.8. Selected residues in the heme environment of DHP. Tyr34, the site suggested to host the principal radical, is in van der Waals interaction with the heme porphyrin. Arg33 is in close proximity and might play a role in substrate binding. The pH 5 radical is proposed to be located on Tyr38 which can make a strong hydrogen bond with the distal His55. The PDB structure file 2QFK\textsuperscript{10} was used to generate the figure.

suggest that the enzyme’s surface area around Tyr34 is the site where substrate oxidation occurs. The existence of an on-surface substrate binding site has been proposed as an alternative to the earlier view where the distal pocket was considered to be the enzyme’s active site\textsuperscript{13-15}. This new hypothesis is in line with the ‘through protein’ mode of substrate oxidation\textsuperscript{38,39}. Contemplating the Tyr34 area as the on-surface substrate oxidation site in DHP A, one may speculate that the residue Arg33, which is close to Tyr34, might play a role
in substrate binding and stabilization. These two residues are both solvent exposed (Fig. 4.8), and the flexible arm of Arg33, with a positively charged guanidinium group that may enable multiple hydrogen bonds, can bind to and/or stabilize the trihalophenol (in the negative phenolate form at physiological pH) in close proximity to the tyrosine which executes the subsequent substrate oxidation. We are preparing mutations of the Arg33 residue to study their effect on the oxidation of halophenols.

**Effects of pH on the reactivity of the oxoferryl heme.** The oxoferryl heme must be in a dynamic equilibrium with its protonated state, which can be represented as the resonance hybrid boxed in Scheme 3.

**Scheme 4.2.** Equilibrium of oxoferryl heme and its protonated state.

\[
\begin{align*}
\text{O}^2^- & \quad \text{Fe}^{4+} & \quad \text{OH}^- \\
\text{Fe}^{4+} & \quad \text{Fe}^{3+}
\end{align*}
\]

A direct correlation of Hb and Mb ferryl reactivity with their ferryl protonation status ($pK_a = 4.7$) has been demonstrated. A close value of the $pK_a$ for this equilibrium might be expected in DHP. Indeed, the state of the oxoferryl heme in DHP at pH 7 has been assessed by cryoreduction of DHP Compound ES with γ-radiation and was concluded to be a deprotonated Fe$^{4+}$=O$^{2-}$ state.

The high oxoferryl reactivity in the protonated state can be understood from its electronic equivalence to a ferric heme iron coordinated by a hydroxyl radical (Scheme 3), the strongest oxidation agent known. The deprotonated oxoferryl heme, on the other hand, can be stable for hours.
Hypothesis: two alternative routes of the reduction of the protonated oxoferryl heme of Compound ES. The oxoferryl heme state, which is part of Compound ES, is chemically reactive and can be converted to the resting ferric state if it is protonated. It is electronically equivalent to a ferric heme iron coordinated by a hydroxyl radical (Scheme 3). A hydroxyl radical will oxidize anything it comes in proximity with. Therefore we suggest that what happens after the oxoferryl is protonated, depends on whether His55 is in the closed or open conformation.

If His55 is in the open conformation (higher populated at the lower pH values, Fig. 4.2), the protonated oxoferryl would abstract a hydrogen atom from the nearest target to be oxidized – the porphyrin. This would leave the porphyrin in a transient neutral free radical state, ready to recombine with the nearby neutral Tyr34 radical (principal radical) thus yielding a covalent cross-link between the tyrosine and the heme. Indeed, our HPLC data show the formation of a cross-link between the heme and the globin (Fig. G.8 and Fig. G.9). Tyr34 is very close to the heme, and it is likely that it could form a cross-link. A homologous residue Tyr103 in horse Mb treated with H$_2$O$_2$ was reported to form a cross-link with the heme $^{43}$, although this assignment has been challenged $^{44}$. Furthermore, an autocatalytic formation of a similar cross-link under H$_2$O$_2$ treatment has been demonstrated in ascorbate peroxidase in which a tyrosine has been engineered close to the heme edge $^{45}$. The yield of the cross-link in DHP is higher at the lower pH values (Fig. 4.7), which is in agreement with a higher concentration of the protonated ferryl form of the heme and a higher concentration of the open His55 conformation. Therefore, we put forward a hypothesis that Compound RH is a ferric type heme with the porphyrin covalently cross-linked to Tyr34. An important
question is whether the formation of such a cross-link might be reversible, since it is known that Compound RH can be reduced by external reductants to the ferrous form of DHP. The possibility that such a link in DHP can be reduced is supported by the fact that similar heme to protein cross-links in horse heart Mb are partially reversible when sodium dithionite is used as a reductant (our unpublished data).

If, however, His55 is in the closed conformation, which in terms of population is a much smaller fraction, the protonated ferryl would certainly attack the distal His55, likely by subtracting an electron from the lone pair of the imidazole’s nitrogen (Fig. 4.9). As a result, a hydroxyl anion will be liberated, the heme will be left in the ferric state, and a neutral radical will be formed on His55. What will happen next? We have assigned the EPR signal of the pH 5 radical to the neutral radical on Tyr38 (see 3.2.3 above) on the basis of the EPR signal simulation and the DHP structure. We also concluded that the pH 5 radical must be strongly hydrogen bonded. Tyr38 is indeed very close to His55 when it is in the closed conformation (Fig. 4.8) and can make a strong hydrogen bond with the nitrogen of the imidazole group. Thus it is reasonable to expect that the Tyr38 radical is formed instantly on His55 oxidation by the protonated oxoferryl (Fig. 4.9). A neutral Tyr38 radical might be in resonance with its cation radical, when the proton is shared between the histidine and the tyrosyl radical, but the population of the latter state must be much smaller than the population of the neutral radical which we observe as the pH 5 radical.
Figure 4.9. Conversion of the protonated oxoferryl heme to a ‘ferric heme + protein radical’ state, when the distal His55 is in the closed conformation. The Tyr38• radical is formed (the pH 5 radical) which might be in an equilibrium with its protonated form, the cation radical.

**Analysis of the optimized rate constants.** The kinetic model used to fit experimental time dependences of the total free radical and total ferric heme states, at three different pH values, holds the following assumptions made on the basis of the experimental results obtained in this work.

a) Formation of Compound ES takes place only when His55 is in the closed conformation.

b) Open/closed His55 equilibrium is governed by a single protonation event.

c) Compound ES decays only if the ferryl heme iron is protonated (although the principal radical associated with Compound ES, can recombine with another radical in the system, independently of the heme oxidation state, thus turning Compound ES into a non-radical ferryl heme species).

d) Ratio of the rates of the two alternative routes of Compound ES decay directly depends on the open/closed His55 conformation: Compound RH is formed in the open conformation but a second, pH 5 radical is formed in the closed conformation. Thus the partial weight of these two routes of reaction \( k_2/k_3 \) should have the same pH profile as the rate constant \( k_1 \).
that reflects the pH dependence of Compound ES formation via reaction (1), that is \( k_2/k_3k_f \) is a constant at pH 5, 6 and 7.

When the kinetic model is optimized with the constraints imposed by the above assumptions, the calculated time dependences are found in satisfactory proximity to the experimental data points (Fig. 4.4 and Fig. 4.5). The conclusions that follow from the analysis of the optimized rate constants further support the feasibility of the kinetic model and the molecular mechanism of the reaction.

Fig. 4.10 shows the pH dependences of the optimized rate constants (Table 2.2). The \( k_1 \) dependence on pH is in agreement with the EPR data on the pH dependence of the R1HS and NAHS equilibrium (Fig. 4.2). Also, the values of \( k_1 \) obtained from the model are in agreement with the Compound ES formation rate constants determined for pH 5 and pH 7 by optical spectroscopy (2.78×10^4 and 3.56×10^4 M\(^{-1}\)s\(^{-1}\) at pH 5 and 7, respectively\(^{17}\)). An estimate of the pK\(_a\) value from the Step parameter defined from the three \( k_1 \) values optimized for pH 5, pH 6 and pH 7 (see SI.G.Results.3) give a value of 6.9. Interestingly, a similar equilibrium between the 5cHS (open) and 6cHS (closed) heme states in Mb, which is characterized by orders of magnitude lower peroxidase activity, is observed at a significantly lower pK\(_a\) of 4.5 \(^{47}\).

We interpret Compound RH of DHP as a ferric heme state with the heme covalently bound to the globin. In agreement with this, the rate constants \( k_2 \) of Compound RH formation at the three pH values (Fig. 4.10, B) correlate with the yield of the heme-to-protein cross-links (Fig. 4.7). The values of \( k_2 \) obtained from the model are approximately 30 times greater than the rates of Compound RH formation determined optically (0.0701 s\(^{-1}\) at pH 5 and 0.0167 s\(^{-1}\) at
Figure 4.10. The optimized values of the second order (A) and first order (B) rate constants used in the kinetic model (see 3.2.5) are plotted as functions of pH. For comparative viewing, \( k_3 \) and \( k_4 \) dependences (B) are multiplied by the factors of 25 and 250, respectively, to bring the pH 5 data point to a similar position on the graph. Rate constants \( k_6 \text{-} k_{10} \) are set in the model to be proportional to \( k_5 \) and therefore they have the shape of the pH dependences identical to that of the \( k_5 \) dependence, and are not shown.

pH 7\(^\text{17} \), although both sets of data have similar pH trends. If these differences are confirmed in the future experiments at common reaction conditions, it might indicate that what we see kinetically as the principal radical disappearance linked to the ferric heme state formation is not formation of Compound RH but rather formation of its precursor, which then transforms to Compound RH with an approximately 30-fold slower rate.
The radical decay rate constants, as determined from the model (Fig. 4.10, A), decrease with increasing pH (with a formal pK$_a$ = 5.2 estimated from the Step parameter for the three ‘basic’ radical decay rate constants $k_5$, see SI.G.Results.3 for details). This is in agreement with a faster loss of the hyperfine structure in the free radical EPR spectrum at the lower pH values. Even quantitative comparison shows a good correlation: while $k_5^{pH6}/k_5^{pH7}$ = 1.8, the rate of loss of the hyperfine structure in the EPR spectra (estimated as inverse time of the lineshape preservation, see 3.2.1) is 2 times greater at pH 6 than at pH 7; also while $k_5^{pH5}/k_5^{pH6}$ = 2.8, the rate of loss of the hyperfine structure at pH 5 is 6 times greater than at pH 6.

We think that such a good quantitative agreement is remarkable particularly since no information about the free radical spectra lineshape is present the kinetic model.

Finally, the overall rate of the oxoferryl heme decay ($k_2+k_3+k_4$) has a pH profile consistent with a pK$_a$=4.35 – a value which is very close to the pK$_a$=4.7 for the globins’ oxoferryl heme protonation 40. Reaction (4), however, contributes very little to the overall sum (0.2-0.4%; note that the $k_4$ dependence is multiplied by a factor of 250 in Fig. 4.10, B).

Overall, the kinetic model that satisfactorily describes the experimental data is dependent on three pK$_a$ values, roughly estimated from the triads of the rate constants optimized at pH 5, pH 6 and pH 7. The first one, pK$_a$ = 6.9, is related to the His55 protonation and thus governs its open/closed conformations. The second, pK$_a$ = 4.4, defines if the oxoferryl heme is protonated and therefore redox active. The third one, pK$_a$ = 5.2, governs the radical decay and is suggested to reflect the radical transfer dependence on the pH.
Conclusions

1. Two HS ferric heme forms are found in the resting enzyme and assigned to the 6cHS (aquo) heme with the distal His55 in the closed conformation and the 5cHS heme with His55 in the open conformation.

2. Two free radicals are identified in DHP activated by H₂O₂. The one associated with Compound ES has been assigned to the on-surface residue, Tyr34. The other radical, better detectable at lower pH values, has been assigned to Tyr38 which is well situated to make a strong hydrogen bond with the distal histidine, His55.

3. Covalent heme-to-protein cross-links are found in DHP treated with H₂O₂.

4. A pH-dependent mechanism of DHP activation by H₂O₂ is proposed and tested by kinetic modelling. A good correspondence between the model and the experimental data, at a range of pH values, allows the following interpretation of the results obtained. Compound ES, the first detectable intermediate of the reaction of DHP with H₂O₂, is a ferryl heme state with a radical on Tyr34. Formation of Compound ES is pH dependent, reflecting the pH dependence of the equilibrium of the distal His55 in the open and closed conformations. Once formed, Compound ES may enter one of the two alternative paths of further transformation. This depends again on whether His55 is in the closed or in the open conformation. In the less populated closed conformation, the Tyr38 radical is formed, but in the major open conformation, the protonated oxoferryl produces a transient porphyrin radical which recombines with Tyr34• and makes a heme-to-protein crosslink. This cross-link might be the Compound RH characterized previously by optical spectroscopy, or it might be its
precursor. Formation of Compound RH is therefore interpreted as the enzyme’s means to terminate the two oxidizing equivalents in a safe way, producing a cross-linked heme, which can be further reduced in a controlled way. Thus, the ability of H$_2$O$_2$-activated DHP to be transformed into a safe state of Compound RH might be important in avoiding unwanted radical propagation in the absence of substrates.

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Supporting Information Available in APPENDIX G: (1) Details of a) new freeze-quenching apparatus, b) EPR spectra correction for variable length of EPR samples, c) spectra shifting in accordance with the microwave frequency, d) assignment of radicals to specific tyrosine residues; (2) supporting figures and tables.
References


APPENDICES
APPENDIX A

New Insights into the Role of Distal Histidine Flexibility in Ligand Stabilization of Dehaloperoxidase-hemoglobin from *Amphitrite ornata*
Abstract

The present work highlights the important role played by the distal histidine in controlling the binding of heme ligands in dehaloperoxidase (DHP) as compared to myoglobin and peroxidases. In DHP the distal histidine is highly mobile and undergoes a conformational change that places it within hydrogen-bonding distance of anionic ligands and water, where strong hydrogen bonding can occur. The detailed resonance Raman (RR) analysis at room temperature shows the presence of an equilibrium between a 5-coordinate and a 6-coordinate (aquo) high spin forms. The equilibrium shifts toward the aquo form at 12 K. These two forms are consistent with the existing X-ray structures where a closed conformation, with His55 positioned in the distal pocket and H-bonded with the distal water molecule (6-coordinate), and an open solvent-exposed conformation, with the His55 displaced from the distal pocket (5-coordinate form), are in equilibrium. Moreover, the comparison between the Raman data at 298 K and 12 K and the results obtained by EPR of DHP in the presence of 4-iodo-phenol highlights the formation of a pure 5-coordinate high spin form (open conformation). The data reported herein support the role of His55 in facilitating the interaction of substrate and inhibitor in the regulation of enzyme function, as previously suggested. The two conformations of His55 in equilibrium at room temperature provide a level of control that permits the distal histidine to act as both the acid-base catalyst in the peroxidase mechanism and the stabilizing amino acid for exogenous heme-coordinated ligands.
Introduction

Dehaloperoxidase (DHP) displays significant peroxidase activity under physiological conditions while having a globin fold (1). Peroxidases are typically characterized by an increased polarity of the distal cavity compared to globins. Accordingly, the postulated mechanism of hydrogen peroxide activation and heterolytic bond cleavage in peroxidases relies on the concerted role played by the conserved distal Arg and His (2) through direct hydrogen bonds and charge stabilization (3-6). However, unlike peroxidases, the distal cavity of DHP shows the presence of only a distal His, without an Arg (7-9). Therefore, it appears that the mechanism of hydrogen peroxide activation in DHP is controlled entirely by the distal His.

In general, heme pocket distal amino acid residues control ligand binding in heme proteins. However, while in hemoglobin and myoglobin, the distal His tunes the ligand affinities via hydrogen-bond stabilization involving its Nε proton (10), the crystal structures and the spectroscopic study of the CN-, NO, CO, and F- adducts of peroxidases revealed that significant changes are induced in the distal cavity upon adduct formation, suggesting that the distal Arg and His residues are significantly perturbed (11, 12). In addition, the comparison of the UV-Vis and RR spectra of the fluoride and hydroxide complexes of various peroxidases and selected mutants has highlighted the complex mechanism of stabilization of anionic ligands exerted by the distal amino acids (13-16). This mechanism resembles that of compound I formation during peroxidase catalysis, where ligand stabilization by the distal arginine is coupled to protonation of the distal histidine (12, 17). Both the distal Arg and His participate, in a concerted manner, in hydrogen-bonding
interactions with the ligand. However, for cytochrome c peroxidase (CCP), it has been shown that the interaction between Arg48 and the anions is possible because the distal Arg undergoes a conformational change that places it within hydrogen-bonding distance of bound fluoride or hydrogen peroxide, which facilitates acid-base catalysis (11, 12).

Since the specific interaction with Arg is missing in DHP, it is of interest to understand whether the different cavity characteristics of DHP, the globins and peroxidases are also reflected in the binding of exogenous ligands. Therefore, we undertook a detailed spectroscopic investigation of the ferric-fluoride and hydroxide ligated forms to highlight how the distal heme protein cavity interacts with the exogenous ligand in comparison with Mb and peroxidases.

The flexibility of the distal histidine is key for determining its ability to interact with heme-coordinated ligands. In many heme proteins, temperature, pH and inhibitor or substrate-binding in the distal pocket are all factors that regulate the conformation of the distal histidine (18, 19). For native DHP, the room temperature X-ray crystal structure showed that there are two conformations of the distal histidine at pH 6.0. Comparison with two X-ray crystal structures at 100 K suggests that these two conformations are associated with an unusual flexibility of the distal His55 (7-9). The 100 K structures consist of a closed conformation with His55 positioned in the distal pocket and H-bonded with the distal water molecule in the metaquo form (PDB 2QFK), and an open solvent-exposed conformation with the His55 displaced from the distal pocket in the five-coordinate deoxy form (PDB 3DR9). The open form is also the only form observed when 4-iodophenol (4IP), binds in the internal binding site (9). The X-ray structural data suggest that His55 is stabilized in the closed
conformation by hydrogen bonding to heme ligands (7). Unlike 4IP, no X-ray structure is available for DHP bound to a 2,4,6-trihalophenol. However, data are available in solution. In particular, 2,4,6-trifluorophenol (TFP) has been found to bind at low temperature (<260 K), but not at room temperature by cryogenic FT-IR, EPR and HYSCORE studies (26, 31). In addition, $^{19}$F NMR and relaxation experiments suggested that 2,4,6-TFP binds externally to the heme distal cavity at ambient temperatures (20). Moreover, recent assignment of the backbone $^{13}$C$_{\alpha}$, $^{13}$C$_{\beta}$, carbonyl $^{13}$C, amide $^1$H and amide $^{15}$N resonance in DHP provide further evidence for the existence of distinct binding sites which allows to distinguish substrates and inhibitors. The substrates 2,4,6-TXP ($X = Br, Cl, F$) bind externally and inhibitors 4-XP bind within the distal pocket. The NMR data show that shifts in the His55 position are coupled to binding of both the substrate and inhibitor. Hence, the flexibility of the distal histidine, His55, appears to have functional relevance for inhibition. The detailed spectroscopic investigation of the ferric form in the presence of different halogenated phenols undertaken in the present work further corroborate the unusual flexibility of the distal His55.

Materials and Methods

Materials. DHP was expressed in *Escherichia coli*, and purified as previously described (29). Purification using CM52 cation exchange cellulose (Whatman, Clifton, NJ) in a 55 mL FLEXCOLUMN (Kimble/Kontes, Vineland, NJ) allowed us to completely remove imidazole contaminant. Isotopically enriched water ($H_2^{18}O$) (95%) and $D_2O$ (99.8%) was purchased from Cambridge Isotope Laboratories, USA, and Merck AG (Darmstadt, Germany), respectively. The substrate analogs 4-iodophenol and 2,4,6-trifluorophenol were purchased
from Acros (New Jersey, USA). All the other chemicals were obtained from Merck AG (Darmstadt, Germany). All chemicals were analytical or reagent grade and were used without further purification.

**Sample Preparation.** Ferric DHP samples were prepared in 150 mM potassium phosphate, pH 6.0. Completely oxidized DHP samples were prepared by oxidation of the significant amount of the oxy-ferrous form present, due to the high protein affinity for oxygen, using excess potassium hexacyanoferrate(III) followed by gel filtration on a Biogel P-6DG column equilibrated with the 150 mM phosphate buffer at pH 6 to remove the oxidant. The DHP-F complex, in 150 mM potassium phosphate at pH 5.0, was prepared by adding a 0.018 M solution of NaF to the sample giving a final concentration of 0.015 M. The samples at pH 5 were obtained by addition of citric acid to the samples at pH 6, reaching a final concentration of 110 mM. The DHP-OH sample was prepared in 150 mM potassium phosphate at pH 9.6. The hydroxyl complexes in isotopically enriched water were prepared by adding 5 µl of DHP, in 150 mM natural abundance potassium phosphate at pH 11, to 45 µL of D$_2$O and H$_2^{18}$O to obtain a final pD 10 and pH 9.6, respectively. The 4IP-DHP (K$_D$: 318 μM) sample was prepared by diluting a 200 μM, pH 6, DHP solution with a saturated 4IP solution (1mM) in 150 mM potassium phosphate at pH 6 to yield final concentrations of 900 μM for 4IPh and 30 μM for DHP. The 4IP:DHP molar ratio of 30:1 was employed since titration of DHP with 4IP revealed progressive variations in the DHP absorption spectrum, reaching a final form for the 30:1 molar ratio (data not shown). The TFP-DHP samples were prepared by using a range from 10 to 320-fold excess of TFP respect to DHP. The concentration of all the
samples was between 15-200 μM. The sample concentration was determined using the molar absorptivity of 116.4 mM\(^{-1}\)cm\(^{-1}\) at 406 nm \((21)\). All samples for low temperature measurements were prepared in 150 mM phosphate and 30% (v/v) of glycerol. It is noted that the room temperature UV-Vis and RR spectra of corresponding samples in the presence and absence of glycerol were identical.

