ABSTRACT

JIANG, SHU. Site-Directed Mutagenesis Studies of the Flexibility of the Distal Histidine in Dehaloperoxidase-Hemoglobin. (Under the direction of Dr. Stefan Franzen).

Dehaloperoxidase-hemoglobin (DHP) is a bioremediation enzyme, which was discovered in the marine worm called *Amphitrite ornata* in 1970s. It was first characterized as the hemoglobin for this worm since DHP has a similar structure to hemoglobins commensurate with its oxygen storage and transport function. However, in 1996, DHP was also found possessing a peroxidase function, which can oxidize the pollutant, 2,4,6-TBP, to less toxic 2,6-DBQ in the natural environment.

Like other peroxidases, the catalytic mechanism for DHP follows the Poulos-Kraut mechanism. However, due to the lack of ancillary amino acids in the distal pocket, the distal histidine, H55, becomes even more crucial in the formation of active intermediates. The exact position of H55 may affect the enzymatic activity. Therefore, T56 mutants were designed to test if the neighbor residues play an important role in the distal histidine conformation. We have confirmed that mutating residues adjacent to H55 may affect the position of H55 as well as the catalytic efficiency of DHP. Mutating T56 to a smaller amino acid appears to permit H55 to rotate more flexibly. The greater flexibility may lead to an increase in catalytic activity. On the other hand, larger neighbor residues appear to restrict the rotation of H55 due to the steric hindrance, which would lead to a decrease in catalytic activity, by the same hypothesis. On the other hand, the greater flexibility leads to more rapid inactivation of the hemoglobin by formation of hemichrome. Thus, our results demonstrate that a balance of enzymatic rate and protein stability appears to be optimum in WT-DHP.
At room temperature, the distal histidine of DHP is observed in two conformations which are defined as ‘open’ and ‘closed’. The motion of the distal histidine appears to be linked to a unique two-site competitive inhibition mechanism. The inhibitor, 4-XP, binds inside the distal pocket, pushing H55 to the solvent exposed ‘open’ conformation. The substrate, 2,4,6-TCP, is hypothesized to bind externally, but the exact position on the protein surface has not been determined. The pKa of 2,4,6-TCP is 6.2, therefore the substrate is mostly in the anionic form at the physiological pH of 7.4. The mutagenesis studies of surface mutants at pH 7.0 confirmed the electrostatic effect between DHP and 2,4,6-TCP. The mutants with more positive charge have increased catalytic efficiency compared with WT-DHP A, while more negatively charged mutants have decreased catalytic efficiency. The neutral double mutants N96D/E31Q and G40K/K51G unexpectedly demonstrate much higher catalytic activity than WT-DHP A, which may suggest that the substrate binding site may be near these residues and close to Y34. These results suggest that the electrostatic contribution is crucial to substrate binding in DHP.
Site-Directed Mutagenesis Studies of the Flexibility of the Distal Histidine in Dehaloperoxidase-Hemoglobin

by
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DEDICATION

To my parents, Lianrong Jiang and Wenyu Zhu, and my husband, Bo Gong.
BIOGRAPHY

Shu Jiang was born in Changchun, China on Aug 16th, 1986. She completed her Bachelor’s degree in the Chemistry Department of Beijing Normal University in 2005. Then she chose to start her master study in the Chemistry Department of North Carolina State University, working with Dr. Stefan Franzen on the Dehaloperoxidase-hemoglobin (DHP) project.
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CHAPTER 1
Overview: Motivation for the Study of Dual Function Proteins

Dehaloperoxidase-hemoglobin (DHP) was first isolated from the marine worm *Amphitrite ornata* [1]. The crystal structure shows that DHP has a globin fold, with eight \(\alpha\)-helices and an embedded heme prosthetic group [2]. DHP was first characterized as the monomeric hemoglobin for oxygen storage and transport in 1977 [1]. However, in 1996, it was also found DHP acts as the dehaloperoxidase in the natural environment [3, 4]. How can a protein with globin structure have a peroxidase function? In order to understand this unique dual functional protein, one first needs insight into the structures and mechanisms of both the globin and the peroxidase families.

1.1 Introduction to Heme Proteins

The heme protein superfamily consists of all of the metalloproteins that contain a heme prosthetic group. Based on the various functions, heme proteins can be classified according to functions of oxygen transport (myoglobin, hemoglobin, etc), catalysis (HRP, cytochrome c peroxidase, etc), defense (catalase), electron transfer (cytochrome a, cytochrome b, etc), active membrane transport (cytochromes) and sensory functions or signal transduction (FixL, CooA, etc). There are characteristic structures associated with each of these functions, although the structures can vary some within each family of proteins.

The common feature for most heme protein is that their active sites contain the ferriprotoporphyrin IX group or ferroprotoporphyrin macrocycles that ligates iron (III) or
iron (II), respectively \[^5\]. In general, regardless of the oxidation state of iron, we name this iron protoporphyrin IX group as ‘heme’ \[^6\]. Figure 1.1 shows the structure of heme b, which is the most abundant heme. The heme structure is made up of the porphyrin, the iron and the side chains. Four pyrrole rings in the porphyrin connect the iron by methine bridges, with the displacement of two hydrogen atoms attached to the pyrrole nitrogens by the iron. There are eight side chains, which consists of four methyl groups at positions 1, 3, 5 and 8, two vinyl groups at position 2 and 4, two propionate groups at position 6 and 7.

**Figure 1.1.** Structure of iron protoporphyrin IX, heme b.

The heme iron is commonly found in five- and six-coordinate adducts. There are four coordination sites with pyrrole nitrogen atoms and the fifth coordination site is with the imidazole side chain of proximal histidine. The only point of covalent attachment of heme to the apoprotein is the bond between the heme iron and the Ne nitrogen atom of the proximal histidine. The sixth coordination site may be vacant or occupied by a range of ligands including \( \pi \)-acid ligands such as \( \text{O}_2 \), \( \text{CO} \) and \( \text{NO} \) and nitrogenous bases such as pyridine and...
imidazole. In most native resting heme peroxidases, the heme iron is five-coordinate. The propionate side chains of heme also form hydrogen bond with neighboring residues. The sixth coordination located on the distal side is vacant in the resting state. However, when the substrate or ligand binds the heme iron during the reaction, the iron changes to six-coordinate.

1.2 Myoglobin and Hemoglobin

1.2.1 Myoglobin

Myoglobin is an important oxygen transport heme protein exists in the muscle tissue of almost all mammals. It is the first protein, for which the three-dimensional structure was determined by X-ray crystallography. In myoglobin, there are eight alpha helices folded in a tertiary structure around the heme prosthetic group. The heme iron (II) in deoxy-ferrous myoglobin is 5-coordinate as discussed above which coordinates with four nitrogens of pyrrole rings and $N_\varepsilon$ of the proximal histidine H93.

The active site is in the distal pocket above the heme iron (II), which is surrounded by hydrophobic amino acids. The major function of myoglobin is reversible binding and transport of diatomic oxygen molecule. Other small diatomic molecules can also enter the distal pocket and bind the heme iron (II) as the sixth coordination ligands. The distal histidine H64 is an important residue in this process. It plays a role in stabilizing the binding of $O_2$ and in preventing the binding of poisonous CO by steric hindrance, hydrogen bonding and other interactions.
1.2.2 Hemoglobin

Like myoglobin, hemoglobin is also an important heme protein for oxygen transport, which transports the oxygen mainly in red blood cells [12]. Hemoglobin and myoglobin evolved from a common ancestor gene. However, unlike the monomeric myoglobin, human hemoglobin is a four-subunit globular protein with quaternary structure [13]. Human hemoglobin is mainly made up of two alpha subunits and two beta subunits (Figure 1.2) [14]. Each of the four subunits is a globular protein with an embedded heme group; therefore, up to four oxygen molecules are allowed to bind the heme in one hemoglobin molecule. Some hemoglobins are dimeric in structure, such as scapharca clam hemoglobin and dehaloperoxidase hemoglobin [2, 15]. In addition to oxygen molecule, hemoglobin is also capable of carrying the metabolic carbon dioxide back to the respiratory organs [16].

![Image of human hemoglobin crystal structure]

Figure 1.2. Crystal structure of human hemoglobin (PDB 1GZX) [14].

The oxygen binding process is more complicated for human hemoglobin than myoglobin. After the first oxygen molecule binds the heme iron (II) in one subunit, there will
be a subtle change on the quaternary structure of hemoglobin to make it much easier to bind the subsequent oxygen molecule. As a result, the hemoglobin changes from the tense (T) state to the relaxed (R) state. This behavior of changing one subunit affecting other subunits is called cooperativity \cite{17}, which significantly increases the efficiency of oxygen transport for hemoglobin. The names T and R arise from the fact that the subunits have stronger interactions in the deoxy form than in the oxy or ligand bound form. The strong interactions induce a tension, which is relaxed when O$_2$ or other diatomic ligands bind to the heme irons.

1.3 Heme Peroxidases

1.3.1 Horseradish Peroxidase and Cytochrome c Peroxidase

Horseradish peroxidase (HRP) and Cytochrome c peroxidase (CcP) are the earliest well-studied heme peroxidases. Their structures and mechanisms have been the model for understanding other peroxidases. Various spectra indicate that the heme iron (III) in the native resting peroxidase is five-coordinate \cite{18}. The key residues around the distal and proximal pocket of HRP and CcP are highly conserved \cite{19}. However, CcP has no heterogeneous carbohydrate attachment as in HRP, which makes it much easier to obtain a crystal structure of CcP \cite{20}.

HRP is extracted from the plant called horseradish. HRP is important in many physiological roles to oxidize organic and inorganic compounds, such as aromatic phenol, amines and sulfonates, etc \cite{21}. The substrate binds at the ‘exposed’ heme edge and gets oxidized in the presence of H$_2$O$_2$ \cite{22}. However, there is an exception for oxidizing indole-3-
acetic acid substrate, in which \( \text{H}_2\text{O}_2 \) is not needed\(^6\). This reaction is widely studied recently with the potential of cancer treatment \(^{23-25}\).

CcP was first discovered in 1940 \(^{26,27}\). Another heme protein, cytochrome c is the natural substrate for CcP in the physiological environment. CcP acts as the electron donor to \( \text{H}_2\text{O}_2 \) and electron acceptor from the heme iron (II) of cytochrome c, which is the classic model to study the electron transfer \(^{27-29}\). The crystal structure of cytochrome c and CcP complex was first determined in 1992 \(^{20}\), which indicates the binding site is external including hydrophobic interactions and electrostatic interactions between the lysine of cytochrome c and the carboxylate groups of CcP. The electron was supposed to transfer from the methy group of cytochrome c through Ala193, Ala194 and Gly192 to Trp 191 radical of CcP \(^{20}\).

1.3.2 Catalytic Mechanism

The reaction cycles for HRP and CcP are similar. Both of them follow the Poulos-Kraut mechanism \(^{30}\), but they have different active intermediates. According to the Poulos-Kraut mechanism, \( \text{H}_2\text{O}_2 \) binds the heme iron of HRP followed by the heterolytic O-O bond cleavage to form Compound I, the iron (IV)-oxo porphyrin \( \pi \)-cation radical species with two-electron-oxidized heme center compared to the ferric form \(^{31}\). However, instead of Compound I, Compound ES was found as the active intermediate for CcP \(^{32}\). Compound ES and Compound I are isoelectronic. EPR data indicates that the radical is located on the edge of the porphyrin for Compound I, while located on the protein residue Trp191 for Compound ES of CcP \(^{33-35}\). Both Compound I and Compound ES can regenerate the rest state via two
sequential one-electron oxidation reactions through Compound II, an iron (IV)-oxo intermediate which is one-electron oxidized compared with the ferric heme (Scheme 1.1) \(^6\).

**Scheme 1.1.** Reaction scheme for HRP Catalysis.

\[
\text{HRP} + \text{H}_2\text{O}_2 \rightarrow \text{Compound I} + \text{H}_2\text{O}
\]

\[
\text{Compound I} + \text{AH}_2 \rightarrow \text{Compound II} + \cdot \text{AH}
\]

\[
\text{Compound II} + \text{AH}_2 \rightarrow \text{HRP} + \cdot \text{AH} + \text{H}_2\text{O}
\]

Distal histidine, distal arginine, proximal histidine and proximal aspartate regulate the formation of Compound I (Compound ES) by ‘pull’ and ‘push’ effect, which is a common feature for most peroxidases. The ‘pull effect’ works on the distal side. Distal histidine serves as an acid-base catalyst accepting the proton from H\textsubscript{2}O\textsubscript{2}. The positively charged distal arginine and distal histidine play a concerted role to cleave the O-O bond of H\textsubscript{2}O\textsubscript{2} to form and stabilize Compound I (Compound ES) \(^{36-39}\). On the proximal side, the negatively charged aspartate provides a charge relay to proximal histidine, leading to a strong interaction between N\textsubscript{ε} of proximal histidine and the heme iron, which is called a ‘push effect’ \(^{19,40-42}\). Both ‘pull’ and ‘push’ effects facilitate the formation of Compound I (Compound ES).