**Spectroscopy**

**Room Temperature.** Electronic absorption spectra were measured with a double-beam Cary 5 spectrophotometer (Varian, Palo Alto, CA). The electronic absorption spectra were obtained using a 5-mm NMR tube or a 1-mm cuvette, and a 600 nm/min scan rate. The RR spectra were obtained using a 5-mm NMR tube and by excitation with the 406.7 and 413.1 nm lines of a Kr\(^+\) laser (Coherent, Innova 300 C, Santa Clara, CA), and the 514.5 nm line of an Ar\(^+\) laser (Coherent, Innova 90/5, Santa Clara, CA). Backscattered light from a slowly rotating NMR tube was collected and focused into a triple spectrometer (consisting of two Acton Research SpectraPro 2300i and a SpectraPro 2500i in the final stage with a 3600 grooves/mm grating) working in the subtractive mode, equipped with a liquid nitrogen-cooled CCD detector. It should be noted that the spectral resolution of the RR spectra cited in the figure captions is that calculated theoretically on the basis of the optical properties of the spectrometer. However, for the moderately broad experimental RR bands observed in the present study (ca. 10 cm\(^{-1}\)), the effective spectral resolution will in general be lower. All RR measurements were repeated several times under the same conditions to ensure reproducibility. To improve the signal/noise ratio, a number of spectra were accumulated and summed only if no spectral differences were noted. The RR spectra were calibrated with
indene, CCl₄, dimethyl sulfoxide as standards to an accuracy of ± 1 cm⁻¹ for intense isolated bands. To determine peak intensities and positions a curve-fitting program (Lab Calc, Galactic) was used to simulate the spectra using a Lorentzian line shape with band widths between 10 and 13 cm⁻¹. In particular, 10 cm⁻¹ has been used to fit the B₁g modes, 11 cm⁻¹ for the vinyl modes, 12 cm⁻¹ for the Eₘ modes, and 13 cm⁻¹ for the A₁g and A₂g modes.

**Low Temperature.** The low-temperature experiments were carried out using an Air Products Displex closed-cycle He refrigerator with automatic temperature control. For the low temperature RR measurements, 20 µl of the protein solution was deposited on the copper cold finger of the refrigerator at 90 K under a nitrogen flow. The temperature was then slowly decreased to 12 K under vacuum, and RR spectra were obtained at this temperature. The back-scattered light was collected and focused into a computer-controlled double monochromator (Jobin-Yvon HG2S) equipped with a cooled photomultiplier (RCA C31034 A) and photon-counting electronics. The RR spectra were calibrated with indene as standard to an accuracy of ±1 cm⁻¹ for intense isolated bands. The glycerol bands were subtracted from the RR spectra of the samples.

EPR spectra were recorded on a Bruker Elexys E500 instrument, equipped with an NMR gaussmeter and a microwave frequency counter. An Oxford Instruments ESR 900 cryostat was used to obtain low temperatures. The spectra were recorded under non-saturating conditions at 5 K, 1 mW microwave power, and 1 mT modulation amplitude. The EPR simulation program used to determine the g values (Xsophe, Bruker) is appropriate for effective S = 1/2 systems.
Results

Room Temperature

Native protein. The absorption spectrum of metaquo DHP at pH 6.0 (Figure A.1, panel A, trace a) is characterized by a Soret band at 406 nm, Q₁ and Q₀ bands at 505 and 529 nm, respectively, and the charge transfer (CT1) band (long wavelength, > 600 nm porphyrin-to-metal charge transfer band) at 637 nm, very similar to the spectrum of metaquo Mb (22). Therefore, the spectrum, almost identical to those previously reported (21, 23), is typical of a high spin species. Accordingly, the corresponding RR spectrum (Figure A.1, panel B, trace a) indicates an equilibrium between a predominant hexa-coordinated high spin species (6cHS) (ν₃ at 1483 cm⁻¹, ν₂ at 1563 cm⁻¹ and ν₁₀ at 1611 cm⁻¹) and a penta-coordinated high spin species (5cHS) (ν₃ at 1494 cm⁻¹). These two forms are consistent with the existing X-ray structures where a closed conformation with His55 positioned in the distal pocket (6-coordinate) and an open solvent-exposed conformation with the His55 displaced from the distal pocket (5-coordinate form) are in equilibrium. (Figure A.2, centre, top) (9). In a previous study, RR spectra showing a mixture of a high spin and a low spin species were obtained (23), the latter due to a bis-imidazole complex resulting from the presence of an imidazole impurity (see Materials and Methods).

Unlike Mb, which is characterized by two coincident ν(C=C) stretching modes at 1621 cm⁻¹ (24), on the basis of depolarization ratio measurements obtained by a curve fitting analysis (Figure A.3, panel A), two polarized bands (Table A.1) are observed for DHP at 1621 and 1632 cm⁻¹ which are, therefore, assigned to two vinyl stretching modes. A direct relationship between the ν(C=C) stretching wavenumber and the orientations of the vinyl
Table A.1. RR frequencies (in cm\(^{-1}\)) in the high wavenumber region of the various ferric DHP species, their normal mode assignments and depolarization ratios (\(\rho\)) (in brackets) compared to those of Mb.

<table>
<thead>
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<th>mode</th>
<th>sym</th>
<th>5cHS</th>
<th>6cHS</th>
<th>5eHS</th>
<th>6eHS</th>
<th>6cHS</th>
<th>6eHS</th>
<th>6cLS</th>
<th>6eLS</th>
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<tr>
<td>(v_{21})</td>
<td>(A_{2g})</td>
<td>(1303^b)</td>
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<td></td>
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<td>(E_g)</td>
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<td></td>
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<td>(v_{6})</td>
<td>(A_{1g})</td>
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<td>(1372 (0.16))</td>
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<td>(1370 (0.15))</td>
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<td>(1445)</td>
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<td>(A_{1g})</td>
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<td>(A_{1g})</td>
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<td>(v_{59})</td>
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<td>(1622 (0.15))</td>
<td></td>
<td></td>
<td>(1620 (0.19))</td>
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<td>(1619 (0.2))</td>
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<td>(v_{(C=O)})</td>
<td>(1632)</td>
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<td>(v_{20})</td>
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<td>(1632 (0.66))</td>
<td>(1611^c)</td>
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<td>(1638^d)</td>
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</table>

\(a\) The underlined frequencies are enhanced with the 514.5 nm excitation wavelength (data not shown). The species written in italics are the most predominant; \(b\) not reported in the figures; \(c\) \(\rho\) cannot be determined due to the overlapping contribution of \(v_{10}\) with the more intense \(v_{(C=O)}\) band at 1621 cm\(^{-1}\); \(d\) overlapped with the \(v_{10}\) at 1630 cm\(^{-1}\); \(e\) \(\rho\) (0.42) cannot be precisely determined due to the overlapping contribution between this mode and the \(v_{(C=O)}\) at about the same frequency; \(f\) \(\rho\) determined with 413.1 nm excitation wavelength.
groups (i.e. their torsional angles), as induced by specific protein interactions, has been established for heme containing peroxidases and myoglobin (25). Therefore, this result is...
consistent with the crystal structure of DHP (8) which shows two different torsional angles of -144° and 158° for 2- and 4-vinyl, respectively.

**Figure A.2.** RR spectra of DHP (centre), DHP-4IP (1:30 molar ratio) (right), and DHP-TFP (1:320 molar ratio) (left) at 298 K (top) and 12 K (bottom) at pH 6. Experimental conditions: λ_{exc}: 406.7 nm, spectral resolution 1.3 cm\(^{-1}\) (298 K) and 5 cm\(^{-1}\) (12 K), power at the sample 5 mW (298 K) and 8 mW (12 K). DHP: average of six spectra with 300 s integration time (298 K), collection interval 8 s/0.5 cm\(^{-1}\) (12 K). 4IP: average of six spectra with 300 s integration time (298 K), collection interval 6 s/0.5 cm\(^{-1}\) (12 K). TFP: average of three spectra with 300 s integration time (298 K), collection interval 6 s/0.5 cm\(^{-1}\) (12 K). The intensities are normalized to that of the v\(_4\). Spectra have been shifted along the ordinate axis to allow better visualization.

The existing heme cavity X-ray structures are also reported. Center, top: structure at room temperature (PDB: 1EW6) (9); center, bottom: 100 K structure (PDB: 2QFK) (8). right, top: room temperature structure of DHP-4IP (PDB: 1EWA) (9).

**Binding of anionic ligands.** Upon addition of fluoride to the protein at pH 5.0 marked changes are observed in the UV-Vis spectrum (Figure A.1, panel A, trace b). In accord with previously reported results (23), the spectrum of the DHP-F adduct is characterized by a Soret band maximum at 406 nm and a CT1 band at 605 nm, which is 5 nm blue-shifted compared to the corresponding band of the Mb-F complex (16). The corresponding RR
spectrum is typical of a 6cHS form (Figure A.1, panel B, trace b) with \( \nu_3 \) at 1478 cm\(^{-1}\), \( \nu_2 \) at 1562 cm\(^{-1}\) and \( \nu_{10} \) at 1605 cm\(^{-1}\). A small amount of a 5cHS unligated protein (\( \nu_3 \) at 1494 cm\(^{-1}\)) is observed. As in the met form, but different from Mb, two \( \nu_{(C=C)} \) vinyl stretching modes are observed at 1620 and 1632 cm\(^{-1}\) (Figure A.3, panel B).

**Figure A.3.** Curve-fitting of the RR spectra of (Fe\(^{3+}\))DHP at pH 6 (A), (Fe\(^{3+}\))DHP-F at pH 5 (B), and (Fe\(^{3+}\))DHP-OH at pH 9.6 (C) in 0.15 M potassium phosphate for parallel (//) and perpendicular (\( \perp \)) polarized light, taken with 406.7 nm (A and B) and 413.1 nm (C) excitation wavelengths.

The UV-Vis spectrum of DHP at alkaline pH (pH 9.6) (Figure A.1, panel A, trace c) is characteristic of a mixture of 6 coordinate low spin (6cLS) (maxima at 414, 541 and 579 nm) and 6cHS (CT at 473 and 598 nm) species with a hydroxyl group bound to the heme iron, as previously observed (23, 26). This behavior is similar to that of Mb except that the
Soret and the CT1 bands are 1 and 2 nm blue-shifted, respectively \((13)\). The corresponding RR spectrum (Figure A.1, panel B, trace c) indicates the presence of a 6cLS \((\nu_3 \text{ at } 1504 \text{ cm}^{-1}, 
 \nu_2 \text{ at } 1581 \text{ cm}^{-1} \text{ and } \nu_{10} \text{ at } 1638 \text{ cm}^{-1})\) and a 6cHS species \((\nu_3 \text{ at } 1477 \text{ cm}^{-1}, 
 \nu_2 \text{ at } 1563 \text{ cm}^{-1})\.

In addition, the bands at 1619 and 1632 cm\(^{-1}\) are assigned to the two \(\nu_{(\text{C=C})}\) vinyl stretches (Figure A.3 panel C). A complete assignment of DHP, DHP-F, and DHP-OH in the high frequency region, compared to Mb, is reported in Table A.1.

Figure A.4 (panel A) compares the low frequency RR spectra of WT-DHP and its adducts with fluoride and hydroxide. The DHP-F RR spectrum (Figure A.4, trace b) shows a new band at 462 cm\(^{-1}\), not present in the metaquo form (Figure A.4, trace a). This band is assigned to the \(\nu_{(\text{Fe-F})}\) stretching mode by analogy with the corresponding band observed for Mb \((27)\). The RR spectrum of DHP-OH shows new bands at 491 and 551 cm\(^{-1}\) (Figure A.4, trace c) which are isotope-sensitive. The band at 491 down-shifts to 482 cm\(^{-1}\) in D\(_2\)O and 471 cm\(^{-1}\) in H\(_2\)\(^{18}\)O, whereas the band at 551 cm\(^{-1}\) shifts to 542 cm\(^{-1}\) in D\(_2\)O and 525 cm\(^{-1}\) in H\(_2\)\(^{18}\)O (Figure A.4, panel B). Therefore, they are assigned to the high spin and low spin Fe-OH stretching modes \(\nu_{(\text{Fe-OH})}\), respectively. Since the frequencies are identical to those previously reported for Mb \((13)\), they indicate that the hydroxide derivative of DHP at room temperature exists in a thermal equilibrium between high and low spin states, as observed for myoglobin and other heme proteins.

The finding of two different \(\nu_{(\text{C=C})}\) vinyl stretching modes in all the ferric forms of DHP allows us to understand the slight difference observed in the electronic absorption spectra of the fluoride and hydroxyl complexes of DHP and Mb. In general, Soret and CT1 maxima shifts can occur when there are differences in the ligand field strength of the anionic
Figure A.4. (Panel A): RR spectra of (Fe$^{3+}$)DHP at pH 6 (a), (Fe$^{3+}$)DHP-F at pH 5 (b), (Fe$^{3+}$)DHP-OH at pH 9.6 (c) in 0.15 M potassium phosphate. Experimental conditions: (a) 406.7 nm excitation wavelength, 5 mW laser power at the sample, average of nine spectra with 300 s integration time, 1.3 cm$^{-1}$ spectral resolution; (b) 406.7 nm excitation wavelength, 5 mW laser power at the sample, average of three spectra with 600 s integration time, 1.3 cm$^{-1}$ spectral resolution; (c) 413.1 nm excitation wavelength, 6 mW laser power at the sample, average of three spectra with 900 s integration time, 1.2 cm$^{-1}$ spectral resolution. The intensities are normalized to that of the $v_7$. (Panel B): Low frequency region RR spectra of (Fe$^{3+}$)DHP-OH. Experimental conditions: excitation wavelength 413.1 nm, 6 mW laser power at the sample, 1.2 cm$^{-1}$ spectral resolution. H$_2$O at pH 9.6 (average of three spectra with 900 s integration time), D$_2$O at pH 10.0 (average of twelve spectra with 900 s integration time) and H$_2$O at pH 9.6 (average of eighteen spectra with 900 s integration time). The intensities are normalized to that of $v_7$ (not shown). Spectra have been shifted along the ordinate axis to allow better visualization.
ligand bound to the iron (16). However, the identity of the frequencies of the $\nu_{(Fe-F)}$ and $\nu_{(Fe-OH)}$ stretching modes between the two proteins allows us to exclude a different interaction between the exogenous ligand and the protein cavity. As a consequence, the blue-shift of the Soret and CT1 bands between the complexes of DHP and Mb with anionic ligands derives from a different orientation of the two vinyl groups. The Soret band results from an electronic transition that involves $\pi$ and $\pi^*$ levels ($\pi \rightarrow \pi^*$), while the CT1 band is due to a transition from the $a_{2u}(\pi)$ porphyrin orbitals to $d\pi$ iron orbitals. The energy of the $\pi$ orbitals depends on the coordination/spin state of the heme and the degree of conjugation between the heme group and its two vinyl substituents. Therefore, the electronic coupling between the vinyl groups and the porphyrin modulates the $\pi \rightarrow \pi^*$ and $a_{2u}(\pi) \rightarrow d\pi$ transitions and furnishes an enhancement mechanism for the vibrational modes of the vinyl groups in the RR spectra (28). In most cases, the vinyl substituents give rise to polarized bands around 1620-1630 cm$^{-1}$. A lower frequency is expected to correspond to a higher degree of conjugation between the vinyl group and the porphyrin $\pi$ system. Increased conjugation with the vinyl group should shift the energy of the $\pi \rightarrow \pi^*$ and $a_{2u}(\pi) \rightarrow d\pi$ transitions to lower energy, thus shifting the Soret and CT1 maxima to the red. In Mb the two $\nu(C=C)$ modes overlap at 1621 cm$^{-1}$ (24). On the contrary, the RR spectra of DHP clearly show the presence of two vinyl stretching modes around 1620 and 1632 cm$^{-1}$. The presence of two vinyl bands in the spectra indicates that the protein matrix imposes different constraints on the two vinyl groups (25). The higher $\nu(C=C)$ frequency in DHP, as compared to Mb, is consistent with a lower
conjugation between the vinyl group and the porphyrin $\pi$ system and, therefore, with the shift to higher energy of Soret and CT1.

The complete assignment of the high frequency region RR modes of the WT-DHP and its complexes with small ligands and 4IP is reported in Table 1.

**Binding of halogenated phenols. Room temperature.** DHP has the capability to catalyze the peroxide-dependent dehalogenation of halogenated phenols (1). Contrasting results have been reported which suggest that substrate binding must precede $\text{H}_2\text{O}_2$ binding to optimize peroxidase activity (29) and vice versa (30). The DHP-4IP crystal structure (9) reveals that 4IP binds in an internal site of the distal heme cavity and forces H55 into a solvent exposed position preventing coordination of the water molecule. Thus it is reasonable to hypothesize that the binding of this molecule affects the heme iron conformation and, therefore, its spin and coordination states. Figure A.2 compares the RR spectra at pH 6 of WT-DHP (centre), DHP-4IP (4IP:DHP =30:1 molar ratio) (right) and DHP-TFP (TFP:DHP = 320:1 molar ratio) (left) complexes at room temperature (top spectra) and 12 K (bottom spectra). The existing X-ray heme cavity structures are also shown. Upon binding 4IP, the predominant species changes from 6cHS ($v_3$ at 1483 cm$^{-1}$, $v_{37}$ at 1583 cm$^{-1}$) to 5cHS ($v_3$ at 1494 cm$^{-1}$, $v_2$ at 1568 cm$^{-1}$, $v_{10}$ at 1630 cm$^{-1}$). Accordingly, the broadening and 6 nm blue-shift of the Soret maximum (399 nm) together with the red-shift of the CT1 (641 nm) (Figure B.1, supplemental) are consistent with the formation of a 5cHS species, as previously suggested by the room temperature crystal structure of the DHP-4IP complex (9) (Figure A.2, right). On the basis of depolarization studies (see Table A.1) the frequencies of the vinyl stretches
are observed to change slightly upon 4IP binding. In particular, one mode is identified at 1622 cm\(^{-1}\) while the second down-shifts and overlaps with the \(v_{10}\) mode at 1630 cm\(^{-1}\). The shift in frequency of the vinyl stretching modes is in accord with the changes of the torsional angles estimated from the crystallographic data of the 4IP-DHP complex (\(I, 9\)).

The binding of TFP to DHP has a quite different effect on the RR spectrum. In the presence of 10-fold excess TFP there are no significant differences at 298 K (and 12 K, see below) with respect to the wild type form, while upon addition of a 40-320 fold excess of TFP at 298 K, a slight increase of 6cHS species is revealed by the intensification of the RR bands at 1483 cm\(^{-1}\) (\(v_3\)) and 1611 cm\(^{-1}\) (\(v_{10}\)) (Figure A.2, left, bottom and Figure B.2 supplemental).

**Low temperature.** The RR spectrum of WT-DHP at 12 K shows a markedly reduced amount of 5cHS confirming the structural data obtained at 100 K by de Serrano et al. (\(8\)) in which the distal histidine is present only in the closed conformation (Figure A.2, centre, bottom). These data are in agreement with the electronic absorption spectrum at 12 K, which shows a 3 nm red-shift of the Soret band and 11 nm blue-shift of the CT1 (data not shown). Unlike WT-DHP, the RR spectrum of the DHP-4IP complex at 12 K indicates the presence of a pure 5cHS species. The small amount of 6cHS (\(v_3\) at 1483 cm\(^{-1}\)) observed at 298 K disappears, indicating a higher affinity of 4IP for DHP at low temperature. As previously noted for other heme proteins at low temperature (\(13\)) an increased frequency (of about 2-6 cm\(^{-1}\)) of the 5cHS core size marker bands is observed at low temperature in the RR spectra as a consequence of a contraction of the heme cavity. The binding of TFP to DHP has a quite
different effect on the RR spectrum. In the presence of 10-fold excess TFP there are no significant differences at both 298 K and 12 K with respect to the wild type form, however, the RR spectrum at 12 K of the DHP-TFP complex for 40-fold excess TFP shows an increase of the 5cHS population [ν₃: 1496 cm⁻¹, ν₂ 1573 cm⁻¹ and ν₁₀ 1636 cm⁻¹] (Figure B.2 supplemental), which becomes the only species present for a 320-fold excess (Figure A.2 left, top). A possible explanation of this effect is that a large excess of TFP can cause non-specific binding of the ligand due to the packing forces exerted by the lower temperature. However, a more plausible explanation is that at room temperature the binding mechanism of TFP is different from that of 4IP. Recently ¹H NMR, ¹⁹F NMR, and relaxation data consistent with an external binding site for TFP have been reported, but the location of that site has not been determined (20). The RR data at 12 K suggest that TFP binds inside the distal cavity side, as 4IP, even if binding at the external site, which might induce allosteric changes to the protein matrix forcing the distal histidine into the open conformation, cannot be completely ruled out. However, binding of TFP at the internal site at 12 K is also in agreement with cryogenic FTIR (26) and EPR experiments (31) which had shown that the substrate affects the distal pocket of DHP at cryogenic temperatures. In particular, cryogenic HYSCORE measurements showed that at 10-fold excess of TFP relative to DHP a heme-bound water molecule in the resting state of the ferric form is displaced when the substrate binds, resulting in a transition from six- to five-coordinated iron (31).

The corresponding EPR spectra of the DHP complexes with 4IP and TFP obtained at 5 K (Figure A.5) are in overall agreement with the RR spectra at 12 K. WT-DHP is characterised by a mixture of 6cHS (g┴ 6.00, g∥ 2.00) and 5cHS (6.09, 5.54, 2.00) forms
The progressive addition of TFP (up to 320-fold excess) leads to an increasing proportion of 5cHS with respect to 6cHS species (Figure A.5, traces b-c). Addition of 4IP (30-fold excess) leads to a pure 5cHS state, characterised by a more rhombic g tensor (6.22, 5.50, 1.99) (Figure A.5, trace d) compared to DHP alone or in the presence of TFP. The EPR bandwidth is also greater in the presence of 4IP, indicating that there is g-strain at these HS sites probably resulting from some structural instability which gives a distribution of values.

**Figure A.5.** X-band EPR spectra showing the low field g⊥ region of DHP (a), DHP-TFP 1:10 (b) and 1:320 molar ratio (c) and DHP-4IP (1:30 molar ratio) (d) at pH 6 in 0.15 M phosphate/glycerol 30 % (v/v). The spectra were recorded at 5 K, 1 mW microwave power, and 10 G modulation amplitude. Spectra have been shifted along the ordinate axis to allow better visualization.
Discussion

The flexibility of the distal histidine in DHP.