**1.4 Comparison of Heme Globin and Heme peroxidase**

Both globins and heme peroxidases are a member of heme protein, with heme prosthetic group in the protein structure. However, the differences in the residue sequence and protein folding result in different functions for these two protein families.
In the native resting state, the heme iron is in ferrous form for globins while in ferric form for heme peroxidases. Only the ferrous heme can bind the oxygen molecule due to the π-back bonding \[^6\]. In order to stabilize the higher oxidation state of the ferric heme iron, the reduction potential of \( \text{Fe}^{3+}/\text{Fe}^{2+} \) is about 50 mV higher in heme peroxidases than globins, which are -194 mV and -220 mV for CcP and HRP, respectively \[^{43-45}\].

On the distal side, the average distance between the Nε of distal histidine and heme iron is longer in heme peroxidases than in globins \[^{30, 46-48}\]. In addition, distal arginine only exists in heme peroxidases. The association of distal arginine with distal histidine is thought to play a key role in the formation of Compound I (Compound ES) \[^{27-30}\]. On the proximal side, the imidazole of proximal histidine binds the heme iron via covalent bond as the fifth coordination for both globins and heme peroxidases, but the bond strength is stronger in peroxidases. All these differences on the distal and proximal side facilitate the formation of compound I (Compound ES) in peroxidases.

The following Chapters are consist of a review of recent studies on DHP by Dr. Franzen group, Dr. Ghiladi group, etc. (Chapter 2), a mutagenesis study of Thr56 in controlling the conformation of the distal histidine in DHP (Chapter 3) and a study on how the electrostatic surface mutants affect the enzymatic rate of DHP (Chapter 4).
REFERENCES


CHAPTER 2
Dehaloperoxidase-Hemoglobin:
A Unique Dual Function Enzyme

2.1 Introduction

Dehaloperoxidase-hemoglobin (DHP) was first discovered in the marine worm, terebellid polychaete Amphitrite ornata, but has been cloned into Escherichia coli and studied as a model for dual function hemoglobin \[^1\]. It is the first hemoglobin found with natural peroxidase function. DHP has a globin structure with eight α-helices and a heme prosthetic group \[^2-4\]. Therefore, it can store and transport diatomic molecules, such as O\(_2\), CO, NO, etc. Although DHP has distinctive structure from other peroxidases such as horseradish peroxidase, it still has a peroxidase function that gives it the capability to oxidize 2,4,6-tribromophenols to the corresponding 2,4-dihaloquinones in the natural environment (Figure 2.1) \[^5\]. The kinetic assay for product formation demonstrates that the peroxidase activity for DHP is 1-2 orders of magnitude greater than myoglobin (Mb) \[^3\], and only 1 order of magnitude less than horseradish peroxidase (HRP) \[^6\].

![Figure 2.1. Dehalogenated oxidization of trihalogenated phenole to yield dihalogenated quinone product as catalyzed by DHP in the presence of hydrogen peroxide.](image-url)
Like other globins and heme peroxidases, the heme iron in DHP has four coordination sites with the pyrrole nitrogen atoms of the heme and the fifth coordination with the imidazole of the proximal histidine. The distal histidine is located above the heme, but does not coordinate with the heme iron (Figure 2.2) \[^7\]. In ferric metaquo DHP, an $\text{H}_2\text{O}$ molecule binds the heme iron as the sixth coordination in the distal pocket \[^6\]. In deoxyferrous DHP, the heme iron is 5-coordinate. Diatomic molecules, such as CO, CN, etc, can enter the distal pocket and bind the heme iron as the sixth coordination \[^8\].

![Crystal structure of DHP A (PDB 2QFK). The distal histidine is located above the heme, but does not coordinate with the heme iron. The imidazole of the proximal histidine coordinates with the heme iron as the fifth coordination.](image)

**Figure 2.2.**

2.2 Poulos-Kraut Mechanism of DHP

Most peroxidases follow the Poulos-Kraut mechanism for activation of bound $\text{H}_2\text{O}_2$ \[^9\]. In the distal pocket, the distal histidine forms hydrogen bond with the hydrogen atom of bond
H$_2$O$_2$ as the proton acceptor, while the positively charged distal arginine forms hydrogen bond with the oxygen atom of bond H$_2$O$_2$. The distal histidine and distal arginine play a concerted role to cleave the O-O bond of H$_2$O$_2$ to form and stabilize Compound I. This process is called ‘pull’ effect [10-13]. On the proximal side, the proximal histidine usually forms hydrogen bond as the proton donor with negatively charged carboxylate group of proximal aspartate. This electronegative proximal histidine can stabilize the heme iron which is in a higher oxidation state in the intermediate compounds. This process on the proximal side is called ‘push’ effect [14-16]. However, DHP lacks distal arginine in the distal pocket. Without arginine, distal histidine H55 becomes the only residue that plays the key role of acid-base catalyst in the formation of Compound I on the distal side [14, 7, 8]. The negatively charged residue in proximal side is also missing in DHP. The N$_\delta$ of proximal histidine interacts with the neutral carbonyl group of L83[7]. As a result, DHP does not have a typical ‘pull’ or ‘push’ effect on the distal and proximal side, respectively.

2.3 Reaction Cycle for DHP

The characterization of DHP intermediates was studied by the research groups of Dr. Ghiladi and Dr. Smirnova based on stop-flow UV-visible spectroscopy and EPR spectroscopy, respectively [17]. The oxidation reaction begins with ferric DHP [UV-visible, 406 (Soret)]. After adding H$_2$O$_2$ to ferric DHP, Compound ES [UV-visible, 419 (Soret), 545, 585 nm; $k_{obs}=(2.78\pm0.01) \times 10^4$ M$^{-1}$ s$^{-1}$] was observed (Figure 2.4 A) [17]. A similar intermediate was also found in CcP [18]. In this process, Compound I may be formed transiently first and then undergoes an endogenous electron transfer to generate Compound
ES. Compound I and Compound ES are isoelectronic; both are two-electron-oxidized intermediates compared to ferric DHP. The first oxidation equivalent is centered on the ferryl moiety and the second oxidation equivalent is located on the porphyrin in Compound I while on a protein side chain in Compound ES\textsuperscript{19-21}. The CW EPR signal (\(g \approx 2.005\), peak-to-peak line width = 21 G) confirmed that the radical in Compound ES is a tyrosyl radical (Figure 2.3)\textsuperscript{17}. Based on the protein structure, this tyrosyl radical may be either Tyr34 or Tyr38\textsuperscript{17}.

![EPR spectra of the radical(s) in DHP Compound ES at pH 7.](image)

**Figure 2.3.** EPR spectra of the radical(s) in DHP Compound ES at pH 7. Samples were prepared from the reaction of 50 mM DHP with a 10-fold molar excess of \(\text{H}_2\text{O}_2\) at 278K and rapidly frozen in isopentane slurry. Spectra were recorded at 77 K at the frequency of 9.2330 GHz\textsuperscript{17}.

In the presence of substrate, Compound ES can be regenerated to ferric DHP by two-electron oxidation or two sequential one-electron oxidations of 2,4,6-TXP (Figure 2.4 A)\textsuperscript{17}, proceeding through Compound II, which is a one-electron-oxidized intermediate relative to ferric DHP. In the absence of substrate, Compound ES converts to a new species Compound
RH [UV-visible, 411 (Soret), 530, 564 nm; $k_{\text{obs}} = 0.0701 \pm 0.0001 \text{ s}^{-1}$] (Figure 2.4 B) \cite{17}.

Compound RH is a protein-heme crosslinked species \cite{22}, which has 6-fold lower peroxidase activity than ferric DHP and can be reduced to the deoxy ferrous state. As a result, deoxy ferrous DHP can bind dioxygen to form the oxy-ferrous intermediate and then regenerate to ferric DHP by autoxidation. It is hypothesized that Compound RH is a protective species to prevent heme bleaching. A new catalytic cycle demonstrates this peroxide-dependent oxidation of ferric DHP in the presence and absence of 2,4,6-TXP. (Figure 2.5) \cite{17}.

Figure 2.4. (A) Calculated UV-visible spectra for ferric DHP incubated with 2,4,6-TCP (black), Compound ES formed (red) and ferric DHP regenerated upon 2,6-DCQ formation (green) (B) Calculated UV-visible spectra for ferric DHP mixed with $\text{H}_2\text{O}_2$ (black), Compound ES formed (red) and Compound RH formed (blue). The calculated data were based on the rapid-scanning data fitted to a double-exponential reaction model using Specfit program \cite{17}.
In the recent study, another reaction cycle started with oxyferrous DHP was proposed by Dr. Ghiladi et al.\textsuperscript{[23]} and Dr. Dawson et al.\textsuperscript{[24]}. It is hypothesized that $\text{H}_2\text{O}_2$ can replace the bound dioxygen molecule in the presence of substrate and Compound II is formed as the active intermediate. Compound II is reduced to ferric DHP via one-electron oxidation of substrate\textsuperscript{[23]}. The product 2,6-DXQ can reduce the ferric DHP to ferrous form to close this cycle\textsuperscript{[25]}.

2.4 Two-site Competitive Inhibition

The distal histidine H55 plays a key role in the substrate and ligand binding. H55 has two conformations in equilibrium at room temperature, ‘open’ conformation ($\sim$40%) and
‘closed’ conformation (~60%) [4]. In the open conformation, H55 swings out of the distal pocket to a solvent exposed position with the heme iron in a 5-coordinate high spin (5cHS) form (Figure 2.6 a). In the closed conformation, H55 is positioned into the distal pocket and stabilized by a hydrogen bond to heme ligand, such as water molecule. In this case, the heme iron is in a 6-coordinate high spin (6cHS) form (Figure 2.6 b) [3, 26].

In a previous study, 4-iodophenol appears in the distal pocket in the crystal structure (PDB 1EWA) [7, 28]. Dr. Thompson, et al. have systematically investigated the binding site for mono- and trihalogenated phenols by crystallography, resonance Raman spectroscopy and UV-visible spectroscopy [27]. Resonance Raman (RR) spectroscopy is a powerful tool to characterize the vibrational frequency of functional groups or particular amino acids and cofactors. RR spectroscopy is complementary to IR spectroscopy, but avoids the interference from water molecules in the sample. RR spectra suggest that the monohalogenated phenols
enter the distal pocket by replacing the water molecule, but do not coordinate to the heme iron \[^{[27]}\]. As a result, the heme iron changes from 6-coordinate high spin \((\nu_3 \text{ at } 1481 \text{ cm}^{-1}, \nu_2 \text{ at } 1562 \text{ cm}^{-1}, \nu_{10} \text{ at } 1611 \text{ cm}^{-1})\) to 5-coordinate high spin \((\nu_3 \text{ at } 1494 \text{ cm}^{-1}, \nu_2 \text{ at } 1568 \text{ cm}^{-1}, \nu_{10} \text{ at } 1632 \text{ cm}^{-1})\) (Figure 2.7) \[^{[27]}\]. However, the binding of substrate 2,4,6-TCP does not affect the heme coordination, which is still 6-coordinate high spin as the same in WT-DHP \[^{[27]}\]. This result indicates that 2,4,6-TCP may bind at the external site. The NMR data also demonstrates that binding of mono- and dihalogenated phenols causes great effect on the distal pocket amino acids and heme group, while binding of 2,4,6-TCP causes the change on the surface residues \[^{[29, 30]}\]. Based on the data above, it has been confirmed that monohalogenated phenols can enter the distal pocket but do not bind the heme iron, leading the distal histidine to the ‘open’ conformation. It is also hypothesized the substrate, 2,4,6-TCP, binds at an external binding site, resulting in the distal histidine in the ‘closed’ conformation. However, the exact position of 2,4,6-TCP binding site has not been determined.
**Figure 2.7.** 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 final concentration of ferric DHP was 100 mM, 4-IP was 1 mM and 4-BP, 4-CP, 4-FP and phenol was 8 mM. Excitation wavelength: 406 nm; resolution: 1.7 cm⁻¹; laser power at the sample: 60mW; acquisition time: 300 s [27].

The dehalogenated oxidation of 2,4,6-TCP substrate to the 2,6-DCQ product can be monitored by time-dependent UV-vis spectra [27]. The spectra suggest that in the presence of 4-IP, 4-BP and 4-CP, both the turnover rate of 2,4,6-TCP substrate (λmax (Soret) = 312 nm) and the formation rate of 2,6-DCQ product (λmax (Soret) = 273 nm) become much slower than in the absence of these monohalogenated phenoles (Figure 2.8). 4-FP does not inhibit the reaction as much as other monohalogenated phenols since only a small fraction of 4-FP binds internally [27]. Therefore, the monohalogenated phenoles inhibit the peroxidase activity as the inhibitor. This is in consistent with the resonance Raman data that monohalogenated
Phenols can enter the distal pocket forcing the distal histidine to the ‘open’ conformation, which inhibits the distal histidine interacting with H₂O₂ in the formation of Compound ES\textsuperscript{[27]}.