WT-(Fe$^{3+}$)DHP. The present spectroscopic characterization carried out in solution is in accord with the crystal structure at 298 K (9) in which His55 was observed to reside simultaneously in the open and closed conformations with nearly equal populations. In one of the two conformations His55 is located in the distal cavity, whereas in the second conformation it is positioned away from the distal pocket towards the solvent. The RR data clearly indicate that at room temperature the protein exists in equilibrium between the 6cHS and 5cHS states. In the 6cHS form a water molecule is coordinated to the iron and hydrogen bonded to a distal histidine (His55), which is orientated towards the heme (closed conformation) whereas in the 5cHS state His55 is exposed to the solvent. Therefore, unlike the resting state of peroxidases, where the heme sixth coordination site is vacant or bound weakly to water (12), at pH 6 ferric wild type DHP contains an metaquo 6cHS species with His55 located in the distal cavity and weakly hydrogen-bonded to the water molecule (closed conformation) (Figure A.6) ($N_6$-O$_{H2O}$ = 3.24 Å). Furthermore, at low temperature the equilibrium is shifted towards the 6cHS form (close conformation), in perfect agreement with the recent structure obtained at 100 K which shows that, similar to the distance observed in myoglobin at neutral pH (32), the His55 is 0.75 Å closer to the heme iron than in the 298 K structure (8, 9).

(Fe$^{2+}$)DHP in the presence halogenated phenols. The internal binding site of DHP has been characterized by X-ray crystallography (9, 32). The structure of the DHP-4IP complex shows that the monohalophenol binding pocket is surrounded largely by hydrophobic residues (F21,
Figure A.6. Comparison between the heme binding pockets of (Fe$^{3+}$)Mb (PDB code 1A6K (35)), (Fe$^{3+}$)DHP (PDB code 2QFK (subunit A) (8)), and (Fe$^{3+}$)CCP (PDB code 1ZBY (41)), showing the key residues and water molecules in each case.

F24, F35, F52, V59, F60 and L100) as well as a tyrosine (Y38). The hydroxyl group of the 4IP substrate can act as a hydrogen bond acceptor for the hydroxyl group of Y38 (distance 3.7 Å), with the distal His positioned out of the cavity (open conformation) (9). As a consequence, no water molecule is observed in the close vicinity of the Fe atom and, accordingly, the RR spectra are characteristic of a mainly 5cHS heme. Moreover, as observed in the X-ray structure at room temperature, the RR data confirms the changes in the vinyl orientation upon 4IP binding. At low temperature, RR core size marker bands and EPR clearly indicate that only the 5cHS species exists. Therefore, at cryogenic temperature the equilibrium of distal histidine conformations shifts towards the open conformation, indicating the stabilization of 4IP in the distal pocket binding site at low temperature. Unlike 4IP, at room temperature TFP binds in an external binding site of the heme (20) maintaining the 6cHS coordination of the resting protein, while at 15 K an increased amount of 5cHS is observed leading us to conclude, in agreement with previous reports, (26, 31) that TFP binds inside the distal cavity, as 4IP, forcing the distal histidine in the open conformation.
**Push-pull effect in (Fe$^{3+}$) DHP compared to peroxidases and globins.**

The heme enzyme DHP is a hemoglobin known to have excellent peroxidase activity under physiological conditions (1, 29). For heme containing peroxidases, the postulated mechanism of peroxide decomposition relies on the concerted roles played by the conserved proximal histidine and the distal histidine and arginine through H-bonds and charge stabilization (12). However, unlike peroxidases, DHP lacks some of the main features which determine the peroxidase activity: on the distal side of the heme in peroxidases, the conserved positively charged guanidinium of an arginine residue and an H-bond from the distal histidine N$_{δ}$ atom to a nearby asparagine (2.4 Å in CCP, Figure A.6, right), depresses the histidine pKa constraining N$_{ε}$ to act as a H-bond acceptor during the catalytic cycle (33). Although the Tyr38 residue of DHP has a strong interaction with H55 (Figure A.6, middle), the pKa of H55 is approximately 4.5, which is identical to that in Mb at room temperature (34). Moreover, the Y38F mutant appears to have a greater initial catalytic rate than wild type. Hence, in DHP there is no obvious analog to the arginine interaction in peroxidases.

In general, the DHP secondary structure is very similar to that of SWMb (35) with the overall disposition of the key α-helices (B, C, D, E, and F) nearly identical. However, on the distal side, His55 in DHP has been reported at different distances from the heme iron, ranging from 5.4 Å (9) to 4.8 Å (8). While the first reported distance is more similar to the distal cavity of peroxidases than globins, the second structure reports a distance closer to that observed in other globins including SWMb, where the distal His64 is 4.3 Å from the heme iron and H-bonded to a distal water molecule (Figure A.6, left). Nevertheless, the close
similarity of the frequencies of the $\nu_{(Fe-F)}$ (462 cm$^{-1}$) and $\nu_{(Fe-OH)}$ (491 and 551 cm$^{-1}$ for the HS and LS forms, respectively) stretching modes in the fluoride and hydroxyl adducts of DHP and Mb (13, 27), but markedly different from the corresponding frequencies observed in peroxidases ($\nu_{(Fe-F)}$ at 385 cm$^{-1}$ for HRP (36) and only a LS $\nu_{(Fe-OH)}$ around 500-507 cm$^{-1}$ for various peroxidases (13, 37-39)) clearly indicate that these ligands bind the heme iron in a similar manner in both DHP and Mb, but quite different from that of peroxidases. In particular, in peroxidases, the Arg is determinant in controlling the ligand binding via a strong hydrogen bond between the positively charged guanidinium group and the anion (13, 15, 16, 40). Recently, a high resolution X-ray structure of resting state CCP revealed that the key residue for the formation of the catalytic intermediate compound, distal Arg48, occupies two positions; one “out” positioned close to the heme propionate and the other “in” positioned close to the heme iron. In the catalytic intermediate compound only the “in” position exists, which enables Arg48 to H-bond with the ferryl O ligand (41). The “in” position has been observed also in the CCP fluoride complex where the Arg48 guanidinium group moves about 2.5 Å toward the ligand to form a hydrogen bond with fluoride (11). Therefore, while in peroxidases, the distal His contributes to the stability of the fluoride complex presumably by accepting a proton from HF and hydrogen-bonding, through a water molecule, to the anion (14, 16, 40) in Mb it is the sole amino acid responsible for stabilization of the heme coordinated ligand. The X-ray structure of the SWMb-F adduct has revealed that the fluoride anion, coordinated to the heme iron, is hydrogen bonded to a water molecule (W195) which, in turn, is hydrogen-bonded to the $N_\varepsilon$ of the distal histidine (10).
On the basis of the spectroscopic results and by analogy with Mb, we suggest that a hydrogen bond network may exist between the His55, a water molecule and the fluoride in DHP. However, this conclusion implies that the distal His, similarly to Arg48 in CCP, may undergo a conformational change that places it within hydrogen-bonding distance of the anionic ligand. In analogy to the role played by the distal Arg in CCP (40) the spectroscopic data suggest that the movement of the distal His55 in DHP is determinate in stabilizing only the anionic ligand binding. In fact, when other ligands without an electrical charge, such as CO and NO, are bound to the heme iron the interaction of the distal His with the heme bound ligand is weaker than in myoglobins. In particular, CO is an excellent probe for investigating the distal cavity of heme proteins [Spiro, T. G., and Wasbotten, I. H. (2005), CO as a vibrational probe of heme protein active sites, J. Inorg. Biochem. 99, 34-44], since back-donation from the Fe d$\pi$ to the CO $\pi^*$ orbitals is increased by polar interactions and formation of H-bonds between the bound CO and the distal protein residues. As a consequence, the Fe-C bond strengthens while the CO bond weakens, thereby increasing the $\nu$(Fe-C) vibrational frequencies and decreasing the $\nu$(C-O) frequencies. Therefore, in the DHP-CO complex the slightly higher $\nu_{CO}$ (1950 vs 1947 cm$^{-1}$) and lower $\nu_{Fe-CO}$ (499 vs 507 cm$^{-1}$) stretching frequencies than SWMb (42-44) indicate a less polar environment around the CO ligand, possibly resulting from a larger distance between the CO and the imidazole. This is in agreement with the Fe-N$_{Distal\ His}$ distance of 4.8-5.4 Å (8, 9) vs 4.3 Å (35) observed in the X-ray structure of ferric DHP and Mb, respectively. In addition, recently it has been shown that the protein interactions with photolyzed NO are weaker in DHP than in the wild-type MbNO (for both SW and horse heart) (45).
The concerted interactions of the distal His and Arg with hydrogen peroxide bound to the heme has been called “the pull effect”, (46-48) Therefore, the pull component in peroxidases is created by the distal histidine functioning as an acid/base in proton transfer to the leaving water molecule with the positively charged arginine stabilizing the developing negative charge (46, 48). On the proximal side of peroxidases, the conserved H-bond between the N₅ atom of the imidazole fifth ligand and the carboxylate of an aspartic side chain, which acts as a H-bond acceptor, imparts an imidazolate character to the histidine ligand (12, 40, 49). The increased electron donation of the proximal imidazole ligand is called “the push effect” since it stabilizes the high oxidation state of the iron intermediate compound, leading to the rapid reaction of peroxidases with hydrogen peroxide (50).

In DHP the N₅ atom of the proximal His interacts with the carbonyl group of a Leu residue (Leu83). On the basis of the ν(Fe-Im) stretching frequency of DHP (233 cm⁻¹), the authors concluded (43) that the H-bond appears to be stronger than in Mb (218-221 cm⁻¹) (51, 52), but weaker than in peroxidases (12). However, a weaker imidazolate character of the proximal Fe-ligand is not expected to impair the peroxidase activity of DHP. In fact, studies of proximal variants of various peroxidases clearly indicate that the “electron push” effect may not be so important for the activity since the strength of the proximal histidine-aspartate hydrogen bond can be modulated without serious effects on the peroxide cleavage step (12).

Therefore, the present results strongly support the view that for DHP the distal His is the only residue capable of performing proton shuttling in the active site. In the presence of
negatively charged ligands, a conformational change places the distal His within hydrogen-bonding distance of the ligands, underlying its important role in anionic ligand stabilization.

**Conclusion**

In conclusion, the present work highlights the different stabilization mechanisms of heme-iron ligands exerted by the distal residues in DHP compared to Mb and peroxidases. For the latter proteins, the Arg is determinant in controlling the ligand binding via a strong hydrogen bond between the positively charged guanidinium group and the anion. The distal His accepts a proton and is hydrogen-bonded (probably through a water molecule) with the iron-coordinated ligand. For DHP (and Mb) the distal His is the only residue responsible for the stabilization of ligands coordinated to the heme iron. However, unlike Mb, in DHP the distal His is highly mobile and undergoes a conformational change to establish a strong hydrogen-bond with ligands. At room temperature, in the ferric state, in the absence of a heme ligand, the distal histidine is in equilibrium between the open solvent-exposed position (5cHS) and the closed conformation (6cHS). The equilibrium shifts to the closed conformation at 12 K. The link between the histidine and heme iron coordination extends to the binding of phenols in the distal pocket. In fact, binding of 4IP in the distal heme cavity shifts the equilibrium toward the open conformation, as the protein in its 5cHS state, while TFP binds externally to the distal side at room temperature (6cHS) but inside the heme cavity at low temperature. When considered in the light of the recent finding that there is an external substrate binding site in DHP (20) the movement of H55 appears to play a role in a regulatory mechanism in DHP function. The spectra presented here show that the flexibility
of the distal histidine leads to the possibility of differing hydrogen bond strength for anionic and neutral ligands to the heme iron. This level of control may, in turn, be important in explaining how a single distal histidine can provide the peroxidase “pull” that is usually thought to require the concerted action of an arginine in proximity to the distal histidine.

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Supplemental Information is provided in APPENDIX B.

They include the UV-vis spectra of \( \text{Fe}^{3+}\text{DHP} \) and \( 4\text{IP}-(\text{Fe}^{3+})\text{DHP} \) at pH 6 in 0.15 M potassium phosphate and RR spectra of \( \text{TFP}-(\text{Fe}^{3+})\text{DHP} \) (40:1 molar ratio) at 298 and 12 K at pH 6 in 150 mM phosphate and 30% (v/v) of glycerol.
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APPENDIX B
Supporting Information for APPENDIX A

New Insights into the Role of Distal Histidine Flexibility in Ligand Stabilization of
Dehaloperoxidase-hemoglobin from Amphitrite ornata.
**Figure B.1.** UV-Vis absorption spectra of (Fe$^{3+}$) DHP (solid line) and 4IP-(Fe$^{3+}$) DHP (dashed line) at pH 6 in 0.15 M potassium phosphate.
Figure B.2. RR spectra of TFP-(Fe$^{3+}$)DHP (40:1 molar ratio) at 12 (bottom) and 298 K (top) at pH 6 in 150 mM phosphate and 30% (v/v) of glycerol. Experimental conditions: $\lambda_{\text{exc}}$: 406.7 nm, spectral resolution 1.3 cm$^{-1}$ (298 K) and 5 cm$^{-1}$ (12 K), power at the sample 5 mW (298 K) and 8 mW (12 K), average of four spectra with 300 s integration time (298 K), collection interval 8 s/0.5 cm$^{-1}$ (12 K). Spectra have been shifted along the ordinate axis to allow better visualization.
APPENDIX C

Kinetic analysis of a naturally occurring bioremediation enzyme: Dehaloperoxidase-hemoglobin from *Amphitrite ornata*
Abstract

The temperature dependence of the rate constant for substrate oxidation by the dehaloperoxidase-hemoglobin of Amphitrite ornata has been measured from 278 K to 308 K. The rate constant is observed to increase over this range by approximately a factor of two each 10 °C temperature increment. An analysis of the initial rates using a phenomenological approach that expresses the peroxidase ping-pong mechanism in the form of the Michaelis-Menten equation leads to an interpretation of the effects in terms of the fundamental rate constants. The analysis of kinetic data considers a combination of diffusion rate constants for substrate and H$_2$O$_2$, elementary steps involving activation and heterolysis of the O-O bond of H$_2$O$_2$, and two electron transfers from the substrate to the iron. In order to complete the analysis from the perspective of turnover of substrate into product, density function theory (DFT) calculations were used to address the fate of phenoxy radical intermediates. The analysis suggests a dominant role for diffusion in the kinetics of DHP.
Introduction

The dehaloperoxidase-hemoglobin from *Amphitrite ornata* (DHP) has recently been shown to have a unique peroxidase enzyme mechanism that may be characterized as non-classical competitive inhibition \(^1\). In the non-classical model, the inhibitor binds remote to the active site and causes a conformational change in the enzyme that prevents substrate binding. In DHP, the substrate binds at an external site and the inhibitor binds in the distal pocket above the heme iron. There are multiple X-ray crystal structures of 4-halophenols binding in the internal site \(^1,2\). The location of the external site has been characterized by spectroscopic measurements. NMR spectroscopy indicates that there is an interaction between the substrate and the distal histidine, H55 \(^3\). Resonance Raman spectroscopy confirms this interaction, using the observation that the distal histidine (H55) position appears to correlate with water in the metquo form such that H55 in the internal position stabilizes the 6-coordinate high spin state and H55 in the external position gives rise to the 5-coordinate high spin state \(^1,4-6\). Models of these structures are shown in Figure C.1. There are two isoforms of DHP in *A. ornata* \(^7\). All previous studies that referred to DHP implicitly referenced DHP A. We have recently cloned and expressed the B isoform of DHP, DHP B, \(^8,9\) so that we now distinguish the specific experiments conducted on DHP A in this study from general features that apply to both isoforms of DHP. The two isoforms are structurally homologous, but differ by five amino acids: I9L, R32K, Y34N, N81S, and S91G. Although there is slight variation in the amino acid sequence, both isoforms are capable of performing the dehalogenation function.
**Figure C.1.** Structures of DHP A with substrate and inhibitor bound to the enzyme. A.) Metaquo form with inhibitor bound (3LB1 is shown, but 3LB2, 3BL3, and 3LB4 are similar in structure); H55 open. B.) Deoxy form of DHP (3DR9); H55 open. 1C.) Metaquo form of DHP (2QFK); H55 closed. D.) Metaquo form with substrate at proposed external substrate binding site; H55 closed.

The external substrate binding site in DHP is related to external binding in peroxidases in that electron transfer from the substrate to the heme edge (or alternatively to an amino acid radical) is the key step in substrate oxidation as shown in Figure C.2. Similar considerations apply to myoglobin mutants that have peroxidase activity. Amino acid radicals, which are formed in myoglobin as well as peroxidases may act as a conduit for electron transfer or might result in protein crosslinking. The oxidized phenol rapidly
deprotonates to form the corresponding phenoxy radical \(^{20,21}\). The standard peroxidase mechanism involves disproportionation of the radical to form the phenoxy radical cation and phenolate as shown on the left side of Figure C.2. Disproportionation is self-electron transfer in this case. Attack by water could occur on either the radical (right side) or the cation (left side). If attack by water occurs on the radical (right side of Figure C.2), then electron transfer occurs subsequently. While the order of electron transfer and attack by water is difficult to ascertain with certainty in a kinetic experiment, it can be addressed using density functional theory (DFT) calculations.

**Figure C.2.** Possible routes from phenol to quinone subsequent electron transfer to the heme oxoferryl intermediates of the DHP A enzyme. These structures were used for DFT calculations described in the text.
Kinetic analysis is needed in order to understand the implication of the various possible pathways for one-electron activation implicit in the external binding site. Substrate binding and oxidation under physiological conditions are the basic physical phenomena required for application of DHP as a bioremediation enzyme. One can regard DHP as a bioremediation enzyme since its natural function is to oxidize 2,4,6-tribromophenol (2,4,6-TBP). While 2,4,6-TBP is a natural pollutant made by organisms such as Notomastus lobatus and Thelepus crispus in benthic ecosystems, 2,4,6-trichlorophenol (2,4,6-TCP) is a man-made pollutant that is widely dispersed. 2,4,6-TCP and related poly-substituted chlorinated phenols are substrates for DHP, while, as we have recently shown, 4-chlorophenols are inhibitors. Since 2,4,6-TCP is an excellent substrate, as well as a bioremediation target, the present study serves as a baseline for future comparison of a range of substrates in DHP and its mutants. Herein, we focus on the kinetics of the uninhibited reaction using an Arrhenius analysis of the rate constants based on kinetic models of the ping-pong mechanism. We show that a phenomenological model permits fit to an equation that has the form of the Michaelis-Menten equation, but ultimately has an interpretation in terms of the fundamental rate constants for peroxidase kinetics. Finally, we consider the fate of the radical generated by the oxidation reactions of DHP. Since this process is not directly probed by the spectroscopic assay, DFT calculations are needed to determine whether the barrier height corresponds to the measured kinetic parameters using an Arrhenius approach.
Materials and Methods

**DHP A Protein growth.** A pET-16b plasmid containing the 6XHis-tagged DHP A DNA insert was transformed into competent BL21(DE3) *E. coli* cells and then plated with 100ug/mL ampicillin and allowed to grow at 37 °C for about 14 hours. Single colonies were isolated and transferred to 2mL starter growths of 2xYT broth containing 75ug/mL of ampicillin at 37 °C with shaking for about 8hours. 1mL of each starter growth was then used to inoculate 6 one liter flasks of 2xYT broth containing 75ug/mL of ampicillin. The 6 liter *E. coli* growth was incubated at 37 °C with shaking for about 15 hours. The cells were collected via centrifugation at 7000 rpm, 4 °C for 20 minutes.

**Purification of 6XHisDHP A.** The *E. coli* cell pellet was resuspended in 2 mL/(gram cell pellet) of lysis buffer (50 mM NaH$_2$PO$_4$, 300 mM NaCl, 10 mM imidazole, pH 8) and lysozyme was added to a final concentration of 1 mg/mL. The cell slurry was stirred at 4 °C for an hour and then sonicated for 30 minutes and 500 uL of DNase I (10 mg/mL) and RNase A (16 mg/mL) were added. The cell slurry was then stirred again at 4 °C for one hour before freezing overnight at -20 °C. After rethawing, the cells were centrifuged at 18000 rpm for half an hour, and the supernatant containing the 6XHisDHP A was collected. The crude DHP A solution was applied to a Ni-NTA agarose column (5 Prime Perfect Pro), washed with washing buffer (50 mM NaH$_2$PO$_4$ 300mM NaCl. 20 mM imidazole, pH 8), and eluted using elution buffer (50 mM NaH$_2$PO$_4$, 300mM, NaCl, 250 mM imidazole, pH 8). The isolated 6XHis DHP A from the column was oxidized by excess 10 mM K$_3$[Fe(CN)$_6$]. Excess K$_3$[Fe(CN)$_6$] was removed by gel filtration on a Sephadex G-25 column, which permitted
simultaneous buffer exchange into 20 mM KH₂PO₄ pH 6 buffer. The oxidized protein was loaded onto a CM-52 column for further purification. Once loaded onto the column, the 6XHis DHP A was washed with 20 mM KH₂PO₄ pH 6 and eluted form the column with 150 mM KH₂PO₄ pH 7 buffer. The concentration of the ferric DHP A was determined using the Soret absorption intensity at 406nm with a molar absorptivity of 116,400 M⁻¹cm⁻¹. The purified 6XHisDHP A was stored at 4 °C for future use.

**Sample preparation.** Purified 6XHisDHP A was oxidized in the presence of 10 mM K₃[Fe(CN)₆], separated from excess K₃[Fe(CN)₆] by gel filtration on Sephadex G-25, and further purified on CM-52 (as above) prior to each experiment. For kinetic assays the elution buffer was 150 mM KH₂PO₄ buffer at pH 7.0. 98% 2,4,6-trichlorophenol from Acros Organics (Lot No.A0245137) was dissolved in 150 mM KH₂PO₄, pH 7 buffer with a final concentration of 3 mM and stored at 4 °C until use. The H₂O₂ solution was prepared from 30% reagent grade H₂O₂ solution from Fisher Chemicals in 150 mM KH₂PO₄, pH 7 buffer to a final concentration of 17.6 mM for the stock solution. The H₂O₂ solution was prepared freshly before use and stored at 4 °C during the course of a series of experiments.