**Figure 2.8.** Kinetic assays showing the catalytic activity of DHP inhibited by 4-BP. Time-dependent UV-vis spectra from 0 s (red) to 120 s (purple). (a) In the absence of 4-BP, the substrate TCP is converted to the product DCQ. (b) In the presence of 4-BP, less substrate is turnover and less product is formed.

Based on the data above, the inhibitor mono-halogenated phenols bind inside the distal pocket, enforcing the distal histidine to the ‘open’ conformation and inhibit enzyme activity. It is also hypothesized that the substrate 2,4,6-TCP binds externally, with the distal histidine in the ‘closed’ conformation. Therefore, the monohalogenated phenols and the substrate 2,4,6-TCP are engaged in two-site competitive inhibition. This two-site competitive binding mechanism is unique for DHP from other globins and peroxidases.

Above all, distal histidine H55 plays a very important role in both ligand binding and substrate oxidation. Extensive mutagenesis studies have been done on distal histidine for globins and peroxidases, which suggest that the position of distal histidine is crucial for the
enzymatic catalysis $^{[31, 32]}$. A series of H55 mutagenesis studies of DHP also suggest the importance of distal histidine $^{[33]}$. However, the questions on how the neighbor residues affect the position of distal histidine have not been fully understood.
REFERENCES


CHAPTER 3

[1] The Role of Thr56 in Controlling the Conformation of the Distal Histidine in Dehaloperoxidase-Hemoglobin from Amphitrite Ornate

3.1 Introduction

The positioning of the distal histidine is crucial for most peroxidases because the distal histidine interacts with H₂O₂ in the formation of the active intermediates \(^{[2,3]}\). In DHP, due to the lack of ancillary amino acids such as an arginine, which is found in the distal pocket for most mono-functional peroxidases, the distal histidine is the only residue that plays the key role in the ‘pull effect’ \(^{[4-6]}\). As a result, the role of the distal histidine has been extensively studied by mutagenesis, such as replacement of histidine by arginine (H55R), valine (H55V) and aspartate (H55D) \(^{[7]}\). However, the question on how neighboring residues affect the conformation of His55 has not been investigated. By mutating residues adjacent to His55, one can affect its position and dynamics without completely changing its function. Such a study is proposed in this work.

In sperm whale myoglobin (SWMb), Phe46 and Arg45 have been suggested to be the critical residues that affect the rotation of distal histidine His64 \(^{[8,9]}\). At pH 4, the side chain of Phe46 moves away from His64. This rearrangement provides greater flexibility to the distal histidine and allows more direct access of diatomic ligands to the distal pocket.

Moreover, at low pH, Arg45 loses the hydrogen bond with side chain of heme porphyrin,
leading to more space for His64 rotation. These results indicate that the residues near distal histidine may affect the \(\text{Ca-C}_\beta\) rotation of this histidine [9].

Since Thr56 is one of the nearest residues to His55 in DHP, we selected Thr56 for systematic mutational studies and altered threonine to glycine (T56G), alanine (T56A), serine (T56S) and valine (T56V). The reason for choosing these conservative mutations is that the structures for these amino acids are similar and form a series of increasing steric bulk. However, if Thr56 is the key residue that modulates His55 rotation, then we would predict that even subtle changes will affect the peroxidase activity significantly. In this study, we conducted kinetic assays and MD simulations and obtained resonance Raman and FTIR spectra to test whether Thr56 plays an important role in the distal histidine conformation.

3.2 Experimental

3.2.1 Material

All chemicals were purchased from Fisher Scientific and Sigma-Aldrich and used without further purification. Deionized water was purified by Barnstead system. The oligonucleotide primers were synthesized by IDT DNA Technologies, Inc. \(^{13}\)CO gas was purchased from Cambridge Isotope Laboratories, Inc.

3.2.2 DHP Mutation and Expression

The T56 mutants, T56A, T56G, T56S, and T56V were obtained by replacing the threonine, at position 56 in the recombinant 6XHis-tagged WT-DHP A, by alanine, glycine, serine and valine, respectively. The primers for these mutants are listed in Table 3.1. Site-
directed mutagenesis was carried out in each case using the QuickChange Multi Site-Directed Mutagenesis Kit (Stratagene). The mutant plasmids were synthesized using 6XHis-tagged WT-DHP A plasmid as the template and upscaled on 2720 Thermo Cycler (Applied Biosystems, Inc) by the thermal cycles consisting of melt (95 °C, 30 s), annealing (55 °C, 60 s), and extension (68 °C, 6 min), for a total of 16 cycles. Dpn I restriction enzyme was added at 37 °C for 1 hour to remove the parental WT-DHP A plasmid. The Dpn-I treated DNA was transformed to XL1-Blue competent cells, which were then incubated on LB-ampicillin (100 μg/ml) agar plates at 37 °C for ~16 hours. A single colony was isolated and transferred to 3 ml 2xYT broth with 100 μg/ml ampicillin and incubated at 37 °C for 8 hours. The plasmid was extracted by E.Z.N.A Plasmid Mini Kit I (Omega Bio-Tek, Inc) and confirmed the presence of the desired mutations by Genewiz, Inc.

The T56 mutant plasmids were transformed to competent BL21 (DE3) cell and incubated on LB-ampicillin (100 μg/ml) agar plates at 37 °C for about 14 hours. A single colony was isolated and transferred to 2 ml 2xYT starter culture with 100 μg/ml ampicillin. The starter culture was shaken at 37 °C for 8 hours. 1 ml from the starter culture was transferred to a 1L flask of 2xYT broth with 100 μg/ml ampicillin. Six such 1 L flasks were shaken at 37 °C for about 15 hours. The cell pellet was collected by centrifuge at 4 °C for 20 minutes at 7000 rpm and stored at -20 °C.
Table 3.1. The primers used in mutagenesis to make T56 mutants.