**Kinetic Assays.** The kinetic assays were conducted in a 0.4 cm path length quartz cuvette with a total volume of 1500 µL. The final concentration of ferric DHP A in the cuvette was [E]ᵣ ~2.4 µM, and substrate, 2,4,6-TCP, concentrations were varied from 100 to 1900 µM. Spectra were obtained using a photodiode UV-VIS spectrometer (Agilent 8453) equipped with a Peltier temperature controller using benchtop mixing of the reagents. In order to reach
thermal equilibrium, DHP A and 2,4,6-TCP (150 mM KH₂PO₄, pH=7 buffer) were allowed to incubate for 5 minutes in the cuvette placed in the thermal cell. The H₂O₂ solution, with a final concentration 500 times DHP A, was injected into the cuvette within one second of initiation of data collection. The data were measured over the wavelength range from 200 – 700 nm with a time resolution of 3.1 seconds. The wavelength monitored during kinetic measurements was 273nm, the peak of the product absorption band. For 2,6-DCQ, ε₂₇₃ is 14,130 M⁻¹ cm⁻¹ at pH 7. Data were extracted using an Excel spreadsheet and analyzed using Igor Pro 5.0.

**Kinetic analysis.** The purpose of this kinetic assay is to study the relationship between enzymatic reaction initial velocity and temperature. The obtained data were first fit to a linear function to get the initial reaction velocity at a given substrate concentration. Then those initial velocities of different substrate concentrations were fit to the Michaelis-Menten equation to obtain relevant parameters k₅th and K_M. Finally, the relationship between these parameters and temperature was analyzed using a phenomenological Arrhenius equation. The ES complex is formed reversibly in the Michaelis-Menten mechanism. However, in peroxidase chemistry there are two complexes, one with the substrate, 2,4,6-TCP, and one with H₂O₂. According to the standard enzyme kinetic scheme, the binding of H₂O₂ leads to formation of compound I and is not reversible. We have recently shown that compound I is not observed in DHP A, and that compound ES is rapidly formed ¹². We recently confirmed similar observations for DHP B ⁹. Both compound I and compound ES consist of a Fe(IV)=O species and a cation radical. The difference is that the radical resides on the heme
ring in compound I, but on an aromatic amino acid in compound ES. We have further shown that the radical is located on one of the five tyrosines in DHP A\textsuperscript{12}. Since compound ES formation appears to be rapid in DHP, we present the peroxidase rate scheme with a compound ES intermediate. Compound ES is reduced in two one-electron steps by a substrate, XAOH, which is 2,4,6-TCP in the present study. The sequential oxidation of two substrates by two different processes with rate constants $k_2$ and $k_3$ is known as the ping-pong mechanism.

**Scheme C.1.** Rate scheme for peroxidase catalysis.

\[
\begin{align*}
\text{DHP} + \text{H}_2\text{O}_2 & \xrightarrow{k_1} \text{Cmp ES} + \text{H}_2\text{O} \\
\text{Cmp ES} + \text{XAOH} & \xrightarrow{k_2} \text{Cmp II} + A^- \\
\text{Cmp II} + \text{XAOH} & \xrightarrow{k_3} \text{DHP} + A^-
\end{align*}
\]

The kinetic model for this rate scheme is given by:

\[
V_o = \frac{V_{\text{max}}[\text{XAOH}]}{K_m + [\text{XAOH}]}
\]

\[
V_{\text{max}} = k_1[H_2O_2][E]_o \quad K_m = \left(\frac{1}{k_2} + \frac{1}{k_3}\right)k_1[H_2O_2]
\]

Dunford does not recommend the use of the Michaelis-Menten model since $k_1 >> k_2$ for well-studied peroxidases such as HRP\textsuperscript{26}. Consequently, compound I (Cmp I) is rapidly
formed and can build up to an appreciable concentration in these peroxidases for a period of minutes or even longer in very pure preparations. According to Eqn. 1, the dependence of $V_o$ on [XAOH] is linear for $k_1 >> k_2$ and saturation should not be observed \(^{26}\). However, DHP A does not behave like HRP in this regard \(^{27,28}\). If we consider that compound ES plays the role of compound I in DHP A \(^{12}\), then $k_1$ and $k_2$ are not vastly different in magnitude in DHP A. Accordingly, the Results presented in this study show that saturation is observed in DHP A. These observations justify the parameterization in Eqn. 1. The product is formed by disproportionation of the radical intermediate $A \cdot$. The $H_2O_2$ concentration was held constant at 1.2 mM in this study so that only the substrate concentration, [XAOH] is considered here.

**DFT Calculations.** Substrates involved in the two pathways, Figure C.2, were constructed using the software package Materials Studio (Accelrys). All quantum chemical calculations were performed in a water environment with the electronic structure package DMol\(^3\) \(^{30-33}\). Ground state geometries of the substrates (without the protein) were optimized using the conjugate gradient method constrained to an energy difference of < $10^{-6}$ Ha, the Perdew-Burke- Ernzerhof (PBE) \(^{29}\) density functional, and with a double numeric basis set with one polarization function. In order to achieve SCF energy convergence for each optimization cycle the thermal treatment of electron occupancy \(^{34}\) with an electronic temperature of 0.02 Ha was implemented. To incorporate solvent effects, the COSMO (conductor-like screening model) module employed by DMol\(^3\) was also implemented. For the solvent used in this study, water, the dielectric constant adopted was, $\varepsilon = 80.4$ \(^{35}\).
Results

Kinetic data were obtained as both time-dependent spectra and single-wavelength kinetics. The single wavelength kinetics were analyzed first to determine the appropriate temperature range for analysis.

The single wavelength (273nm) kinetics shown in Figure C.3 give the change in the concentration of the product 2,6-dichlorophenol-1,4-benzoquinone (DCQ) at seven different temperatures. The change in the product concentration is assumed to be proportional to the enzymatic rate. However, the concentration of DCQ reaches a maximum and begins to decrease at longer times when the temperature is greater than 20 °C. The decrease in absorbance on time scales of 50 seconds and longer is due to secondary hydrolysis reactions of the DCQ product 36. Because of the secondary reaction, only the short time kinetics were used to estimate the catalytic rate for the process. This choice is consistent with the common practice of confining analysis of Michaelis-Menten kinetics to the initial rates.

The initial rates of appearance of the product, 2,6-DCQ at 273 nm at five different temperatures are shown in the Figure C.4 as a function of substrate, 2,4,6-TCP, concentration. As expected, based on the kinetics in Figure C.3, Figure C.4 shows that the initial rate of the enzyme reaction between DHP A and 2,4,6-TCP at pH 7 has a strong temperature dependence. The initial rate data were fit to Eqn. 1 and from the curve fitting, we can determine the phenomenological Michaelis constant and the catalytic constant, \( K_M \) and \( k_{cat} \), respectively. Table C.1 provides a summary of the Michaelis-Menten fit parameters for the initial rate data.
**Figure C.3.** Single wavelength kinetics at 273 nm of the oxidation reaction of TCP by DHP as a function of time and product DCQ concentration obtained using a UV-VIS spectrometer. Assay conditions were [Ferric DHP] ~2.4μM, [TCP] ~ 150μM, [H₂O₂] ~ 1.2 mM, pH 7 KP buffer.

**Figure C.4.** Single wavelength kinetics of oxidation of TCP by DHP as a function of substrate TCP concentration and enzymatic reaction initial velocity. The assay conditions were Ferric DHP ~2.1 μM, [TCP] ~ 150μM, [H₂O₂] ~ 0.1-1.9 mM, pH 7 KB buffer.
Table C.1. Kinetic parameters from the curve fitting to the phenomenological Michaelis-Menten equation

<table>
<thead>
<tr>
<th>T/K</th>
<th>V\text{max}/\mu\text{M}s^{-1}</th>
<th>\text{k}\text{cat}/s^{-1}</th>
<th>K_M/mM</th>
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<td>283</td>
<td>6.32</td>
<td>2.68</td>
<td>1.58</td>
</tr>
<tr>
<td>288</td>
<td>9.16</td>
<td>3.88</td>
<td>1.16</td>
</tr>
<tr>
<td>293</td>
<td>14.2</td>
<td>6.00</td>
<td>1.13</td>
</tr>
<tr>
<td>298</td>
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<tr>
<td>303</td>
<td>21.7</td>
<td>9.19</td>
<td>1.06</td>
</tr>
</tbody>
</table>

Using the phenomenological Michaelis-Menten equation the turnover number is, \( k_{\text{cat}} = \frac{V_{\text{max}}}{[E]_T} = k_1[H_2O_2] \). According to the parameterization presented in Eqn. 1, the phenomenological \( k_{\text{cat}} \) is a pseudo-first order rate constant (i.e. \([H_2O_2]\) is constant) that represents the activation of the heme iron to form compound ES. Figure C.5 presents an analysis of \( k_{\text{cat}} \) as a function of temperature based on the Arrhenius equation, \( k = A e^{-E_A/RT} \).

The fit shown in Figure C.5 is from the linearized form \( \ln k = -\frac{E_A}{RT} + \ln A \). Here the parameter \( A \) is defined as the pre-exponential or Arrhenius factor. \( E_A \) is called the activation energy and \( R \) is the universal gas constant. Figure C.5 shows the plot of \( \ln k \) against \( 1/T \) is reasonably approximated by a straight line with slope equal to \(-E_A/R\) and intercept equal to \( \ln A \). The slope of the line found from the fit is \(-5295\), which corresponds to an activation energy of \( E_A = 44 \text{ kJ/mol} \).
Figure C.5. Plot of \( \ln(k_{\text{cat}}) \) vs. \( 1/T \) with a fit to a line according to the Arrhenius Equation \( \ln k_{\text{cat}} = -E_a/RT + \ln A \). \( k_{\text{cat}} \) was obtained from the fit of the initial velocity data to Eqn. 1.

Figure C.6 shows a plot of the logarithm of \( \ln(k_{\text{cat}}/K_M) \) as a function of \( 1/T \). Although \( k_{\text{cat}}/K_M \) in the Michaelis-Menten scheme is a second order rate constant related to enzymatic efficiency when substrate concentration is high, the phenomenological expression according to Eqn. 1 is proportional to \( k_2k_3/(k_2 + k_3) \), which represents the combined rate constant for substrate oxidation. Figure C.6 shows that the activation energy for this process is \( E_a = 56.3 \) kJ/mol.
Using DFT calculations, the change in Gibb’s Free energy for each step of the two pathways shown in Figure C.2 were calculated. Table C.2 provides the calculated Gibb’s Free energy for each of the reaction participants in the proposed mechanism. According to the reactions, the first mechanism after the formation of a radical involves disproportionation (-0.8 kJ/mol) followed by nucleophilic attack of a cation by water (+37.4 kJ/mol). The second mechanism involves nucleophilic attack of a radical by water (+35.0 kJ/mol) followed by electron transfer (+1.7 kJ/mol). Thus the net combined barrier height is calculated to be +36.6 and +36.7 kJ/mol for the left and right side pathways shown in Figure C.2, respectively. The final step involving rearrangement to form the product 2,6-DCQ is exergonic with $\Delta_{\text{quinone}}G^0 = -33.8$ kJ/mol.
Table C.2. Calculated Gibb’s Free energies.

<table>
<thead>
<tr>
<th>Compound</th>
<th>( G_r, M ) (kJ/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disproportion</td>
<td></td>
</tr>
<tr>
<td>TCPR</td>
<td>-93.696</td>
</tr>
<tr>
<td>TCP+</td>
<td>-94.036</td>
</tr>
<tr>
<td>TCP-</td>
<td>-94.156</td>
</tr>
<tr>
<td>Nucleophilic attack of radical</td>
<td></td>
</tr>
<tr>
<td>TCPR</td>
<td>-93.642</td>
</tr>
<tr>
<td>TCPR+H(_2)O</td>
<td>-106.80</td>
</tr>
<tr>
<td>Nucleophilic attack of cation</td>
<td></td>
</tr>
<tr>
<td>TCPRC</td>
<td>-93.889</td>
</tr>
<tr>
<td>TCPRC+H(_2)O</td>
<td>-104.64</td>
</tr>
<tr>
<td>Formation of quinone</td>
<td></td>
</tr>
<tr>
<td>DCQ</td>
<td>-91.377</td>
</tr>
<tr>
<td>HCl</td>
<td>-47.081</td>
</tr>
<tr>
<td>H(_2)O</td>
<td>-48.173</td>
</tr>
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</table>

Discussion

The analysis of the temperature dependence of the rate constants in the peroxidase scheme provides activation energies and mechanistic information on the steps leading to catalysis in DHP A. We have used 2,4,6-TCP as a substrate because it is more soluble than the native substrate, 2,4,6-tribromophenol (2,4,6-TBP). First we consider secondary reactions that occur subsequent to the rate constants analyzed here. Secondly, we will discuss the interpretation of the activation energies of the rate constants, \( k_1 \), \( k_2 \) and \( k_3 \) in the peroxidase kinetic scheme. Next, we will discuss 2,6-DCQ product formation via a radical pathway,
which appears to be rapid compared to any subsequent secondary reactions, and finally we present DFT calculations to address the mechanism of the disproportion of radicals to give the product.

We have identified a second reaction on longer time scales, which depletes the product 2,6-DCQ. This reaction is highly activated and has a negligible rate below 20 °C. It is approximately one order of magnitude slower than product formation even at the highest temperature. It has been shown elsewhere, and we have independently confirmed, that the second process is the reaction of 2,6-DCQ with H₂O₂ to produce 3-hydroxy-2,6-DCQ, which proceeds even uncatalyzed in solution 36. Although the further reactions of 2,6-DCQ do not interfere with the analysis presented here, they are of interest as steps on the path to the total dechlorination of 2,4,6-TCP and will be considered elsewhere. Figure C.3 shows that these processes are significant only at T > 20 °C. However, since the initial rate constant for 2,4,6-TCP oxidation also increases over this same temperature range, the separation of time scales between the first and subsequent steps is maintained such that the initial rate approximation still holds for determination of the enzyme kinetic parameters. The formation of a less active form of DHP A, known as compound RH, is a second process that occurs on a slower time scale 12. The inactivation of catalyst has also been considered in a kinetic model for oxidation of 2,4,6-TCP by Fe(III) meso-tetra(4-sulfonatophenyl)porphine that has strong parallels with DHP A catalysis 36. Specifically, there is a slow process (conversion to RH) that alters catalytic efficiency of the catalyst without necessarily completely abolishing that activity.
The phenomenological analysis in terms of a Michaelis-Menten kinetic scheme provides insight into the two fundamental processes required for catalysis by DHP. The catalytic rate constant, $k_{\text{cat}}$, is apparently proportional to $k_1$, the rate constant for formation of compound ES, the first active species. The efficiency, $k_{\text{cat}}/K_M$, is proportional to the overall substrate oxidation rate constant $k_2k_3/(k_2 + k_3)$. The oxidized substrate rapidly loses $\text{H}^+$ to become a 2,4,6-trichlorophenoxy radical (2,4,6-TCPR). The radical can react by disproportionation to make 2,4,6-TCP and 2,4,6-trichlorophenoxy radical cation (2,4,6-TCPRC). Here we do not consider the possibility that a second electron transfer to the heme iron by 2,4,6-TCPR immediately leads to product, but rather, follow the well-established precedent in the peroxidase literature shown in Scheme I that the mechanism consists of two one-electron processes similar to the peroxidase family of enzymes $^{37}$. Previous consideration of a two-electron model for DHP was an attempt to rationalize the hypothesis that the substrate binding site is in the distal pocket $^{27,28}$. However, we now know that the distal pocket is an inhibitor binding site and the substrate binding site is external as is commonly observed in peroxidases $^{1,3,38-40}$.

According to the mechanism in Scheme 1, the rate constant, $k_1$, is a bimolecular rate constant for the formation of compound ES. The temperature dependence of the pseudo-first-order rate constant, $k_1[H_2O_2]$, arises primarily from the steps required to break the O-O bond. The distal histidine, H55, acts as an acid-base catalyst for these steps. The energy barrier of 44 kJ/mol determined from the Arrhenius analysis applies to the bond breaking step.
The rate constants $k_2$ and $k_3$ are bimolecular rate constants that represent the combination of the diffusion rate constant, $k_d$, for formation of the enzyme-substrate complex and the electron transfer rate constant, $k_{ET}^{ES,II}$, from substrate to compounds ES and II, respectively $^{41}$.

$$k_{2,3} = k_d/(1 + K_D k_d / k_{ET}^{ES,II})$$

Here, $K_D$ is the dissociation equilibrium constant, where $K_D = k_d^{-1}$. The electron transfer barrier height has a major contribution from the outer sphere reorganization energy, $\lambda$, which has been estimated to be in the range of 0.5 - 1.25 eV for peroxidases $^{41,42}$. If the electron transfer driving force were $\varepsilon = 0$ and the reorganization energy were 1.25 eV, then the barrier height would be $E = (\lambda - \varepsilon)^2/4\lambda = 0.375$ eV. This is a maximal value since smaller values of the energy gap, $\varepsilon$, or reorganization energy, $\lambda$, will decrease $E$. Based on this estimate, electron transfer can only account for part of the 0.58 eV (56 kJ/mol) barrier height obtained from the kinetic analysis of the Michaelis-Menten equations, and the diffusion rate constant, $k_d$, may be rate limiting. However, it is reasonable to assume that $k_d$ increases with temperature. Hence, the origin of the kinetic effect is likely to arise from subsequent diffusion-controlled steps such as disproportionation and attack by water to produce the quinone product (Figure C.2).

The unusual oxidation kinetics of 2,4,6-TCP by metalloporphyrins arise from the fact that the catalyst concentration determines the amount of oxidized product but not the rate of oxidation $^{36}$. This type of behavior also suggests that the electron transfer from substrate to catalyst (i.e. heme iron or tyrosine) is not rate limiting. The catalyst concentration may be a limiting reagent due to diffusion control as well as other factors such as inactivation by conversion to a less active form. This type of process is not entirely unexpected for reactions
involving strong oxidants such as H₂O₂. Nonetheless, it is consistent with rate limiting steps elsewhere in the sequence of steps that lead to product.

Figure C.2 shows two pathways that involve attack by H₂O₂, either on the radical or the radical cation, to displace chloride. DFT calculations have been employed to determine which of the reaction pathways in Figure C.2 occurs by calculating the change in Gibb’s free energy for each step. First, the analysis for the disproportionation reaction yields Δ\text{disprop}G^o = -0.8 kJ/mol followed by attack on the cation radical, Δ\text{phenoxy_cat}G^o = +37.4 to lead to an overall energy barrier of +36.6 kJ/mol shown as the blue pathway in the energy level diagram in Figure C.7. The alternative pathway consists of attack on the phenoxy radical by a water with a free energy change of Δ\text{phenoxy_rad}G^o = +35.0 kJ/mol followed by electron transfer, Δ\text{elec_trans}G^o = +1.7 kJ/mol, to give an overall energy barrier of +36.7 kJ/mol (shown as the red pathway in Figure C.7. Although the overall barrier heights are so similar that the two
Figure C.7. Energy level diagram based on DFT calculations.

pathways appear equally likely, Figure C.7 makes it clear that disproportionation will dominate in a thermodynamic sense. Since water is present in much greater concentration than the substrate, required for the bimolecular disproportionation, there is still a kinetic issue to consider. Given the consideration above, the electron transfer from the heme (or amino acid radicals in the protein) is unlikely to account for the measured barrier heights in the Arrhenius kinetic analysis. Therefore, we suggest that the calculated barrier height of 36.6 kJ/mol is the dominant contribution to the measured barrier height of 56 kJ/mol for $k_{\text{cat}}/K_M$. Finally, we propose the hypothesis that the solution portion of the reaction consists of a disproportionation followed by attack of a phenoxy cation by water. Although we have
given a complete analysis of the fundamental rate constants in terms of a pseudo-Michaelis-Menten scheme, and demonstrated saturation, our analysis suggests that the rate limiting steps for product formation may occur apart from the enzyme itself.

Conclusion

DHP is a unique dual function enzyme. Since it functions as both a hemoglobin and a dehaloperoxidase, it has features that distinguish it from other peroxidases. Two specific unique features are the internal binding site and a switch in function that may be linked to the flexibility of H55. To understand the activation energy for the various steps in the oxidation of substrate, we have measured the temperature dependence of the oxidation of 2,4,6-TCP to 2,6-DCQ. We applied the analysis of Dunford and compared it to a Michaelis-Menten analysis to show that the rate constants, $k_1$, $k_2$, and $k_3$ of the ping-pong mechanism can be understood in a mechanism that gives saturation of the rate at high substrate concentration. This type of kinetic result, which is valid for DHP, is distinct from enzymes such as HRP, which have very large $k_1$ and do not exhibit saturation. Based on the fit to an Arrhenius model, we were able to understand the temperature of phenomenological $k_{cat}$ and $k_{cat}/K_M$, which correspond to $k_1[H_2O_2]$ and $k_2k_3/(k_2 + k_3)$, respectively. The activation energies are 44 and 56 kJ/mol, respectively, for these two processes. The rate limiting process is $k_2k_3/(k_2 + k_3)$, which has an activation energy that is fairly typical for chemical reactions. An anecdotal rule of thumb suggests that the rate constant for many diffusion-controlled reactions increases by a factor of 2 for each 10 °C, which corresponds to an activation energy of 54 kJ/mol. The rate constant for substrate oxidation in DHP is close to that value, probably
because of the diffusion-controlled nature of the solution reactions involving the radical XAO’ that lead to product formation.

We have analyzed the mechanism of the respective steps in order to understand the origins of the activation energy. The rate constant for formation of compound ES, $k_1[H_2O_2]$, reflects the rate limiting step in the catalytic rearrangement of $H_2O_2$ bound to the heme iron followed by electron transfer from a tyrosine. The second and third processes, $k_2k_3/(k_2 + k_3)$, both result in oxidation of substrate but involve both diffusion to the active site and electron transfer. Although $k_2$ and $k_3$ are nominally electron transfer steps that generate the radical intermediate, the observed rate constant appears to be dominated by the diffusional dissociation from the binding site and subsequent attack water. The analysis suggests a dominant role for a bimolecular solution component in the kinetics of substrate oxidation by DHP and has implications for the mechanism of the entire peroxidase family.

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Supplemental Information is provided in APPENDIX D.
References


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APPENDIX D

Supporting Information for Appendix C

Kinetic analysis of a naturally occurring bioremediation enzyme: Dehaloperoxidase-hemoglobin from Amphitrite ornata
Derivation of Equation 1.

The peroxidase rate scheme (also known as the ping-pong mechanism) is given as:

\[ \text{DHP} + H_2O_2 \xrightarrow{k_1} \text{CmpI} + H_2O \]

\[ \text{CmpI} + AH \xrightarrow{k_2} \text{CmpII} + A \cdot \]

\[ \text{CmpII} + AH \xrightarrow{k_3} \text{DHP} + A \cdot \]

First we write rate equations for each component in the peroxidase rate scheme. We have defined \( A \cdot \) as \( P \), the product, and \( \text{DHP} \) as the ferric form of the enzyme in the rate equations below.

\[ \frac{d[DHP]}{dt} = -k_1[DHP][H_2O_2] \]

\[ \frac{d[CmpI]}{dt} = k_1[DHP][H_2O_2] - k_2[CmpI][AH] \]

\[ \frac{d[CmpII]}{dt} = k_2[CmpI][AH] - k_3[CmpII][AH] \]

\[ \frac{d[P]}{dt} = (k_2[CmpI] + k_3[CmpII])[AH] \]
Next we apply the steady state approximation to both the CmpI and CmpII intermediates.

\[ 0 \approx k_1[DHP][H_2O_2] - k_2[CmpI][AH] \]
\[ k_1[DHP][H_2O_2] \approx k_2[CmpI][AH] \]

and

\[ 0 \approx k_2[CmpI][AH] - k_3[CmpII][AH] \]
\[ k_2[CmpI][AH] \approx k_3[CmpII][AH] \]

Thus,

\[ [CmpI] \approx \frac{k_1[DHP][H_2O_2]}{k_2[AH]} \]

and

\[ [CmpII] \approx \frac{k_1[DHP][H_2O_2]}{k_3[AH]} \]

Substituting the equations for CmpI and CmpII back into the rate equation for product formation, we have,

\[ v_o \approx \frac{d[P]}{dt} = (k_2[CmpI] + k_3[CmpII])[AH] \]

\[ v_o \approx \left( k_2 \left[ \frac{k_1[DHP][H_2O_2]}{k_2[AH]} \right] + k_3 \left[ \frac{k_1[DHP][H_2O_2]}{k_3[AH]} \right] \right)[AH] \]

\[ v_o \approx k_1[DHP][H_2O_2] \]

For the peroxidase scheme presented above,

\[ [E]_o = [DHP] + [CmpI] + [CmpII] \]

Substituting the equations for CmpI and CmpII into the above expression, we have,

\[ [E]_o = [DHP] + \frac{k_1[DHP][H_2O_2]}{k_2[AH]} + \frac{k_1[DHP][H_2O_2]}{k_3[AH]} \]

\[ [E]_o = [DHP] \left( 1 + \frac{k_1[H_2O_2]}{k_2[AH]} + \frac{k_1[H_2O_2]}{k_3[AH]} \right) \]
\[ [E]_o = [DHP] \left( 1 + \frac{k_1[H_2O_2]}{[AH]} \left( \frac{1}{k_2} + \frac{1}{k_3} \right) \right) \]

For traditional Michaelis Menton kinetics,

\[ \frac{v_o}{[E]_o} = \frac{k_2[S]}{\frac{k_{-1} + k_2}{k_1} + [S]} \]

\[ v_o = \frac{k_2[E]_o[S]}{\frac{k_{-1} + k_2}{k_1} + [S]} \]

\[ v_o = \frac{V_{max}[S]}{K_M + [S]} \]

\[ V_{max} = k_2[E]_o \]

\[ K_M = \frac{k_{-1} + k_2}{k_1} \]

Mapping the peroxidase rate scheme onto the Michaelis Menton equation yields,

\[ \frac{v_o}{[E]_o} = \frac{k_1[DHP][H_2O_2]}{[DHP] \left( 1 + \frac{k_1[H_2O_2]}{[AH]} \left( \frac{1}{k_2} + \frac{1}{k_3} \right) \right)} \]

\[ \frac{v_o}{[E]_o} = \frac{k_1[H_2O_2]}{\left( 1 + \frac{k_1[H_2O_2]}{[AH]} \left( \frac{1}{k_2} + \frac{1}{k_3} \right) \right)} \]

\[ \frac{v_o}{[E]_o} = \frac{k_1[H_2O_2]}{\left( 1 + \frac{k_1[H_2O_2]}{[AH]} \left( \frac{1}{k_2} + \frac{1}{k_3} \right) \right)} \]
By analogy to the traditional Michaelis Menton equation,

\[ V_{\text{max}} = k_1[H_2O_2][E]_o \]

and

\[ K_M = \left( \frac{1}{k_2} + \frac{1}{k_3} \right) k_1[H_2O_2] \]

Thus,

\[ v_o = \frac{V_{\text{max}}[AH]}{(K_M + [AH])} \]

Here, [AH] is equivalent to [XAOH] from the main text of the manuscript.
APPENDIX E

Oxidative dechlorination of halogenated phenols catalyzed by two distinct enzymes: horseradish peroxidase and dehaloperoxidase
Abstract

The mechanism of the dehalogenation step catalyzed by dehaloperoxidase (DHP) from *Amphitrite ornata*, an unusual heme-containing protein with a globin fold and peroxidase activity, has remarkable similarity with that of the classical heme peroxidase, horseradish peroxidase (HRP). Based on quantum mechanical/molecular mechanical (QM/MM) modeling and experimentally determined chlorine kinetic isotope effects we have concluded that two sequential one electron oxidations of the halogenated phenol substrate leads to a cationic intermediate that strongly resembles a Meisenheimer intermediate – a commonly formed reactive complex during nucleophilic aromatic substitution reactions especially in the case of arenes carrying electron withdrawing groups.
Introduction

Application of enzymes in order to remove environmental and industrial contaminants has been studied for the last twenty years with increasing intensity as novel mechanisms are discovered in living organisms [1-7]. The reactions carried out using enzyme-based techniques are characterized by high efficiency and selectivity and are significantly more benign for the environment [8-10] when compared to purely chemical methods of environmental remediation.

One of the most important groups of pollutants in industrial wastewaters are chlorinated phenols. They originate primarily from the pulp and paper industries and are toxic carcinogens. In addition to their artificial origin, about 4000 organohalogenated compounds are produced biologically or by natural abiogenic processes such as volcanic eruptions, forest fires, and other geothermal processes [11]. Marine organisms such as seaweeds, sponges, corals, tunicates, and bacteria constitute the major biological producers of organohalogenated compounds, while terrestrial plants, fungi, lichen, bacteria, insects, and even some higher animals including humans produce a small fraction [12].

Thus taking into account the vast number of different sources of halogenated compounds, Nature has developed several mechanisms in order to reduce the levels of these toxic molecules. Many groups of enzymes are capable of degrading the halogenated compounds [13-15]. For example, hydrolytic dehalogenases replace the halogen with a hydroxyl group, reductive dehalogenases replace the halogen with a hydrogen, and oxidative dehalogenases exchange the halogen for oxygen with concomitant substrate oxidation (Scheme 1) [12].
Scheme E.1. Selected examples of enzymatic dehalogenation: (a) hydrolytic, (b) reductive, and (c) oxidative.

\[
\begin{align*}
R - X + H_2O & \rightarrow R - OH + H^+ + X^- \quad \text{(a)} \\
R_2 - C - X \quad \text{R}_1 + e^- & \rightarrow R_2 - C^\cdot + X^- \quad \text{(b)} \\
R_2 - C - X \quad \text{R}_1 & \rightarrow R_2 - C - X \quad \text{R}_1 + e^- \rightarrow R_2 - C = O + HX \quad \text{(c)}
\end{align*}
\]

Many mechanistic studies on selected hydrolytic dehalogenases have been presented [16-17], and even such a narrow class of enzymes shows a huge variety of possible pathways. Thus, research leading to detailed analysis of biological reactions is necessary in order to implement them more universally into detoxification processes and employ them on an industrial scale. However, enzymes as biocatalysts suffer from certain disadvantages (e.g. high costs of producing enzymes in large amounts, inactivation under specific conditions, possibility of utilizing them under very toxic environment, full characterization of all reaction products, etc.). Therefore, before they can be used efficiently, a number of studies are required in order to tune their performance accordingly. The enzymatic processes such as peroxidase- and phenol oxidase-catalyzed treatment of phenols and aromatic amines are likely the most comprehensively studied systems for waste treatment so far. Peroxidases present in plants, such as horseradish and soybean, and fungi, such as white-rot fungi, as well
as phenol oxidases from mushrooms (tyrosinase) and white-rot fungi (laccase) have all been found to have some potential for environmental applications, and some studies toward this direction have been undertaken [1-7,19-20]. Species living in environments where halogenated contaminants are found have been forced to develop appropriate protection pathways. One example of such a species is the terebellid polychaete *Amphitrite ornata*, a marine worm found to contain the dehalogenating enzyme, dehaloperoxidase (DHP). DHP is a dimeric hemoglobin that was identified as the active agent in the oxidation of the naturally occurring repellent, 2,4,6-tribromophenol (TBP). The repellents TBP, 2,4-dibromophenol (DBP) and 4-bromophenol (4-BP) are synthesized by a host of organisms in benthic ecosystems. The prevalence of TBP, DBP, and 4-BP in coastal wetland environments suggests that a natural regulation of their catabolism must exist.

Like horseradish peroxidase (HRP), DHP possesses a heme cofactor with a distal and proximal histidine on either side and catalyzes the oxidative dehalogenation of halogenated phenols to their corresponding quinones when activated by \( \text{H}_2\text{O}_2 \) (Scheme 2) [21-23]. The mechanism involves two consecutive one-electron transfers in order to oxidize organic haloderivatives similar to other heme-containing peroxidases including HRP. However, although the net reaction is considered to proceed according to the same mechanism, the enzyme intermediates (oxidizing species) formed during the reaction differ. In HRP, the reaction proceeds via the two enzyme intermediates, compound I and compound II (Scheme 3), whereas in DHP, the formation of a compound ES intermediate similar to the tryptophan cation radical found in cytochrome c peroxidase (CcP) has been reported [24]. While compound I of HRP is characterized by an oxoferryl heme with a porphyrin cation radical,
compound ES of DHP is characterized by an oxoferryl heme with a tyrosine cation radical [24]. Therefore, the two enzymes, a prototypical heme-containing peroxidase (HRP) and hemoglobin with significantly enhanced peroxidase activity (DHP), share a similar mechanism of oxidation but differ in the details of the electron transfer pathway between the substrate and heme iron.

The distal side of the heme near the active site of HRP is considered to be too narrow for organic substrates to enter and directly interact with the heme iron center. Instead, they bind at the heme edge where they are in contact with surrounding solvent water molecules that aid in dehalogenation. Unlike HRP, DHP has been shown to possess a well-defined binding pocket on the distal side of the heme large enough to accommodate mono-halogenated organic substrates [25-29]. This led to the hypothesis that oxidation of halogenated phenols occurred in the interior of DHP. While HRP can catalyze the oxidation of all known derivatives of halophenols (mono-, di-, and tri-), DHP is primarily active toward trihalophenols with only moderate activity toward dihalophenols and no activity toward monohalophenols. Further investigation of internal binding of monohalophenols led to the discovery that internal binding inhibits the peroxidase function of DHP [30]. Therefore, DHP must have at least two binding sites, the internal site on the distal side of the heme [31-32] and an external site similar to that of HRP where substrate oxidation occurs. Two possible external sites have been proposed on the basis of $^1$H$^{15}$N HSQC experiments on $^{13}$C/$^{15}$N labeled DHP; one at the external heme edge near the distal His55 similar to HRP, and one near the dimer interface [33]. Thus in the line with the previous studies of dehalogenating enzymes [16-17], we were very interested to check whether the
dehalogenation step itself is accompanied by interactions with these enzymes in any way. And if so, what are their mechanisms? For this purpose we have employed kinetic isotope effect analysis as their values that can be determined both experimentally and theoretically have already proven many times to be a successful tool in elucidating mechanisms of enzymatic reactions [35-37]. In this particular case, we have focused on chlorine kinetic isotope effects as they are a good probe to differentiate between alternative reaction pathways (i.e. water attack on a dissociable phenoxy radical vs. a cationic intermediate). Herein we present our efforts in describing the role of the two distinctive enzymes in the degradation process of organic pollutants like halogenated phenols. Our QM/MM results suggest that the reaction pathway involving the cationic intermediate is preferred for the dehalogenation reaction. To the best of our knowledge these results represent the only chlorine isotopic fractionation data available for dehalogenation reaction catalyzed by peroxidases.
Scheme E.2. The catalytic cycle of DHP with 2,4,6-trichlorophenol (TCP) as a substrate.
Scheme E.3. The catalytic cycle of HRP with TCP as a substrate.
Experimental Section

Reagents

Nitric acid (pure p.a.), silver nitrite (pure p.a.), ethanol (special pure), from POCh Gliwice, mercuric (II) thiocyanate (pure p.a., Fluka), ferric ammonium sulfate (L.P.P-H "OCh" Lublin, Poland), concentrated sulphuric acid (suprapur, Merck), 2,6-dimethyl-4-chlorophenol (DMCP) (97%, Acros Organics), 2,4,6-trichlorophenol (TCP) (98%), 4-chlorophenol (4-CP) (99+%), potassium phosphate dibasic (Reagent Grade ≥ 98%), potassium phosphate monobasic (Reagent Grade ≥ 99%), HRP, Type IV, from horseradish, sodium biphenyl reagent, solution in 2-ethoxyethyl ether, from Sigma-Aldrich, toluene (pure p.a.), n-hexane (pure p.a.) from Chempur were used without further purification.

Dehaloperoxidase – protein expression, purification and oxidation

Recombinant his-tagged wild type protein was expressed in E. coli and purified as previously described [28,33]. The isolated His-DHP was oxidized with excess of potassium ferricyanide, K₃[Fe(CN)₆] and buffer exchanged into 20 mM KH₂PO₄, pH 6 solution using a Sephadex G-25 column. The oxidized protein was loaded onto a CM 52 ion exchange column for further purification. It was washed on the column with 20 mM KH₂PO₄, pH-6 buffer and eluted from the column with 100 mM KH₂PO₄, pH 7 buffer. The concentration of the protein was determined using intensity of the Soret absorption at 406 nm and a molar absorptivity of 116400 M⁻¹ cm⁻¹.

Dehalogenation of TCP catalyzed by DHP and HRP

We have used a 5 mM solution of TCP in 100 mM potassium phosphate buffer at pH 7. Because of the low solubility of DMCP in pure water, a small amount of ethanol (1% v:v)
was added to the solution. The protein concentrations were 500 and ~260 μM for DHP and HRP, respectively. The reactions were initiated by addition of a calculated amount of H₂O₂ (assuming 1/1 stoichiometry for H₂O₂/substrate turnover) required to make the reaction proceed to ~20% and 100% for KIE analysis.

The progress of the reaction was monitored spectrophotometrically as shown in Fig. E.1.

**Dehalogenation of 4-CP catalyzed by HRP**

A 7.5 mM solution of 4-CP in 100 mM potassium phosphate buffer, pH 5.5 was used in the reaction with ~380 μM solution of HRP. We changed the pH conditions for the reaction with 4-CP based on study showing that optimum pH for p-chlorophenol is about 6 [34]. The reaction was initiated and controlled by accurate dosage of hydrogen peroxide (about 143 μmoles per 100 ml of the reaction mixture) via a homemade reaction apparatus. Reaction progress to ~20% and 100% for KIE analysis was also monitored spectrophotometrically.

**Dehalogenation of DMCP catalyzed by DHP**

Reaction of DHP with DMCP was conducted in the same way as that for DHP with TCP. We have used a 1.9 mM solution of 2,6-dimethyl-4-chlorophenol (DMCP) as an analog in 100 mM potassium phosphate buffer, pH 7. The concentration of DHP in each of the reactions was 300 μM. Again, the reactions were initiated by addition of a calculated amount of H₂O₂ required to make the reaction proceed to ~20% and 100% for KIE analysis. Preparation of this solution also required addition of ethanol due to low solubility in pure water.
Figure E.1. The example of UV-Vis monitoring of the substrate conversion catalyzed by DHP. Panel A shows the spectrum of TCP disappearance and panel B presents the dependence of the absorbance at 313 nm on the degree of substrate conversion. The reactions were conducted in 100 mM potassium phosphate buffer (pH 7.0) at 4°C.

In order to avoid accidental quinone product degradation, as was observed at higher temperatures [38], all reactions were carried out at 4°C.

**Samples preparation for Cl KIEs measurements**

After stopping the reaction at the desired reaction progress (19-20%), the mixture was filtered and silver chloride was precipitated from the solutions with 0.2 M AgNO₃ at room temperature. Next, the precipitates of AgCl were washed with water three times and were left to dry in a desiccator in the dark. Chlorine kinetic isotope effect was calculated from the following equation [39]:

\[
\frac{k_{35}}{k_{37}} = \frac{\ln(1-f)}{\ln(1-f_{R=0})}
\]  

(1)
where $f$ is the reaction progress, $R_f$ is the chlorine isotopic ratio of the product at the reaction progress $f$, and $R_\infty$ is the chlorine isotopic ratio of the completely converted chlorophenol. In the case of 4-CP, this ratio was determined using Lassigne method [40] based on the reaction of organic compound with metallic sodium, whereas in the case of TCP, the complete conversion by the enzyme was used. For DMCP, the Liggett method based on the reaction with sodium biphenyl [41] was applied. Chlorine isotopic ratios were measured using a hybrid FAB-IR mass spectrometer as described previously [42] with a modified sample support that results in reduced sample requirement. All of the measurements were repeated independently three times.

**Computational Methods**

**General Approach**

In order to approach the mechanistic details of the dehalogenation step catalyzed by DHP and HRP, the quantum mechanical/molecular mechanical (QM/MM) method implemented in QSite [43] has been used. The relaxed potential energy surfaces were modeled. The QM parts of these systems consisting of substrate molecule, either 4-chlorophenol (4-CP) or 2,4,6-trichlorophenol (TCP), and selected water molecules were treated with B3LYP [44-45] functional and lacvp* [46] basis set. Our choice of method for modeling the reactions of interest was the compromise between the methods which were found to be appropriate for heme-containing enzymes on one hand and able to predict chlorine isotope effects correctly on the other. Based on the data available in the literature [47-48] the combination of B3LYP with lacvp basis set seemed to be a good choice including open shell species [49]. On the
other hand the previous model studies of Fang et al. on chlorine KIEs on S_N2 reactions showed that both B1LYP and B3LYP functional can predict the values of KIEs correctly [50] and hence can be successfully used for modeling dehalogenation reaction. The MM part was simulated using the OPLS-AA force field. All QM/MM simulations have been performed in QSite. In order to include solvent initial models have been soaked in 40x40x40 Å water box and optimized to a minimum using the default convergence and optimization criteria. Calculations of KIEs were performed using ISOEFF98 program [51]. KIEs were obtained from the complete Bigeleisen equation [39] at 300 K for the transition from proximity complexes of both reactants to the corresponding transition states.

\[
\frac{k_1}{k_h} = \frac{\nu_h^\text{\#}}{\nu_h^\text{\#}} \prod_i \frac{\sinh \left( \frac{\hbar \nu_{i,\text{h}}^\text{\#}}{k_B T} \right) \sinh \left( \frac{\hbar \nu_{i,\text{h}}^\text{\#}}{k_B T} \right)}{\sinh \left( \frac{\hbar \nu_{i,\text{h}}}{k_B T} \right) \sinh \left( \frac{\hbar \nu_{i,\text{h}}}{k_B T} \right)}
\]

where \( u = \hbar \nu/k_BT \), \( h \) and \( k_B \) are Planck and Boltzmann constants, respectively, and \( T \) is the absolute temperature. Also, \( n \) is the number of atoms, and \( \nu_i \) are the frequencies of normal modes of vibrations. The superscript “\#” indicates the properties of the transition state.

To recognize the role of enzyme in the dechlorination step, three different forms of substrate (phenolate, phenoxy radical and cation) were considered, and the respective reactions in the water environment using the same QM/MM scheme with TIP3P water molecules as MM part representatives were modeled. Open shell species were treated using unrestricted Hartree-Fock (UHF) method [52]. Molecular geometries of all species were fully optimized and vibrational analysis was carried out to confirm identity of the stationary points (3n - 6 real vibrations in the case of reactants or products and one imaginary frequency corresponding to
the desired reaction coordinate in the case of transition state structures). The transition state
structures have been located along the lowest Hessian eigenvector using mixed Murtagh-
Saragant/Powell method [54] as implemented in Jaguar program [55]. The radical
intermediates, especially when 4-CP binds to enzymes, have a tendency to form dimers or
polymerize and either shut the reaction down or escape from the reaction site. So although
this route as the alternative to water molecule attack on a cationic form of the substrate
sounds rather unlikely, we have decided to test this pathway in our attempts on the
mechanism elucidation.