<table>
<thead>
<tr>
<th>T56A</th>
<th>5'-C CAA GTT CGG TGA TCA <strong>CGC</strong> TGA GAA AGT GTT CAA C-3'</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5'-G TTG AAC ACT TTC TCA <strong>GCG</strong> TGA TCA CCG AAC TTG G-3'</td>
</tr>
<tr>
<td>T56G</td>
<td>5'-G GCC AAG TTC GGT GAT CAC <strong>GGT</strong> GAG AAA GTG TTC AAC CTG-3'</td>
</tr>
<tr>
<td></td>
<td>5'-CAG GTT GAA CAC TTT CTC <strong>ACC</strong> GTG ATC ACC GAA CTT GGC C-3'</td>
</tr>
<tr>
<td>T56S</td>
<td>5'-CC AAG TTC GGT GAT CAC <strong>TCT</strong> GAG AAA GTG TTC AAC-3'</td>
</tr>
<tr>
<td></td>
<td>5'-GTT GAA CAC TTT CTC <strong>AGA</strong> GTG ATC ACC GAA CTT GG-3'</td>
</tr>
<tr>
<td>T56V</td>
<td>5'-G GCC AAG TTC GGT GAT CAC <strong>GGT</strong> GAG AAA GTG TTC AAC CTG-3'</td>
</tr>
<tr>
<td></td>
<td>5'-CAG GTT GAA CAC TTT CTC <strong>AAC</strong> GTG ATC ACC GAA CTT GGC C-3'</td>
</tr>
</tbody>
</table>

3.2.3 DHP Purification

After thawing, the cell pellet was resuspended in 2 ml/ (g of cell pellet) lysis buffer (50 mM NaH$_2$PO$_4$, 300 mM NaCl, 10 mM imidazole, pH 8) with 1 mg/ml lysozyme and stirred at 4 °C for 1 hour. Then 500 μl Dnase I (10 mg/ml) and Rnase A (16 mg/ml) were added. The cell slurry was stirred at 4 °C for another 1 hour until it was no longer viscous. After sonication for about 15 minutes, the cell slurry was centrifuged at 4 °C for 45 minutes at 18000 rpm. The protein was released from the cells and subsequently was dissolved in the supernatant. The crude DHP protein solution was loaded on a Ni-NTA column pretreated with lysis buffer, washed by washing buffer (50 mM NaH$_2$PO$_4$, 300 mM NaCl, 20 mM imidazole, pH 8), and eluted by elution buffer (50 mM NaH$_2$PO$_4$, 300 mM NaCl, 250 mM imidazole, pH 8). Next, the collected DHP from the Ni-NTA column was oxidized by excess K$_3$[Fe(CN)$_6$] and loaded on a Sephadex G-25 column to remove extra K$_3$[Fe(CN)$_6$] as well as change the buffer to 20 mM KP buffer (20 mM KH$_2$PO$_4$, 20 mM K$_2$HPO$_4$, pH 6). Finally, the
oxidized DHP was further purified using a CM-52 column, washed by 20 mM potassium phosphate buffer at pH 6, and eluted by 100 mM potassium phosphate buffer (100 mM KH$_2$PO$_4$, 100 mM K$_2$HPO$_4$, pH 7). The purified DHP was stored at 4 °C or at -20 °C mixed with 50%/50% glycerol/KP buffer. The purity of DHP was determined by the ratio of A$_{406}$/A$_{280}$, which is over 4 for purified protein.

3.2.4 Kinetic Assays

The kinetic assays for Michaelis-Menten analysis were conducted in 100 mM potassium phosphate buffer (100 mM KH$_2$PO$_4$, 100 mM K$_2$HPO$_4$, pH 7) using a UV-vis spectrometer (Agilent 8453) equipped with a Peltier temperature controller. The final concentration of ferric DHP was 0.5 μM for T56V and 2 μM for WT and other mutants. 2,4,6-TCP substrate concentrations were varied from 100 to 1400 μM. DHP and 2,4,6-TCP were incubated at 25 °C for 3 minutes in a cuvette in the temperature-controlled sample holder to reach thermal equilibrium. Excess H$_2$O$_2$ (~2000 μM) was added to the cuvette to initiate the assay. The spectra were collected every 0.5 seconds for 60 seconds, monitoring the formation of the product 2,6-DCQ ($\lambda_{\text{max}} = 273\text{nm}$, $\varepsilon_{273} = 13200 \text{ M}^{-1} \text{ cm}^{-1}$) at pH 7 [10]. The initial turnover velocity, $v_0$, was obtained for each concentration of 2,4,6-TCP substrate. The data of $v_0$ versus 2,4,6-TCP concentration were fitted independently using the enzyme kinetics equations provided in GraFit4.03.
3.2.5 Resonance Raman Spectroscopy

All ferric DHP samples were prepared as above. To prepare the DHP-CO samples, purified DHP samples were reduced to the ferrous form using a syringe needle that had been rinsed by 1 M of anaerobically prepared sodium dithionite solution to add a slight excess of sodium dithionite to deoxy DHP dissolved in degassed buffer. Ferrous DHP samples were placed under $^{12}\text{CO}$ or $^{13}\text{CO}$ atmosphere for about 10 minutes to form DHP-$^{12}\text{CO}$ or DHP-$^{13}\text{CO}$ samples, respectively \[^{10,11}\].

For all resonance Raman experiments, the final protein concentration was 100 $\mu\text{M}$ in 100 mM potassium phosphate buffer (100 mM KH$_2$PO$_4$, 100 mM K$_2$HPO$_4$, pH 7). The samples were placed into HP-507 NMR tubes and excited into the Soret band (~410 nm) using a Coherent Mira 900 titanium sapphire (Ti:sapphire) laser, which was pumped by a Coherent Verdi 10 frequency-doubled diode-pumped Nd:YLF laser generating 10 W at 532 nm. The fundamental beam from the Ti:sapphire, tunable in the range ~700–1000 nm, was sent through a Coherent 5-050 doubler to generate a working range of 400–430 nm for Soret band excitation. The beam was focused cylindrically on the sample. Scattered photons were collected by a Spex 1877 triple spectrometer (2400 grooves/mm final stage grating) connected with an ISA SPEX liquid nitrogen-cooled CCD with ~1.7 cm$^{-1}$ resolution. Data was obtained by SpectraMax 2.0 software. The spectra were calibrated using the standard spectrum of indene, toluene, and cyclohexene for ferric DHP samples and toluene and cyclohexene for DHP-CO samples.
3.2.6 FTIR Spectroscopy

The deoxy-ferrous DHP samples and DHP-\textsuperscript{12}CO samples for WT and each mutant were prepared as above at a final concentration of 2-3 mM. The cell used for measurement in the FTIR spectrometer was a split cell with a path length of 25 microns between two 1” CaF\textsubscript{2} windows. For each mutant or WT DHP, the deoxy-ferrous and DHP-\textsuperscript{12}CO samples were injected into each half of the split cell. The spectra were collected on the Digilabs FTS-6000 FTIR spectrometer purged with dry air. Each spectrum is accumulated with 1024 scans by a Mercury Cadmium Telluride (MCT) detector with the resolution of 2 cm\textsuperscript{-1}. The \( \nu_{\text{co}} \) frequency was analyzed in Origin using the absorption difference spectrum, which was obtained by the subtraction of the deoxy-ferrous DHP spectrum from the DHP-\textsuperscript{12}CO spectrum.