**Modeling TCP dehalogenation in water environment**

Each model studied consisted of a substrate molecule and 201 surrounding water molecules.
Altogether the model comprised 615 atoms. The solvent molecules were included by soaking
the solute molecule into the 18×18×18 Å water box. After initial minimization the solute and
the four nearest water molecules were defined as a QM part and were modeled using
B3LYP/lacvp* method in the subsequent QM/MM energy profile calculations. During each
step of the calculations, atoms beyond 8 Å from the solute molecule were frozen. The
reaction coordinate along which the energy scan was performed was defined as a decreasing
distance between the oxygen atom of the attacking water molecule and the *para* position
carbon of the substrate to which chlorine was attached. For the case of phenoxy radical and
cation pathways, attack of a water molecule at *ortho* position was also considered. The
default convergence and optimization criteria were applied.
**HRP catalyzed reaction**

A model for the dehalogenation reaction catalyzed by HRP was prepared based on the 1HCH X-ray structure [53]. The substrate molecule (4-CP) was docked to the active site based on the position of the first structure of a peroxidase-substrate complex demonstrating the existence of an aromatic binding pocket [56]. This particular enzyme-substrate complex seemed to be a good candidate for initial substrate position as the substrate, benzhydroamic acid is a similar size molecule. However, the 1HCH structure is of better resolution (1.57 Å vs 2.00 Å in case of this reference structure) thus we have decided to use it for our model preparation. After docking, the entire structure was preminimized using OPLS-AA force field [57-59] implemented in Impact program. Then, in order to evaluate which position (whether chlorine substituent or hydroxyl group facing the heme iron) is favorable, the interactions analysis between substrate molecule and surrounding residues has been performed. The following amino acids have been chosen: Phe179, Gln176, Gly69, Ala140, Leu138, Pro139, His42, Arg38, Phe142, Arg178, Phe143, Ser73, Phe41 and Pro141. Those were the residues found within 7 Å sphere from the substrate moiety. The interaction energies were calculated based on fully optimized structures where the substrate molecule, in either of the positions considered, was treated quantum mechanically in the surrounding of MM protein and water atoms. Next, the optimized geometries of respective substrate-amino acid pairs were taken out from the system and the single point gas phase energies using the same level of theory were calculated for a pair and separated components (100 Å away to assure the total decay of interactions). The more negative values, the larger the contribution to the stabilization of the substrate.
The pre-minimized system, with 4-CP in the proper orientation, was soaked into the 40×40×40 Å water box. The total model consisted of 7102 atoms, out of which 30 (4-CP plus 6 water molecules) were treated quantum mechanically. In an attempt to describe the dehalogenation reaction catalyzed by HRP at the molecular level, a reaction coordinate has been defined as the distance between the chlorinated carbon and oxygen from the attacking water molecule. Two alternative pathways have been taken into consideration: via radical and via cation. During the energy scan along the reaction coordinate, atoms beyond 15 Å from the substrate molecule were frozen.

**DHP catalyzed reaction**

For the case of DHP, although there are enzyme crystal structures available, the task of preparing the appropriate enzyme model for the reaction was not so straightforward, as the binding site of trihalophenols or any tri-substituted derivatives of phenol is not known and internally bound monohalogenated phenols were shown to act as inhibitors. Thus we have decided to explore the idea of external binding site which has been recently proposed [33]. For this purpose we have used the 2QFK structure [31] and the two possible binding sites from Davis et al. have been taken into consideration as shown in Fig. E.2. One of them presents a heme edge binding site whereas the other is located in the dimer interface. Models with the bound substrate molecule were prepared using QSite program. Water molecule was coordinated to the iron ion of heme. The resulting models comprised 2693 (heme edge) and 5308 (dimer interface) atoms, respectively. In order to estimate the binding energy of the substrate in each of the structures, we have also added solvent water molecules. We have used Poisson-Boltzmann (PBF) [60-62] continuum model, and in each case, the system was
optimized using the default convergence and optimization criteria. Only TCP molecule in its anionic form was defined as a QM part in the QM/MM simulation. DFT functional M06-2X [63-64] with lacvp* basis set was applied for QM calculations whereas the rest of the system was treated with OPLS-AA force field. We have decided to use M06-2X functional instead of B3LYP because it has been shown recently to be superior to the latter one in studying non-covalent interactions and calculating interaction energies [64]. In order to compare the two external binding sites on DHP with the internal distal site above the heme, we used the 1EWA X-ray structure of DHP [65] that has a 4-iodophenol molecule bound internally so we could estimate binding energies for both 4-CP and TCP. Then, using the optimized structures, the substrate molecule was removed from each of them by gradually elongating the distance between it and the rest of the system until the total energy of the system stopped changing. The binding energy was then estimated as the difference between the substrate-enzyme complex and the separated species, where the substrate was already separated by 100 Å.
Figure E.2. Two possible external binding sites of DHP: panel A – near the heme edge, panel B – dimer interface.

Results and Discussion

The values for chlorine kinetic isotope effects determined for 4-chlorophenol (4-CP) and 2,4,6-trichlorophenol (TCP) dehalogenation catalyzed by HRP and for TCP and 2,4-dimethyl-4-chlorophenol (DMCP) catalyzed by DHP are shown in Table E.1. Reaction of DHP with 4-CP was not possible because 4-CP acts as an inhibitor to DHP peroxidase.
activity (*vida infra*). Preliminary reactions of DHP with TCP suggested that all chlorine atoms could be removed which could affect the KIE results. For this reason, DMCP was selected as a second substrate for DHP in order to ensure only the *para*-halogen was extracted for KIE measurements.

The values clearly indicate that there is no kinetic isotope effect on the reaction rate during the studied reactions. Several factors may lead to such observations: 1) there is no isotopic fractionation on this particular step of the reaction, 2) there is isotopic fractionation but the dehalogenation step is masked by other steps of the overall reaction, 3) the mechanism of this step is different than one would expect for the reaction which seems to be a nucleophilic attack on aromatic carbon atom, or 4) dehalogenation occurs during a polymerization reaction as has been reported for other reactions catalyzed by peroxidases [66].

In order to accommodate different mechanistic hypotheses we have combined the experimentally determined values with theoretical prediction of isotope effects. Molecular modeling studies allowed us on one hand to get more detailed insight into the reaction mechanism and on the other helped us to estimate the role of the protein in the oxidation process under study.
Table E.1. Experimental Cl KIEs determined for different chlorophenols degradation catalyzed by HRP and DHP at 277 K.

<table>
<thead>
<tr>
<th></th>
<th>HRP</th>
<th>DHP</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCP</td>
<td>1.0006±0.0005</td>
<td>nd</td>
</tr>
<tr>
<td>TCP</td>
<td>0.9996±0.0002</td>
<td>1.0005±0.0001</td>
</tr>
<tr>
<td>DMCP</td>
<td>nd</td>
<td>0.9995±0.0004</td>
</tr>
</tbody>
</table>

TCP dehalogenation in water environment

The energy profiles for water attack at the aromatic carbon have been modeled, and transition state structures have been determined for the phenolate and radical species. The geometries of the saddle points and imaginary frequencies, as well as energetics and isotope effects, are presented in the Table E.2.

Table E.2. Calculated kinetic isotope effects and saddle point characteristics for dehalogenation of TCP in anionic and radical forms in water at 300 K

<table>
<thead>
<tr>
<th></th>
<th>phenolate</th>
<th>phenoxy radical</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>p-position</td>
<td>p-position</td>
</tr>
<tr>
<td>$^3$Cl KIE</td>
<td>1.0037</td>
<td>1.0085</td>
</tr>
<tr>
<td>$^{18}$O KIE</td>
<td>0.9998</td>
<td>0.9875</td>
</tr>
<tr>
<td>$^{13}$C KIE</td>
<td>1.0226</td>
<td>1.0364</td>
</tr>
<tr>
<td>Saddle point characteristics:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>geometry (Å):</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-Cl</td>
<td>1.866</td>
<td>1.981</td>
</tr>
<tr>
<td>C-O</td>
<td>1.740</td>
<td>1.666</td>
</tr>
<tr>
<td>O-H</td>
<td>1.021/0.974</td>
<td>1.025/1.017</td>
</tr>
<tr>
<td>Frequency (cm$^{-1}$)</td>
<td>-229</td>
<td>-353</td>
</tr>
<tr>
<td>Barrier (kcal/mol):</td>
<td></td>
<td></td>
</tr>
<tr>
<td>QM/MM</td>
<td>57.3</td>
<td>31.3</td>
</tr>
</tbody>
</table>
We have not calculated isotope effects for water attack at the *ortho* aromatic carbon position for the radical species, but judging from the geometry of the transition state, the KIE should not be different from the results obtained for the *para* position. For both the anion and radical species, significant water deprotonation has been observed during water attack which is reflected in both O-H distances and the inverse oxygen KIE. The high barrier heights for the phenolate and phenoxy radical pathways suggest that these two pathways are likely unfavorable for dechlorination. In fact, the unrealistic value of 57.3 kcal/mol for the phenolate pathway is expected since it is well known that oxidation of the phenolate form occurs prior to the dechlorination step.

However, in the case of the cationic form of the oxidized substrate, the overall picture is quite different. No transition state (TS) was found for the water attack as there was no barrier for this process at either the *ortho* or *para* positions. The system forms a kind of Meisenheimer complex (Mc) with a C-Cl bond length of 1.833/1.846 Å and C-O distance of 1.417/1.411 Å for *ortho*/*para* positions, respectively. The QM/MM energies for this process are -48.3/-28.7 kcal/mol for the *ortho* and *para* positions, and the lack of barrier for this process indicates that it is spontaneous. Comparison of the initial state with the formed stable complex reveals significant water deprotonation as shown in the example of the reaction at the *para* position in Fig. E.3. This deprotonation seems to occur just before the complex formation and may play a stabilizing role in the reaction.
Figure E.3. Overlaid structures of reactants shown in magenta and a Meisenheimer complex (Mc) in yellow. Numbers indicate O-H bond lengths in Å in an attacking water molecule.

Analysis of atomic charges derived from the electrostatic potential with monopoles located at the atomic centers [67-69] shows significant charge migration from the benzene ring of the substrate to the water molecules. In the reactants state, 0.87e was distributed within the aromatic ring whereas after Mc formation, this charge is redistributed among water molecules. The biggest charge changes are observed for C1 (from -0.44e in the reactant state to 0.00e in the Mc intermediate), C2 (from -0.44e to -0.28e), C5 (from 0.41e to 0.12e), and C6 (from -0.30e to 0.10e). The oxygen atom from the attacking water molecule gained 0.32e
and all of the chlorine atoms show negative charge buildup, in particular the departing one in the para position (Cl12) (atom numbering shown in Fig. E.4). This observed charge migration is accompanied by single and double bond changes within the benzene ring toward a quinone-like structure: bonds C1-C2 and C4-C5 get shortened by 0.03 and 0.02 Å, respectively, and bonds C6-C1 and C6-C5 get elongated by 0.08 and 0.07 Å, respectively. Thus, the calculated cationic pathway yields a charge redistribution and bond length changes that closely resemble the quinone product structure.

Starting from the Meisenheimer complex, we have further elongated the C-Cl bond to explore the nature of chlorine ion expulsion in order to elucidate the mechanistic pathway that leads to removal of the halogen substituents responsible for toxicity. In a water environment, two energetic maxima were observed. The first maximum, at about 7.6 kcal/mol, was recorded at a C-Cl bond length of 2.34 Å, but it could not be optimized into any transition state. Therefore, we concluded that it is most likely not related with any bond

Figure E.4. Atom numbering in TCP molecule.
breaking/forming act or such act proceeds via either very flat barrier or without any at all, as the majority of this barrier is in the MM term (4.4 kcal/mol) – Fig. E.5, panel A. The second maximum, with 14.9 kcal/mol at a C-\text{Cl} bond length of 3.5 Å, can likely be attributed to \text{Cl}⁻ complexation with one of the water molecules as illustrated in Fig. E.5, panel B, green structure.

Within the QM/MM scheme implemented in QSite the total energy of the system is calculated based on the following equation:

\[
E(\text{QM/MM})_{\text{tot}} = E(\text{QM}) + E(\text{MM}) + E(\text{QM/MM})_{\text{int}}
\]  

(3)

Where \(E(\text{QM})\) represents the gas phase energy of the QM subsystem, \(E(\text{MM})\) is the classical energy of the remaining part of the entire system, and \(E(\text{QM/MM})_{\text{int}}\) is the interaction energy between these two defined subsystems. Since only the total and the MM energy values are reported during QM/MM calculations in QSite, we have decided to explore the origin of the above findings. For this purpose the QM subsystem has been extracted, and its gas phase single point energy has been calculated using the same level of theory as in the full QM/MM calculations. Based on the obtained values, we could calculate the QM/MM interaction energy. All energy values obtained within this analysis for the two located maxima with respect to the initial state, which was the Meisenheimer complex, are listed in Table E.3. The QM gas phase energy differences should not be treated as the exact barrier heights for the theoretical gas phase process since they were not obtained based on optimization but rather on single point energies of the structures optimized using full QM/MM calculation. However, they can be treated along with derived interaction energies as a clear indication of strong stabilization of the formed product. These QM gas phase single point energies are heavily
suppressed by the QM/MM interactions which, as a result, give almost barrierless processes or processes with a very small, flat barrier.

### Table E.3.

Relative energies (kcal/mol) of maxima located during C-Cl elongation in a water environment with respect to the initial Meisenheimer complex.

<table>
<thead>
<tr>
<th></th>
<th>QM/\text{MM}_{\text{tot}}</th>
<th>MM</th>
<th>QM</th>
<th>QM/\text{MM}_{\text{int}}</th>
</tr>
</thead>
<tbody>
<tr>
<td>1$^{\text{st}}$ maximum</td>
<td>7.7</td>
<td>4.4</td>
<td>15.7</td>
<td>-12.4</td>
</tr>
<tr>
<td>2$^{\text{nd}}$ maximum</td>
<td>14.9</td>
<td>8.9</td>
<td>45.1</td>
<td>-39.1</td>
</tr>
</tbody>
</table>

### Figure E.5.

Snapshots of the intermediate structures obtained during elongating C-Cl bond in water. Panel A: three major structures related to the first observed maximum on the energy profile, panel B: three major structures related to the second observed maximum on the energy profile; in both cases, the starting point is shown in magenta, the maximum in orange and the first point right after it in green.
HRP-catalyzed dehalogenation

In order to evaluate which binding orientation (whether chlorine substituent or hydroxyl group facing the heme iron) is favorable, an interaction analysis between the 4-chlorophenol molecule and surrounding amino acid residues has been performed. The following amino acids have been chosen: Phe179, Gln176, Gly69, Ala140, Leu138, Pro139, His42, Arg38, Phe142, Arg178, Phe143, Ser73, Phe41 and Pro141. The calculated interaction energy for each substrate-AA pair is shown in the Fig. E.6. On the basis of the obtained values (-41.0 vs -32.9 kcal/mol for O→Fe and Cl→Fe position, respectively) it has been accepted that the orientation with the oxygen facing the heme iron will be more favorable and should be used in further mechanistic studies.
In order to provide mechanistic details of the dehalogenation reaction of 4-CP catalyzed by HRP, we have computed the energy profiles for the phenoxy radical and cationic pathways. Results for the phenoxy radical pathway of 4-CP catalyzed by HRP are presented in Table E.4. They are very similar to the results for the phenoxy radical pathway of TCP in a water environment. The chlorine KIE is significant for the phenoxy radical pathway, which is in disagreement with the experimentally observed value of 1.0006. The calculated value of 1.0074 is within the typical range of 1.006-1.008 [17] for chlorine KIEs with moderately elongated C-Cl bond length of about 0.3 Å. Although the experimental values were
determined at lower temperature, no significant change is expected at room temperature since no isotope effect was ever observed. Also KIEs calculated at 277 K (TCP in water and HRP-catalyzed 4-CP) do not show any significant difference. For the water mediated reaction, we obtained a KIE of 1.0092 at 4°C and for the HRP-catalyzed reaction, we obtained a KIE of 1.0080. Moreover, the energy barrier for the phenoxy radical pathway catalyzed by HRP is high when compared to the uncatalyzed reaction (31.3 kcal/mol). Thus we suspect that the disagreement of the KIEs and the high energy barrier are further indications that the phenoxy radical oxidation route is probably not adopted by peroxidases.

Table E.4. Calculated kinetic isotope effects and saddle point characteristics for dehalogenation of 4-CP in radical form catalyzed by HRP at 300 K.

<table>
<thead>
<tr>
<th></th>
<th>phenoxy radical</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{37}$Cl KIE</td>
<td>1.0074</td>
</tr>
<tr>
<td>$^{18}$O KIE</td>
<td>0.9860</td>
</tr>
<tr>
<td>$^{13}$C KIE</td>
<td>1.0379</td>
</tr>
<tr>
<td>Saddle point characteristics:</td>
<td></td>
</tr>
<tr>
<td>geometry (Å):</td>
<td></td>
</tr>
<tr>
<td>C-Cl</td>
<td>1.951</td>
</tr>
<tr>
<td>C-O</td>
<td>1.664</td>
</tr>
<tr>
<td>O-H</td>
<td>1.032/1.018</td>
</tr>
<tr>
<td>Frequency (cm$^{-1}$)</td>
<td>-370</td>
</tr>
<tr>
<td>Barrier (kcal/mol):</td>
<td></td>
</tr>
<tr>
<td>QM/MM</td>
<td>47.0</td>
</tr>
</tbody>
</table>
The formation of an enzyme Meisenheimer complex (EMc) has also been observed for the cationic pathway (Fig. E.7). A stabilization of -52 kcal/mol was observed for the cationic intermediate bound to the enzyme model relative to that in a water environment (-29 kcal/mol for TCP in water vs. -81 for 4-CP in the HRP active site). However, this additional stabilization did not introduce a barrier for this process, nor was there a calculated chlorine kinetic isotope effect. Thus, the calculation for the cationic pathway agrees with experiment.

We have also analyzed the interactions of the Meisenheimer complex with the neighboring residues in the active site and compared them with those seen for the enzyme-substrate complex. The results of such comparison are shown in Fig. E.8. It is evident that the enzyme stabilizes the EMc complex and such stabilization, by about 30 kcal/mol, is pronounced within both increasing positive interaction and decreasing negative interaction with residues that are interacting with 4-CP in a similar manner. While we are aware that such an analysis should be treated with care and only qualitatively, it definitely indicates to some important patterns during transformation of the ES into the EMc complex. The key geometrical parameters for the enzyme-substrate complex as well as the Meisenheimer complex are shown in Fig. E.7.
**Figure E.7.** The geometries of the enzyme-substrate (ES) and the enzyme-Meisenheimer complex (EMc) formed during the attack of water molecule on the cationic form of 4-CP in the active site of HRP. The numbers indicate the key geometrical parameters.
Further analysis of the EMc formation reveals similar observations as the ones for the Mc intermediate in water. An analogous charge migration has been observed from the aromatic ring to the surrounding water molecules. The only difference is the charge redistribution among the accompanying water molecules. For the uncatalyzed reaction, most of the charge is transferred to the oxygen of the attacking water, whereas for the catalyzed reaction, the charge from the benzene ring is moved and redistributed on at least three water molecules, forming a hydrogen-bonded, proton donor-acceptor chain.

The subsequent exploration of Cl\textsuperscript{-} departure revealed a lack of a barrier for this process as well, which suggests that it may either happen spontaneously or via a very small barrier. The most important structures related to chlorine expulsion are shown in Fig. E.9. By analogy to the reaction studied in water environment, we also performed a similar analysis of all the energy terms for this observed maximum. With respect to the Meisenheimer complex, the maximum at 2.89 Å is characterized by the total QM/MM energy of 22 kcal/mol out of which 15.5 kcal/mol is the classical energy, 19.8 kcal/mol is the QM gas phase single point energy and the interaction energy between the QM and MM subsystems is -13.3 kcal/mol.
Figure E.8. The comparison of interaction energies between bound 4-chlorophenol (S) and formed Meisenheimer complex (Mc) and the neighboring residues in the active site of horseradish peroxidase. Red and blue bars denote substrate- and Meisenheimer complex-aminoacid interaction energy, respectively.
Figure E.9. Overlaid structures obtained on C-Cl elongation pathway for MCP catalyzed by HRP. EMc complex is shown in green, the maximum on the energy profile in orange and the final – product structure in magenta.

DHP-catalyzed dehalogenation

Recently, Thompson et al. [30] reported a unique two-site competitive binding mechanism in DHP in which 4-CP and TCP compete for an internal and an external binding site in/on DHP, respectively. Binding of each molecule to its respective site produces a conformational change in the distal His55 amino acid. Internal binding of 4-CP expels the iron-coordinated water molecule and pushes His55 to a solvent exposed open conformation resulting in a 5-coordinate high spin heme iron. Thus internal binding of 4-CP blocks access to the heme iron and disrupts the peroxidase acid/base catalyst. This is in fact the mechanism by which 4-CP inhibits the peroxidase activity of DHP and why we were unable to use 4-CP for KIE analysis. External binding of TCP pushes His55 into a closed conformation resulting
in a predominately 6-coordinate high spin heme and positioning His55 to serve as the peroxidase acid/base catalyst. Thus external binding primes peroxidase function.

In order to explore the idea of an external binding site, the 2QFK structure was used with His55 in its closed confirmation. Two models comprising two possible external binding sites, obtained from locations presented by Davis et al. [33], were prepared and compared to internal binding in the distal pocket of the heme. Using the M06-2X/OPLS-AA calculation scheme implemented in QSite, we have estimated the binding energy for each of the sites. We decided to performed the analysis using a newly (at that time) developed functional (M06-2X) as it was recommended to be more appropriate for nonconvalent interactions [64]. The results are presented in Fig. E.10.

![Graph showing binding energies for MCP and TCP in different sites of DHP.](image)

**Figure E.10.** The estimated binding energies for MCP and TCP bound in the different sites of DHP.
The discrimination between monohalophenols and trihalophenols in the distal pocket is clearly evident, which is likely a partial confirmation of the inhibiting properties of the former.

Although, on the basis of the obtained data, it is not possible to differentiate between the two speculated external binding sites as this issue requires further exploration using different techniques, we have decided to use the heme edge site, in agreement with HRP mechanism, in order to model the solvent water attack on the cationic form of the substrate. We have prepared the appropriate model from the 2QFK structure consisting of almost 14000 atoms in an analogous way as we did for the case of HRP. 21 atoms were assigned to the QM part of the system: TCP plus three water molecules. The reaction coordinate was defined in the same way as previously. Again, a stable cationic intermediate formation was observed without any barrier at a C-O distance of 1.4 Å and a C-Cl bond length of 1.81 Å. The QM/MM energy of this complex is -68.5 kcal/mol compared to the starting enzyme-substrate complex. Further exploration of the nature of this stabilization as compared to uncatalyzed and HRP-catalyzed reactions revealed some differences between the two enzymes. An all energy terms analysis showed that, in case of HRP, the stabilization of the QM subsystem is expressed in both the QM and QM/MM\textsubscript{int} energies whereas in the case of DHP, although the sum of these two terms gives almost the same value, it differs significantly in the contribution of each of the terms as illustrated in Fig. E.11. However, this observation should be treated with care and can only be discussed at the qualitative level since in the case of DHP it is a preliminary result, and it is based on a speculative binding site. Despite these obvious limitations, this finding, if confirmed, can be another indication of
the pattern observed so far for oxidoreductase-catalyzed dehalogenation. Two studied enzymes, a classical peroxidase and a globin with enhanced peroxidase activity, show the same action on halogenated substrates. During the initial steps of their reaction via two single electron transfer events, they prepare the organic substrate to be ready for chloride ion expulsion which then seems to happen without any energetic cost to the protein. The intermediate formed along the reaction pathway resembles the so called Meisenheimer complex which has already been reported for aliphatic dehalogenases, e.g. for the reaction catalyzed by 4-chlorobenzoyl-CoA dehalogenase [70-71].

**Figure E.11.** Relative energies (kcal/mol) of Mc complex formed in water, DHP and HRP with respect to the corresponding substrate (in cation form) state.
The QM/MM calculations for chlorophenol dehalogenation strongly suggest that the cationic pathway is the most favorable for chlorine expulsion. The results are in reasonable agreement with a van’t Hoff analysis of experimental Michalis-Menton kinetics of DHP catalyzed TCP oxidation that showed an overall activation energy of 13.4 kcal/mol [38]. Although the calculated energetics cannot be compared directly to the experimental activation energy as the latter one comprises all the steps from substrate binding to the product release, the mechanism description obtained within modeling should be easily accommodated within the scenario drawn based on this experimental kinetic data.

Conclusions

In the presented study we have investigated the catalytic power of two enzymes of different origin but having very similar activity toward halophenols. Using a complementary approach of kinetic isotope effects and QM/MM calculations, a picture of the mechanism of removing halogens by this group of enzymes, peroxidases, can be drawn. The obtained results allow us to make a working hypothesis that the peroxidase activity of the presented enzymes is mostly focused on a transformation of the toxic halogenated substrate into a cationic intermediate form which can easily expel a halogen substituent to form a lesser toxic compound. For both HRP and DHP, the dehalogenation reaction takes place at an external site, and the QM/MM calculations suggest that the HRP- and DHP-catalyzed dehalogenation reactions happen in a very similar fashion as the same reaction in solution without the enzyme. The methodology used in the presented study clearly indicates that the
dehalogenation reactions catalyzed by these enzymes are side reactions or other reaction channels initiated by electron transfer that do not demand much effort from the enzyme. Biocatalysts functioning in this way might be useful tools in the fight against halogenated contaminants, especially when their range of reactivity has a tendency to expand, and there are examples of multifunctional enzymes such as DHP, C. fumage chloroperoxidase [72-73], and lignin and manganese peroxidase [15] that have dehalogenating activity in addition to other basic functions.

Acknowledgements

This work was done in collaboration with Stefan Franzen (NCSU), Lukasz Szatkowski (Technical University of Łódź, Poland), Rafal Kaminski (Technical University of Łódź, Poland), and Agnieszka Dybala-Defratyka (Technical University of Łódź, Poland) and was the subject of a publication, L. Szatkowski, M. K. Thompson, R. Kaminski, S. Franzen, and A. Dybala-Defratyka. Arch Biochem Biophys, 2010, 505:22-32. M.K.T. was supported by Army Research Office grant 52278-LS.
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235


237


APPENDIX F

Supporting Information for Inhibition Chapter 3

Internal binding of halogenated phenols in dehaloperoxidase-hemoglobin inhibits peroxidase function
Figure F.1. Electronic absorption spectra of (black) WT-DHP and DHP with (yellow) phenol, (purple) 4-FP, (blue) 4-CP, (green) 4-BP, and (red) 4-IP. The Soret maximum undergoes a systematic blue shift as the substrate halogen is changed and follows the halogen series. The blue shift and a lowered extinction coefficient of the Soret are consistent with a shift from 6cHS to 5cHS heme. For the UV-vis spectroscopic data presented here, DHP concentration was 8 µM and the final concentration of 4-XP was 1 mM (maximum solubility) for 4-IP and 8 mM for 4-BP, 4-CP, 4-FP, and phenol in 150 mM potassium phosphate buffer, pH 6.
Figure F.2. SVD component analysis. a) Column eigenvectors of SVD showing the grand mean (U1) of the data set and first difference eigenvector (U2) plotted versus wavenumber. The first difference spectrum indicates which peaks shift during the substrate titration. b) Row eigenvectors of SVD displaying well-defined curves corresponding to each of the two column eigenvectors. The VT2 row vector corresponds to the intensity changes and peak shifts of U2 and is used to establish the apparent dissociation constant.
Figure F.3. Electronic absorption spectra of WT-DHP (black), DHP with 2,4,6-TBP (green), DHP with 2,4,6-TCP (blue), and DHP with 2,4,6-TFP (red). The Soret band narrows and the CT1 band blue shifts upon the addition of 2,4,6-TBP and 2,4,6-TCP, consistent with conversion to aquo-6cHS heme. The blue shift of the Soret band upon addition of 2,4,6-TFP can be attributed to the 5cHS population observed in the Raman spectrum. The final concentration of 2,4,6-TBP was 200 µM, and the final concentrations of 2,4,6-TCP and 2,4,6-TFP were 4 mM in 150 mM potassium phosphate buffer, pH 6.
Figure F.4. Resonance Raman spectrum DHP with 3 mM 2,4,6-TCP only (red), DHP with 3 mM 2,4,6-TCP followed by the addition of 5 mM 4-BP (lower black), DHP with 5 mM 4-BP followed by the addition of 3 mM 2,4,6-TCP (upper black), and DHP with 5 mM 4-BP only (blue). The two black spectra, obtained by adding either the substrate or the inhibitor first to DHP, are identical, indicating that the substrate and the inhibitor are in equilibrium with the enzyme. Spectra were obtained in 150 mM potassium phosphate buffer, pH 6. Excitation wavelength was 406 nm; 1.7 cm\(^{-1}\) resolution; laser power at the sample 60mW, and 300 s acquisition times.
**Figure F.5.** Time-dependent UV-vis spectra from 0 seconds (red) to 120 seconds (purple). Assay conditions were 2.4 μM DHP, 250 μM 4-FP, and 240 μM H₂O₂ in 100 mM potassium phosphate buffer at pH 7. Unlike 4-IP, 4-BP, and 4-CP, 4-FP has a low affinity to bind internally in DHP and is clearly oxidized to 1,4-benzoquinone.
Figure F.6. Time-dependent UV-vis spectra from 0 seconds (blue) to 120 seconds (teal). Assay conditions were 2.4 μM DHP, 240 μM H₂O₂, and 120 μM 2,4,6-TBP, pH 7 without (a) and with (b) addition of 240 μM 4-BP. (a) In the absence of 4-BP, the substrate (316 nm) is converted to product (291 nm). (b) In the presence of 4-BP, little product is formed with no significant decrease in the substrate band.
Figure F.7. (a) X-ray crystal structure of the heme active site showing hydrophobic amino acid residues surrounding the distal cavity (PDB 3FNW) (b) The location of the substrate halogen is analogous to the Xe4 binding site in sperm whale met-Mb (1).
Figure F.8. X-ray crystal structure of the DHP monomer (PDB 2QFK). Blue residues are the distal pocket residues affected by internal binding of the inhibitor (see Figure F.7). Green residues are the dimer interface residues affected by substrate binding. Highlighted residues are based on $^1$H-$^{15}$N HSQC experiments of Davis et al (2).
Figure F.9. (a and b) Time-dependent UV-vis spectra from 0 seconds (red) to 120 seconds (purple). Assay conditions were 2.4 μM DHP, 250 μM 2,4,6-TFP, and 240 μM H₂O₂ in 100 mM potassium phosphate buffer at pH 7 (a) and 2.4 μM DHP, 250 μM 2,4,6-TFP, 250 μM 2,4,6-TCP, and 240 μM H₂O₂ in 100 mM potassium phosphate buffer at pH 7 (b). Time traces of product formation (c).
References


APPENDIX G

Supporting Information for Chapter 4

Compound ES of dehaloperoxidase decays via two alternative pathways depending on
the conformation of the distal histidine
SI. Methods. 1. The apparatus for freeze-quenching EPR samples on a rotating cold surface

Figure G.1. The freeze-quench attachment to the Update Instrument apparatus. The insets show the top view (with the chamber lid up), the bottom view (detached from the base) and the chamber lid.

The chamber (1) with the sample collection rotating disk (2) is filled with liquid nitrogen from the supply Dewar (3); the process is controlled by the solenoid valve (4) operated by the temperature sensor in the chamber. The chamber is sealed by the lid (5) with the sliding door (6) to the disk access opening (7). Continuous evacuation of the chamber creates a pressure of 0.2 atm inside, when the chamber is at room temperature, and prevents
from the pressure building up above 1 atm, when liquid nitrogen is supplied. Vacuum is maintained by the vacuum regulator solenoid valve (equipped with a heater (8)) and the pressure sensor. The stepper motor (9), is equipped with a stepper motor controller to ensure a smooth running at low frequencies. The motor rotates the sample collection disk with a high precision (e.g. 1 turn per ejection time). The seals between the chamber and rotating shaft (10) prevent the liquid nitrogen in the chamber from leaking. The part of the shaft that is outside the chamber is permanently heated to 40°C to avoid water condensation, which otherwise would immediately got frozen and would block the motor once it is stopped. The process of cooling the chamber with the sample collection disk, under continuous pumping, takes not more than 2 minutes. During this time the chamber is kept away from the nozzle of the Update Instrument mixing apparatus, in order to keep the latter warm. Once ready to execute, the whole apparatus is turned around the stand (11) so that the disk access opening is found exactly under the ejection nozzle. The apparatus is then lifted 3 mm up, using a lever, to make a seal between the disk access opening seal (12) and the nozzle of the Update Instrument (the ‘rotate+lift’ mechanism). The sliding door is then open and the shot is executed.

SI. Methods.2. Correction for variable length of EPR samples

A very small sample (a tiny piece of burnt match which gives a strong free radical EPR signal) was placed at the bottom of an EPR tube. The dependence was measured of the EPR signal intensity of this ‘point’ sample as function of the vertical position of the bottom of the EPR tube in the resonator (Fig. G.2)
Figure G.2. The EPR signal intensity of a ‘point’ sample as function of the sample vertical position in the resonator SP9703 (Bruker).

The dependence shown in Fig. G.2 was integrated on an array of different ranges, and the integral value for the range of 0 – 32 mm was set to unity (Fig. G.3). Thus the coefficients by which EPR signal intensity should be divided to account for variable length (sample size) have been obtained.
Figure G.3. Correction factors for EPR samples shorter that the active zone of the resonator SP9703, which has been found to be 32 mm.

By using this dependence, we can now make correction for the EPR signals obtained from a sample which is shorter that the active zone of the resonator. For example, if an EPR sample is 17 mm long, the experimentally measured EPR signal should be divided by the value of 0.8 (see the graph above).
Figure G.4 gives an example of the degree to which the correction procedure can affect the raw data.

![Graph of FR concentration over time](image)

**Figure G.4.** An experimentally obtained kinetic dependence of free radical concentration before (top) and after (bottom) applying the procedure of EPR signal intensity correction for variable size of EPR samples.
SI.Methods.3. Shifting the EPR spectra along the magnetic field axis in accordance with the microwave frequency used to record the spectra

Figure G.5. Free radical EPR spectra of DHP reacting with H₂O₂ as recorded in six different samples (freeze-quenched). A – spectra as measured; B – the same spectra after each one was shifted to the left or to the right in accordance with the microwave frequency used to record each spectrum.
Figure G.6. Free radical EPR spectra of the reaction mixtures of 40 μM DHP + 120 μM H₂O₂ (final concentrations) at three different pH values freeze-quenched at the time indicated. DHP was freshly re-oxidised and cleaned of ferricianide for each of the pH value series. One cryotube of stock H₂O₂ solution (see section 2.3) was used for a session of making not more than 3-4 samples. All the spectra are presented normalised to the same intensity to highlight the lineshape change over the reaction time as well as differences in the shape at different pH values. The spectra were recorded at 10 K, the microwave frequency ν_MW was slightly different for different EPR samples and was within the range of 9.469-9.473 GHz. All spectra were shifted along the magnetic field axis to correspond to some standard frequency (9.472169 GHz) as described in SI. Methods.3. Other instrumental conditions were; microwave power P = 0.05 mW, modulation frequency ν_m = 100 kHz, modulation amplitude A_m = 3 G, time constant τ = 82 ms, scan rate ν = 0.596 G s⁻¹, number of scans per spectrum NS = 1.
Figure G.7. The $g=6$ region of the EPR signals of the two rhombic HS ferric heme forms of DHP. Similar spectra were obtained for slow-freeze and RFQ samples. Presented here is the result of spectral deconvolution of the slow-freeze samples, i.e. when a reaction mixture was placed in an EPR tube, and the tube was immersed in methanol kept on dry ice. The dotted line is the rhombic form (type 1 high spin form or $R_1$HS) detected in the resting DHP (control). The continuous line is the rhombic form detected in the samples treated with $H_2O_2$ (type 2 high spin form or $R_2$HS).
The control spectrum (dotted line) was obtained as the following spectral algebra: \( \text{(average of two independent samples of 60} \ \mu\text{M DHP, pH 7)} - \ 1.6 \times \text{(average of two independent samples of 30} \ \mu\text{M DHP, pH 7)} \). The reason for this subtraction is in the fact that the two averaged spectra contained different proportions of the rhombic and axial HS ferric heme forms.

The spectrum of DHP treated with \( \text{H}_2\text{O}_2 \) (continuous line) was obtained as a difference of two spectra A and B, \( \text{(Spectrum A)} - 0.38 \times \text{(Spectrum B)} \), where Spectrum A is an average spectrum of four slow-freeze samples:

\[
\begin{align*}
30 \ \mu\text{M DHP} + 30 \ \mu\text{M H}_2\text{O}_2 + 15 \ \text{s} \\
30 \ \mu\text{M DHP} + 30 \ \mu\text{M H}_2\text{O}_2 + 45 \ \text{s} \\
60 \ \mu\text{M DHP} + 60 \ \mu\text{M H}_2\text{O}_2 + 15 \ \text{s} \\
60 \ \mu\text{M DHP} + 60 \ \mu\text{M H}_2\text{O}_2 + 45 \ \text{s},
\end{align*}
\]

and Spectrum B, is the axial HS ferric heme in the control obtained as: \( \text{(average of two independent samples of 30} \ \mu\text{M DHP, pH 7)} - 0.36 \text{(average of two independent samples of 60} \ \mu\text{M DHP, pH 7)} \).
Figure G.8. HPLC of dehaloperoxidase before and after reaction with hydrogen peroxide measured at 280 nm (blue line) and 400 nm (red line). (A) HPLC chromatogram of dehaloperoxidase (80 µM). Acidic solvents used for HPLC analysis causes the separate elution of the heme moiety (peak i) and the apoprotein (peak ii). (B) Heme and protein oxidation products from the reaction between dehaloperoxidase (80 µM) and H₂O₂ (160 µM) at pH 5. Here peaks i and ii are decreased due to oxidative modifications that include heme to protein cross-linking (peak iii) and other oxidatively modified heme not covalently bound to the protein (peaks iv).
Figure G.9. Optical properties of heme to protein cross-linked species (blue line), unmodified heme (red line), oxidatively modified free hemes (green and purple lines). Spectra were taken from the chromatogram in Figure G.8, normalized with respect to the Soret peak and offset for clarity. Both unmodified and heme to protein cross-linked hemes have a typical ‘heme b’ spectra, corresponding to iron-protoporphyrin-IX, whereas the oxidatively modified hemes have a 720 nm band, characteristic of a ‘heme d’ chlorine heme spectra where one of the pyrrol rings is converted to a pyrroline.
Table G.1. The TRSSA generated parameters used to simulate spectrum B in Fig. 4.6 (*principal tyrosyl radical)*

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<thead>
<tr>
<th>gx</th>
<th>gy</th>
<th>gz</th>
</tr>
</thead>
<tbody>
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<td>2.00424</td>
<td>2.00220</td>
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Euler angle

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<th>Aβ1z, MHz</th>
<th>φβ1, degree</th>
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<table>
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<table>
<thead>
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<table>
<thead>
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<td>-22.0</td>
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<thead>
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<th>AC2z, MHz</th>
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<table>
<thead>
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<tbody>
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<table>
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<td>4.93</td>
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* TRSSA input: rC1 = 0.400 and θ = 45°; cAm = -0.3 G; Angle between β-protons = 118°
Table G.2. The TRSSA generated EPR simulation parameters used to simulate spectrum C in Fig. 4.6 (pH 5 tyrosyl radical)**

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<th>$A^{i_2}z$, MHz</th>
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<table>
<thead>
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<th>$A^{C_3}y$, MHz</th>
<th>$A^{C_3}z$, MHz</th>
<th>$\phi^{C_3}$, degree</th>
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<tbody>
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<table>
<thead>
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<th>$A^{C_5}y$, MHz</th>
<th>$A^{C_5}z$, MHz</th>
<th>$\phi^{C_5}$, degree</th>
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<tbody>
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<table>
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<th>$A^{C_2}y$, MHz</th>
<th>$A^{C_2}z$, MHz</th>
<th>$\phi^{C_2}$, degree</th>
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<tbody>
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<td>45.0</td>
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<table>
<thead>
<tr>
<th>$A^{C_6}x$, MHz</th>
<th>$A^{C_6}y$, MHz</th>
<th>$A^{C_6}z$, MHz</th>
<th>$\phi^{C_6}$, degree</th>
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<tbody>
<tr>
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<td>7.5</td>
<td>1.5</td>
<td>-45.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>$\Delta H_x$, G</th>
<th>$\Delta H_y$, G</th>
<th>$\Delta H_z$, G</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.38</td>
<td>5.62</td>
<td>5.20</td>
</tr>
</tbody>
</table>

** TRSSA input: $\rho_{C_1} = 0.420$ and $\theta = -8^\circ$; $c_{Am} = -0.666$ G; Angle between $\beta$-protons = $122^\circ$
SI.G. Results.2. Assignment of the principal and the pH 5 radicals to tyrosine residues in DHP

The following analysis was facilitated by use of the website “Tyrosine residues in different proteins: Phenol ring rotation angle database” (http://privatewww.essex.ac.uk/~svist/lev1/tyrdb/home.shtml).

The EPR signal of the principal tyrosyl radical in DHP has been simulated (Fig. 4.6) for the value of the phenoxy ring rotation angle \( \theta \) being either 45° or 75° (Table 4.1). TRSSA yields identical sets of simulation parameters for these two angles, as explained in Fig. G.10.

**Figure G.10.** Two rotational conformations of the phenoxy ring in a tyrosyl radical with \( \theta = 45^\circ \) and \( \theta = 75^\circ \) (the sum of these complementing angles is 120°) have the same values of the hyperfine splitting constant for the two methylene protons.

Therefore we have to analyse the proximity of the ring rotation angle in different tyrosines to both 45° and 75°. There are 5 tyrosines in each of the two subunits of the DHP structure file 1EW6.
When the target angle is set to $\theta_{\text{target}} = 45^\circ$ and the search results are sorted by the angle difference $|\theta - \theta_{\text{target}}|^\circ$ (ascending), Tyr28 and Tyr34 are the best candidates (the differences with the target angle are all less than 4°):

| Tyrosine sort by chain by number | $\theta ^\circ$ sort | $|\theta - \theta_{\text{target}}|^\circ$ sorted |
|----------------------------------|---------------------|-----------------------------------------------|
| B Tyr28                          | 44.945              | 0.1                                           |
| B Tyr34                          | 47.08               | 2.1                                           |
| A Tyr34                          | 41.42               | 3.6                                           |
| A Tyr28                          | 41.24               | 3.8                                           |
| A Tyr16                          | 36.155              | 8.8                                           |
| B Tyr16                          | 28.415              | 16.6                                          |
| A Tyr107                         | 62.15               | 17.2                                          |
| B Tyr107                         | 65.66               | 20.7                                          |
| A Tyr38                          | -16.515             | 61.5                                          |
| B Tyr38                          | -16.935             | 61.9                                          |

For the target angle set to $\theta_{\text{target}} = 75^\circ$, Tyr107 in the two chains have the closest $\theta$-values:

| Tyrosine sort by chain by number | $\theta ^\circ$ sort | $|\theta - \theta_{\text{target}}|^\circ$ sorted |
|----------------------------------|---------------------|-----------------------------------------------|
| B Tyr107                         | 65.66               | 9.3                                           |
| A Tyr107                         | 62.15               | 12.9                                          |
| B Tyr34                          | 47.08               | 27.9                                          |
| B Tyr28                          | 44.945              | 30.1                                          |
| A Tyr34                          | 41.42               | 33.6                                          |
| A Tyr28                          | 41.24               | 33.8                                          |
| A Tyr16                          | 36.155              | 38.8                                          |
| B Tyr16                          | 28.415              | 46.6                                          |
| A Tyr38                          | -16.515             | 91.5                                          |
| B Tyr38                          | -16.935             | 91.9                                          |

However, the closeness of $\theta$ in Tyr107 to $75^\circ$ ($9^\circ$ and $13^\circ$ in B- and A-chains respectively) is far worse than the closeness of $\theta$ in Tyr28 and Tyr34 to $45^\circ$. So we discard Tyr107 as a
possible candidate and consider Tyr28 and Tyr34 to be the most likely sites to host the principal tyrosyl radical in DHP.

The EPR signal of the pH 5 tyrosyl radical has been simulated for $\theta = -8^\circ$ or $-52^\circ$ (Fig. 4.6 and Table 4.1). TRSSA generates the same set of simulation parameters for these angles, as Fig. G.11 illustrates.