3.2.7 Molecular Dynamics Simulations

Molecular Dynamics (MD) simulations were performed using the scalable molecular dynamics program, NAMD\textsuperscript{12}, which is designed for high-performance simulation of large biomolecular systems. The X-ray crystallographic coordinates for WT-DHP were obtained from the Protein Data Bank (PDB 2QFK)\textsuperscript{5}. Mutations were created using the ‘Mutate Residue’ module of the visual molecular dynamics program, VMD\textsuperscript{13}. NAMD simulations were carried out with periodic boundary conditions with 10 Å water molecules and 0.5 M Na\textsuperscript{+} and 0.5 M Cl\textsuperscript{-} ions. A quantitative estimate of fluctuations and distance between the heme iron and distal histidine was obtained by analysis of the output files for a period of 10 ns.
3.3 Results

3.3.1 Kinetic Assays

The initial velocities of the formation of the product, 2,6-DCQ ($\lambda_{\text{max}} = 273$nm) versus different 2,4,6-TCP concentrations are shown in Figure 3.1 for the mutants T56A, T56G, T56S and T56V compared to WT-DHP A. The initial velocity of T56G reaches a maximum and begins to decrease when the concentration of 2,4,6-TCP is greater than 1000 $\mu$M. We will not further consider the reasons for this unusual behavior in this study. However, we note that we have preliminary evidence that the substrate may enter the distal pocket and act as an inhibitor at a high concentration of substrate in certain mutants where the distal cavity is expanded relative to WT [Franzen et al. unpublished data]. Therefore, for T56G, only the kinetics at low 2,4,6-TCP concentrations were considered in the determination of the Michaelis-Menten parameters. The other three mutants and WT-DHP follow the standard Michaelis-Menten kinetics up to the maximum concentration of 2,4,6-TCP studied (1400 $\mu$M). We also note that the fitting curve for T56V is almost linear with a quite large $v_0$ at high 2,4,6-TCP concentration if 2 $\mu$M enzyme concentration was used. In order to conveniently demonstrate the kinetics using the same apparatus for all mutants, 0.5 $\mu$M enzyme concentration was used for T56V kinetic assays.
**Figure 3.1.** Michaelis-Menten analysis of WT-DHP A (black), T56A (orange), T56G (green), T56S (blue) and T56V (red), showing the initial reaction velocity versus substrate 2,4,6-TCP concentrations. The assay conditions were [T56V] ~ 0.5 μM, [WT-DHP] and [other T56 mutants] ~ 2 μM, [TCP] = 0.1 mM-1.4 mM and [H₂O₂] ~ 2 mM in 100 μM pH 7 KP buffer at 298K. The Michaelis-Menten fit parameters are given in Table 3.2.

Table 3.2 summarizes the Michaelis-Menten fit parameters for WT-DHP A and four mutants, T56A, T56G, T56S and T56V. Based on Michaelis-Menten fitting, kₙₐₜ was found in the order T56G < WT-DHP A < T56S ≈ T56A < T56V. The Kₘ values were observed in the reverse order T56V > WT-DHP A > T56S > T56A > T56G. The catalytic efficiency, kₙₐₜ/Kₘ, follows the trend T56G > T56A > T56S > T56V ≈ WT DHP. This trend is opposite to the trend in the size of amino acid 56, V > T > S > A > G. The trends above indicate that a smaller amino acid in position 56 may lead to smaller Kₘ value and greater catalytic efficiency. The greatest change in rate constant was observed for T56V, which has kₙₐₜ value roughly three times higher than WT-DHP A. In fact, the behavior of T56V is also somewhat
anomalous since its $K_m$ value is greater than the maximum 2,4,6-TCP concentration studied. This can be seen in Figure 3.1 from the fact that the T56V plot is nearly linear.

**Table 3.2.** Michaelis-Menten fit parameters for WT and T56 mutants.

<table>
<thead>
<tr>
<th></th>
<th>$V_{\text{max}}$ (μM/s$^{-1}$)</th>
<th>$k_{\text{cat}}$ (s$^{-1}$)</th>
<th>$K_m$ (mM)</th>
<th>$k_{\text{cat}}/K_m$ (mM$^{-1}$ s$^{-1}$)</th>
</tr>
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<tr>
<td>T56A</td>
<td>28.61±3.69</td>
<td>14.30±1.85</td>
<td>0.46±0.16</td>
<td>31.09±11.54</td>
</tr>
<tr>
<td>T56G</td>
<td>15.06±1.16</td>
<td>7.53±0.58</td>
<td>0.22±0.05</td>
<td>34.23±8.21</td>
</tr>
<tr>
<td>T56S</td>
<td>28.24±2.38</td>
<td>14.10±1.19</td>
<td>0.75±0.14</td>
<td>18.80±3.85</td>
</tr>
<tr>
<td>T56V</td>
<td>13.12±2.25</td>
<td>26.20±4.50</td>
<td>2.00±0.51</td>
<td>13.10±4.03</td>
</tr>
<tr>
<td>WT-A</td>
<td>21.14±3.13</td>
<td>10.56±1.57</td>
<td>1.05±0.28</td>
<td>10.06±3.07</td>
</tr>
</tbody>
</table>

### 3.3.2 Electronic Absorption Spectroscopy

Figure 3.2 shows the Soret and Q bands for WT-DHP A and the T56 mutants. Compared with the Soret band at 406 nm for WT-DHP, T56A and T56S are slightly shifted to 407 nm and 408 nm, respectively. A small component of low-spin heme, which is observed at ~ 420 nm, may be the cause of this red shift. For the Q band of the mutants, T56A and T56G demonstrate a significant shoulder around 440 nm and T56S shows a broad band between 500 nm and 550 nm. The red shift of the Q bands may also result from a component of low-spin heme for these three mutants. The Q band of T56V is similar to WT, with no obvious shift. The formation of hemichrome may explain the appearance of the low-spin heme in some mutants as discussed below $^{14}$.
3.3.3 Resonance Raman Spectroscopy