**Figure G.11.** Two rotational conformations of the phenoxyl ring in a tyrosyl radical with $\theta = -52^\circ$ and $\theta = -8^\circ$ (the sum of these complementing angles is $-60^\circ$) have the same values of the hyperfine splitting constant for the two methylene protons.
The database search result for $\theta_{\text{target}} = -8^\circ$ is as follows:

| Tyrosine sort | $\theta$ $^\circ$ sort | $|\theta - \theta_{\text{target}}|$ $^\circ$ sorted |
|--------------|-------------------------|----------------------------------|
| A Tyr38      | -16.515                 | 8.5                              |
| B Tyr38      | -16.935                 | 8.9                              |
| B Tyr16      | 28.415                  | 36.4                             |
| A Tyr16      | 36.155                  | 44.2                             |
| A Tyr28      | 41.24                   | 49.2                             |
| A Tyr34      | 41.42                   | 49.4                             |
| B Tyr28      | 44.945                  | 52.9                             |
| B Tyr34      | 47.08                   | 55.1                             |
| A Tyr107     | 62.15                   | 70.2                             |
| B Tyr107     | 65.66                   | 73.7                             |

And the search for $\theta_{\text{target}} = -52^\circ$ shows that no tyrosine in both DHP subunits has the ring rotation angle $\theta$ closer than $35^\circ$ to the target, even though Tyr38 comes up at the top of the table again:

| Tyrosine sort | $\theta$ $^\circ$ sort | $|\theta - \theta_{\text{target}}|$ $^\circ$ sorted |
|--------------|-------------------------|----------------------------------|
| B Tyr38      | -16.935                 | 35.1                             |
| A Tyr38      | -16.515                 | 35.5                             |
| B Tyr16      | 28.415                  | 80.4                             |
| A Tyr16      | 36.155                  | 88.2                             |
| A Tyr28      | 41.24                   | 93.2                             |
| A Tyr34      | 41.42                   | 93.4                             |
| B Tyr28      | 44.945                  | 96.9                             |
| B Tyr34      | 47.08                   | 99.1                             |
| A Tyr107     | 62.15                   | 114.2                            |
| B Tyr107     | 65.66                   | 117.7                            |

In conclusion, the principal radical is most likely located on Tyr28 or Tyr34. The site of the pH 5 radical is most likely to be Tyr38 with any alternative being far less likely.
SI.G. Results.3. Kinetic model description

The following kinetic model was used in order to simulate experimentally determined kinetic dependences of the ferric heme state and free radical concentrations on reaction time.

Table G.3. Six reaction components participating in the reactions.

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O₂</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>Fe(III)</td>
<td>DHP with the heme iron in the ferric state and no radicals on the polypeptide moiety of the enzyme</td>
</tr>
<tr>
<td>Fe(IV)</td>
<td>DHP with the heme iron in the ferryl state and no radicals on the polypeptide moiety of the enzyme</td>
</tr>
<tr>
<td>•Fe(III)</td>
<td>DHP with the heme iron in the ferric state and a radical on the protein moiety of the enzyme; location of the radical on DHP is not important</td>
</tr>
<tr>
<td>•Fe(III)•</td>
<td>Ferric DHP bi-radical - DHP with the heme iron in the ferric state and two radicals on the protein moiety of enzyme; location of the two radicals on DHP is not important</td>
</tr>
<tr>
<td>•Fe(IV)</td>
<td>DHP with heme iron in the ferryl state and a radical on the protein moiety of enzyme; as before, the location of the radical is not important, as far as the model is concerned</td>
</tr>
</tbody>
</table>
Table G.4. Ten reactions considered in the model.

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Description and assumptions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe(III) + H₂O₂ → •Fe(IV)</td>
<td>$(k_1)$ Formation of Compound ES – the ferryl heme DHP with a Tyr34 radical. It is assumed that this reaction only takes place if the distal histidine His55 is in the closed conformation. Since the populations of the ‘open’ and ‘closed’ conformations are pH dependent, $k_1$ should be also pH dependent: $k_1$ should be higher at the higher pH values, when the partial population of the closed conformation is increased.</td>
</tr>
<tr>
<td>•Fe(IV) → Fe(III)</td>
<td>$(k_2)$ First order decay of Compound ES to Compound RH - the ferric heme DHP with the heme cross-linked to Tyr34. Although Fe(III) in (2) is different from Fe(III) in (1), the model considers these species being the same because they should have similar EPR spectra, possibly with only subtle differences. This reaction goes ahead only if the ferryl heme is protonated. If not – the ferryl heme state is stable.</td>
</tr>
<tr>
<td>•Fe(IV) → •Fe(III)•</td>
<td>$(k_3)$ First order decay of Compound ES to ferric DHP bi-radical, when the pH 5 radical (the Tyr38 radical) is formed on reduction of the protonated oxoferryl, while the Tyr34 radical is still on the enzyme. This reaction goes ahead only if the ferryl heme is protonated. If not – the ferryl state is stable.</td>
</tr>
<tr>
<td>Fe(IV) → •Fe(III)</td>
<td>$(k_4)$ First order ferryl reduction to the ferric heme plus radical state. The location of the radical on the enzyme is not important for the model. This reaction goes ahead only if the ferryl heme is protonated. If not – the ferryl state is stable.</td>
</tr>
</tbody>
</table>

Table continues below

Three types of free radical species are formed in reactions (1-4): •Fe(IV), •Fe(III) and •Fe(III)•. Decay of protein bound free radical is a complex process. These radicals can be transferred from their original location on the enzyme to a new location via either intra- or inter-molecular mechanism. But a radical will disappear only when recombining with another radical. Therefore, to understand the overall radical decay process, let us first consider the radical transfer and then the radical-to-radical recombination reactions.
**Radical transfer.** Any polypeptide based radical, e.g. the unpaired spin density on Tyr34, can be moved to a different location on the same DHP molecule or to a location on a different DHP molecule. This newly formed radical can be also moved further producing a third type of radical, and so on. This process would not affect the total radical kinetics since it does not result in a change of the total number of radicals. This is why the kinetic model does not specify the location of radicals on DHP: as far as the model is concerned, the DHP molecules with a *ferric heme and a radical on Tyr34* and with a *ferric heme and a radical on Tyr38* belong to the same kinetic variable •Fe(III). The process of radical transfer would affect, however, the EPR spectrum: an increase in number of different types of EPR signals from different types of free radicals, when overlaid, would result in a loss of the individual lineshape of the primary radical signal. Thus, if an EPR signal of the primary radical had a hyperfine structure, the process of a cascade transfer of this radical to new locations will result in a loss of this structure, and the EPR spectrum would evolve into a plain singlet without any hyperfine structure features. The loss of the hyperfine structure of a free radical EPR spectrum with time seems to be a fundamental feature of all haem proteins reacting with peroxide (section 3.2.1 in the paper and Fig. G.6 in this document supports this statement for the DHP case).

Does the overall radical transfer rate depend on pH? We believe it does. A protonated (cation) radical on a residue would oxidise another residue faster than a neutral radical. On the other hand, the higher the degree of protonation of a non-radical residue, the less readily it could be oxidised. These two opposite trends would result in a pH dependence of the rate of radical transfer.
Radical recombination. When any two radicals in their wander around and between DHP molecules meet – they annihilate. Two radicals can recombine either within the same DHP molecule or in an inter-molecular reaction. In either case the chemical structure of the enzyme will be modified. A simplification adopted in this model is that the chemical modification of DHP does not have any effect on the kinetic dependences. The model also does not consider any radical termination reactions that involve a change in the heme oxidation state (apart from Compound RH formation via reaction (2)). These assumptions, justifiable, in our view, at the moderate excess of H_2O_2 over heme used, have allowed us to keep the number of variables in the model at a reasonable level.

Table G.5. Ten reactions considered in the model (continued).

<table>
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<tr>
<th>Reaction</th>
<th>Description and assumptions</th>
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<tbody>
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<td>•Fe(IV) + •Fe(IV) $\xrightarrow{k_5}$ Fe(IV) + Fe(IV)</td>
<td>(5)</td>
</tr>
<tr>
<td>•Fe(IV) + •Fe(III) $\xrightarrow{k_6}$ Fe(IV) + Fe(III)</td>
<td>(6)</td>
</tr>
<tr>
<td>•Fe(IV) + •Fe(III) • $\xrightarrow{k_7}$ Fe(IV) + •Fe(III)</td>
<td>(7)</td>
</tr>
<tr>
<td>•Fe(III) + •Fe(III) $\xrightarrow{k_8}$ Fe(III) + Fe(III)</td>
<td>(8)</td>
</tr>
<tr>
<td>•Fe(III) + •Fe(III) • $\xrightarrow{k_9}$ Fe(III) + •Fe(III)</td>
<td>(9)</td>
</tr>
<tr>
<td>•Fe(III) • + •Fe(III) • $\xrightarrow{k_{10}}$ Fe(III) + •Fe(III)</td>
<td>(10)</td>
</tr>
</tbody>
</table>

The three types of radicals, •Fe(IV), •Fe(III) and •Fe(III)•, can recombine in six possible ways. The rate constants $k_5$ – $k_{10}$ are different but all have the same pH dependence – the same as the radical transfer rate constants (not explicitly used in this model).
**The model: User’s notes.** Reactions (1-10) were included in the model for the case of initial DHP concentration of 40 μM and initial H₂O₂ concentration of 120 μM; the data used as the experiment reference (kinetic dependences of the ferric heme state and total free radical at pH 5, 6 and 7) are presented in Figures 4.4 and 4.5 of the paper. In the Excel file (*Kinetic_model.xlsx* available in SI), the time axis was set on N=1200 time points with a variable increment on four equal ranges:

\[
\begin{align*}
t_i &= t_{i-1} + \delta_1 & i &= 1 \ldots N/4 \\
t_i &= t_{i-1} + \delta_2 & i &= (N/4 + 1) \ldots N/2 \\
t_i &= t_{i-1} + \delta_3 & i &= (N/2 + 1) \ldots 3N/4 \\
t_i &= t_{i-1} + \delta_4 & i &= (3N/4 + 1) \ldots N
\end{align*}
\]

Concentration of a reaction component \(X\) at a time point \(i\) was calculated by the formula

\[
[X]_i = [X]_{i-1} + \left(\frac{d[X]}{dt}\right)_{i-1}(t_{i-1} - t_i)
\]

where the rate for \(X\) at the time point \(i-1\), \(\left(\frac{d[X]}{dt}\right)_{i-1}\), was expressed via the concentrations of the reaction components at the time point \(i-1\) and the rate constants in reactions (1-10). The spreadsheets in *Kinetic_model.xlsx* are constructed in the way that varying any or all four time increments \(\delta_i\) does not affect the sums in the target cells (see below), even though when a \(\delta_i\) is changed, the line number in the spreadsheet that corresponds to particular time point (that must correspond to the experimental time point) might change. This in-built facility was useful to quickly overcome any oscillation or “grass” effects caused by a bad correspondence of a function derivative and the step of integration.
The graphs showing the kinetic dependences in the Excel spreadsheets were combined as follows:

\[
[\text{Free radical}](t) = \cdot \text{Fe(III)}(t) + 2 \cdot \text{Fe(III)}^\cdot (t) + \cdot \text{Fe(IV)}(t)
\]

\[
[\text{Ferric heme}](t) = \text{Fe(III)}(t) + \cdot \text{Fe(III)}^\cdot (t) + \cdot \text{Fe(III)}^\cdot (t)
\]

\[
[\text{Ferryl heme}](t) = \text{Fe(IV)}(t) + \cdot \text{Fe(IV)}(t)
\]

The Solver tool of Excel 2007 was used to minimize the ‘target’ parameters – an average absolute difference between experimental and calculated concentration values, added together for the ferric heme and the free radical:

\[
T_{pHi} = 1/n_i \sum_{j}^{n_i} (\text{ABS}([\text{Free radical}]_j^{\text{experiment}} - [\text{Free radical}]_j^{\text{model}}) + 1/n_i \sum_{j}^{n_i} (\text{ABS}([\text{Ferric heme}]_j^{\text{experiment}} - [\text{Ferric heme}]_j^{\text{model}}))
\]

where \( pHi \) indicates the pH-series pH 5, pH 6 and pH 7; \( n_i \) – number of data points in the \( pHi \)-th series; \( 1 \leq j \leq n_i \). The aggregate target parameter \( T = T_{pH5} + T_{pH6} + T_{pH7} \) was used in the global optimization.

**Constraints on the radical decay rate constants.** We will try now to diminish the number of rate constants to be optimized by making hypotheses about relationships between the constants \( k_5-k_{10} \) that describe the radical decay. We postulate, that at any given pH value, the slowest of the six constants is \( k_5 \) because it describes recombination of two species with maximal repulsive couple of charges, +4 and +4. We will call this rate constant the basic rate constant of radical recombination and, for the time being assign it a value of 1. Other five
constants should be greater and there are two assumptions we make that allows us to calculate the factors that affect the increase in each case.

Firstly, we assume that the charge of the interacting radicals has an effect on how fast they might approach each other. Ferric and ferric (3+ and 3+) molecules will experience less repulsive coulomb force than ferryl and ferryl molecules (+4 and +4) by a factor of (4x4)/(3x3) which is 1.78. This factor for a faster recombination of a ferric and a ferryl radical species, with respect to the ferryl–ferryl case ($k_5$), will be 1.33 as it is equal to the ratio of (4x4)/(4x3).

The second assumption we make is that a bi-radical has twice higher chances to recombine with a regular radical. From this it follows that the rate of two bi-radicals recombination to two regular radicals (reaction (10)) should be 4 times faster than the rate of two regular radical recombination.

Thus the aggregate factors linking the rate constants $k_6$-$k_{10}$ to the basic rate constant of radical recombination $k_5$ are the products of the factors caused by the iron charge and by the number of radicals on the protein and are given in the last column of the table below. Therefore, only $k_5$ was varied in the model, other constants were simply calculated as follows: $k_6 = 1.333k_5$, $k_7 = 2.667k_5$ and so on. This diminishes the number of variable by $5\times3=15$, leaving only 15 parameters to vary.
Table G.6. Corrections to rate constants.

<table>
<thead>
<tr>
<th>Rate constant</th>
<th>Number of radicals on two interacting species</th>
<th>Charges of two interacting species</th>
<th>Increase (factor) of rate constants, with respect to $k_5$, caused by iron charge</th>
<th>Increase (factor) of rate constants, with respect to $k_5$, caused by number of radicals on interacting species</th>
<th>Aggregate factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_5$</td>
<td>1--1</td>
<td>IV--IV</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>$k_6$</td>
<td>1--1</td>
<td>IV--III</td>
<td>$(4^*4)/(4^*3)=1.3333$</td>
<td>1</td>
<td>1.3333</td>
</tr>
<tr>
<td>$k_7$</td>
<td>1--2</td>
<td>IV--III</td>
<td>$(4^*4)/(4^*3)=1.3333$</td>
<td>2</td>
<td>2.6667</td>
</tr>
<tr>
<td>$k_8$</td>
<td>1--1</td>
<td>III--III</td>
<td>$4^*4/(3^*3)=1.7778$</td>
<td>1</td>
<td>1.7778</td>
</tr>
<tr>
<td>$k_9$</td>
<td>1--2</td>
<td>III--III</td>
<td>$4^*4/(3^*3)=1.7778$</td>
<td>2</td>
<td>3.5556</td>
</tr>
<tr>
<td>$k_{10}$</td>
<td>2--2</td>
<td>III--III</td>
<td>$4^*4/(3^*3)=1.7778$</td>
<td>4</td>
<td>7.1111</td>
</tr>
</tbody>
</table>

Constraints on the rate constants of Compound ES formation and decay.

**Constraint on $k_1$.** The rate constant of Compound ES formation was assumed to be dependent directly on the protonation/deprotonation status of the distal His55 and therefore on its either open or closed conformation. Thus our assumption is that Compound ES is formed via reaction (1) only if the distal His55 is in the closed conformation. If so, $k_1$ must depend on pH in the same way (with the same pK$_a$) as the populations of deprotonated and protonated His55. Generally speaking, $k_1$ should increase with pH increasing. More specifically, if a single protonation event defines the open/closed equilibrium (as we assume), then there should be the following constraint on how $k_1$ may change from pH 5, to pH 6 and to pH 7.

We define the “Step” parameter for three fixed pH values, pH 5, pH 6 and pH 7: a ratio of the increase in $k_1$ from pH 6 to pH 7 to the increase from pH 5 to pH 6:

$$Step = (k_1^{pH7} - k_1^{pH6})/(k_1^{pH6} - k_1^{pH5})$$
If pK_a of the proton equilibrium in question is significantly lower than 6, Step will be just above 0.1. If pK_a is significantly higher than 6, Step will be just below 10. If Step is exactly 1, it indicates that pK_a=6. Thus the constraint imposed on the three values of k_1 (at the three pH values) is as follows: the k_1 values at pH 5, 6 and 7 should be such that the Step parameter could be found in the interval 0.1>Step>10.

If the kinetic model cannot yield a satisfactory fit when this condition is met, it would indicate that the Compound ES formation cannot be governed by a single protonation / deprotonation equilibrium.

The usefulness of the Step parameter is in the possibility to estimate the pK_a valued of the single protonation / deprotonation equilibrium that defines the pH dependence of a rate constant. If Step has a value too close to either 0.1 or 10, the accuracy of the estimate collapses (since any pK_a value much less than 6 would give Step very close to 10 and any pK_a value much greater than 6 would give Step close to 0.1). But otherwise it is quite possible to make a judgment of what the pK_a value is. There are two spreadsheets in the Excel file Kinetic_model.xlsx for determination of the pK_a values from the three rate constant values at pH 5, 6 and 7 (spreadsheets Protonated and Deprotonated).

**Constraints on k_2 and k_3.** From the experiments performed we concluded that Compound ES, with the oxoferryl heme state protonated, decays via two alternative routes, forming Compound RH (reaction (2)) or the ferric DHP bi-radical (reaction (3)). We also concluded that which of the two reactions takes place depends of the conformation of the distal His55: reaction (2) takes place in the open conformation and reaction (3) – when His55 is in the
closed conformation. But the open/closed equilibrium is governed by the same protonation/deprotonation equilibrium that defines the pH dependences of the rate constant $k_1$ (see above). Although overall decay of Compound ES depends on whether oxoferryl is protonated or not (should be overall faster at the lower pH values), the relative weight of $k_2$ and $k_3$ should be dependent on the open/closed equilibrium, i.e. the ratio $k_2/k_3$ should be increasing on pH increase in the same way $k_1$ is increasing. Therefore the constraints were imposed as follows:

$$\frac{k_2^{pH5}}{k_1^{pH5}} = \frac{k_2^{pH6}}{k_1^{pH6}} = \frac{k_2^{pH7}}{k_1^{pH7}} = A,$$

where $A$ is a constant. These three new equations allowed us to eliminate three rate constants from the variables and make them dependent on a new common for all parameter $A$. Thus the values of $k_2^{pH5}$, $k_2^{pH6}$, and $k_3^{pH7}$ were calculated from $k_1$ and $k_2$ at corresponding pH values and from a new common variable $A$. This resulted in a further decrease of the number of variables in the model from 15 to 13.

**Optimised rate constants and model’s sensitivity to their values.**

The sensitivity of the minimized target parameter $T$ to changes in individual rate constants has been studied at the optimized point. When optimized, $T=12.728$. This combines the target parameters for individual pH series as follows:
Table G.7. Rate optimization target parameters.

<table>
<thead>
<tr>
<th></th>
<th>$T_{pH5}$</th>
<th>$T_{pH6}$</th>
<th>$T_{pH7}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free radical</td>
<td>1.628</td>
<td>2.238</td>
<td>1.867</td>
</tr>
<tr>
<td>Ferric heme</td>
<td>3.267</td>
<td>2.776</td>
<td>0.952</td>
</tr>
<tr>
<td>Combined T</td>
<td>4.895</td>
<td>5.014</td>
<td>2.818</td>
</tr>
<tr>
<td>Global $T$</td>
<td></td>
<td></td>
<td><strong>12.728</strong></td>
</tr>
</tbody>
</table>

Partial derivatives of the global parameter $T(k_{i}^{pH5}, k_{i}^{pH6}, \ldots, k_{i}^{pH7}, k_{j}^{pH5}, k_{j}^{pH6}, \ldots, k_{j}^{pH7}, k_{k}^{pH5}, k_{k}^{pH6}, \ldots, k_{k}^{pH7})$ by each rate constant, while the other rate constants were kept constant (at the optimized values), have been calculated as ratios of the difference of the $T$ values for $k_{i}=1.03k_{i}^{optimized}$ and $k_{i}=0.97k_{i}^{optimized}$ to the difference of $1.03k_{i}^{optimized} - 0.97k_{i}^{optimized}$, which is $0.06k_{i}^{optimized}$.

\[
\frac{\partial T}{\partial k_{i}} = \frac{T(1.03k_{i}^{optimized}) - T(0.97k_{i}^{optimized})}{0.06k_{i}^{optimized}}
\]
Table G.8. Optimized rate constants.

<table>
<thead>
<tr>
<th>Optimized rate constants</th>
<th>Partial derivatives</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>units</td>
</tr>
<tr>
<td>$k_1$ $\mu$M$^{-1}$s$^{-1}$</td>
<td>0.025</td>
</tr>
<tr>
<td>$k_2$ s$^{-1}$</td>
<td>2.291</td>
</tr>
<tr>
<td>$k_3$ s$^{-1}$</td>
<td>0.093</td>
</tr>
<tr>
<td>$k_4$ s$^{-1}$</td>
<td>0.009</td>
</tr>
<tr>
<td>$k_5$ $\mu$M$^{-1}$s$^{-1}$</td>
<td>0.145</td>
</tr>
<tr>
<td>$k_6$ $\mu$M$^{-1}$s$^{-1}$</td>
<td>0.194</td>
</tr>
<tr>
<td>$k_7$ $\mu$M$^{-1}$s$^{-1}$</td>
<td>0.387</td>
</tr>
<tr>
<td>$k_8$ $\mu$M$^{-1}$s$^{-1}$</td>
<td>0.258</td>
</tr>
<tr>
<td>$k_9$ $\mu$M$^{-1}$s$^{-1}$</td>
<td>0.516</td>
</tr>
<tr>
<td>$k_{10}$ $\mu$M$^{-1}$s$^{-1}$</td>
<td>1.033</td>
</tr>
</tbody>
</table>