3.3.3.1 Ferric WT-DHP and T56 Mutants

All protein samples were freshly oxidized using K₃[Fe(CN)₆] as described in the experimental section prior to loading in a spinning cell. Figure 3.3 compares the RR core size marker bands region for WT-DHP A and the T56 mutants. By the end of RR measurement, the Soret bands of WT-DHP and all T56 mutants were still observed at ~ 406nm, with no observable shift. According to a previous study, the ferric heme in WT-DHP A shows both 6cHS form (ν₃ at 1481 cm⁻¹, ν₂ at 1562 cm⁻¹, ν₁₀ at 1611 cm⁻¹) and 5cHS form (ν₃ at 1494 cm⁻¹, ν₂ at 1568 cm⁻¹, ν₁₀ at 1632 cm⁻¹) [15]. The ν₃ band around 1505 cm⁻¹, observed particularly
in T56S and T56A, is indicative of a 6cLS form of heme iron\textsuperscript{[16]}. Since no exogenously added ligands were added to these samples, this may be an indication that hemichrome has been formed in these samples. Using the $v_3$ core size marker band for comparison, T56S has almost the same amount of 6cLS as 6cHS heme, while T56A only has a small component of 6cLS heme. This evidence for LS heme is in agreement with the electronic absorbance data discussed above. However, T56G does not have an obvious 6cLS component in the Raman spectrum despite the possible indication of 6cLS in the Q band spectrum.

\textbf{Figure 3.3.} Resonance Raman core size marker band region of 100 $\mu$M ferric WT-DHP A (black), T56A (orange), T56G (green), T56S (blue) and T56V (red) in 100 mM pH 7 KP buffer at 298 K. Excitation wavelength: $\sim$410 nm; resolution: 1.7 cm$^{-1}$; laser power at the sample: 60 mW; acquisition time: 180 s.
3.3.3.2 DHP-$^{12}\text{CO}$ and DHP-$^{13}\text{CO}$

Isotopic substitution is a powerful tool to identify vibrational bands by the change in the reduced mass. $^{13}\text{C}$ substitution causes the $\nu_{\text{Fe-c}}$ band to shift to a lower wavenumber because of the increase in the carbon mass. Figure 3.4 (A-E) demonstrate the $\nu_{\text{Fe-c}}$ band for DHP – $^{12}\text{CO}$ and DHP – $^{13}\text{CO}$ samples in the range from 450 cm$^{-1}$ to 650 cm$^{-1}$ for WT-DHP A and each T56 mutant. However, the isotopic shift is difficult to determine precisely from the spectrum, therefore, a difference spectrum is needed. Another benefit of a difference spectrum is that it can eliminate the interference from the porphyrin ring and systematic error, so the bands in a difference spectrum are only from the Fe-CO stretching and Fe-C-O bending modes. Difference spectra and Gaussian fitting are shown in Figure 3.5 (A-E) for WT-DHP and each T56 mutant.

In DHP-$^{12}\text{CO}$ spectra, only WT-DHP and T56V have relatively sharp $\nu_{\text{Fe-c}}$ bands, which are observed at 498.5 cm$^{-1}$ and 493.4 cm$^{-1}$, respectively. A previous measurement identified the $\nu_{\text{Fe-c}}$ frequency of WT DHP-$^{12}\text{CO}$ is 507 cm$^{-1}$ at pH 5$^{[11]}$. The difference in the observed $\nu_{\text{Fe-c}}$ frequency may result from the fact that our resonance Raman spectra were obtained at pH 7, but at pH 5 in the previous study. The lower $\nu_{\text{Fe-c}}$ frequency for T56V is also consistent with the higher $\nu_{\text{co}}$ frequency in the FTIR data, since $\nu_{\text{Fe-c}}$ and $\nu_{\text{co}}$ are inversely correlated due to $\pi$-back bonding donation from the heme iron$^{[17]}$.

Gaussian fitting was applied to the difference spectra for WT-DHP and each mutant. The band composition and isotope shift are listed in Table 3.3. T56S failed to be fitted in the difference spectra because the isotopic shifted data are of insufficient quality to determine an
accurate difference, although there is clearly an isotope effect. Therefore, the fitting for the T56S mutant is on the T56S-^{12}CO resonance Raman spectrum itself rather than the difference spectrum. The difference spectrum of WT-DHP was fit using a model with one Fe-C stretching band and one Fe-C-O bending band. However, T56A, T56G, T56S and T56V all have multiple Fe-C stretching bands, which may indicate multiple conformations of the distal histidine as observed in the FTIR spectra below. The isotope shift for each band is in the range of 1 cm\(^{-1}\) to 2 cm\(^{-1}\), which strongly supports the assignments of the bands as the Fe-C stretch and bend.
Figure 3.4. Resonance Raman spectra in the range of 450 cm\(^{-1}\) - 650 cm\(^{-1}\), showing Fe-C stretching band and Fe-C-O bending band of 100 \(\mu\)M ferrous DHP\(^{12}\)CO (red line) and ferrous DHP\(^{13}\)CO (blue line) for WT-DHP (A), T56A (B), T56G (C), T56S (D) and T56V (E) in 100 mM KP buffer at pH 7.
Figure 3.5. The difference spectra (purple line) of ferrous DHP-$^{13}$CO spectrum minus ferrous DHP-$^{12}$CO spectrum in the range of 440 cm$^{-1}$ - 600 cm$^{-1}$. Gaussian fitting (black line) was applied on difference spectra for WT (A), T56A (B), T56G (C), T56S (D) and T56V (E).
Table 3.3. Fe-C stretching frequency, Fe-C-O bending frequency and the shift between ferrous DHP-\(^{12}\)CO spectrum and ferrous DHP-\(^{13}\)CO spectrum at each frequency, obtained from the Gaussian fitting of the difference spectra for WT and T56 mutants.

<table>
<thead>
<tr>
<th></th>
<th>Fe-C Stretch</th>
<th></th>
<th>Fe-C-O Bend</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wavenumber (cm(^{-1}))</td>
<td>Shift (cm(^{-1}))</td>
<td>Wavenumber (cm(^{-1}))</td>
</tr>
<tr>
<td>WT</td>
<td>497.6</td>
<td>1.7</td>
<td></td>
</tr>
<tr>
<td>T56A</td>
<td>470.1</td>
<td>1.2</td>
<td>492.1</td>
</tr>
<tr>
<td>T56G</td>
<td>489.8</td>
<td>1.0</td>
<td>505.2</td>
</tr>
<tr>
<td>T56S*</td>
<td>485.6</td>
<td>NA</td>
<td>502.1</td>
</tr>
<tr>
<td>T56V</td>
<td>495.0</td>
<td>1.4</td>
<td>515.0</td>
</tr>
</tbody>
</table>

*Value is based on fitting to the T56S-\(^{12}\)CO resonance Raman spectrum itself rather than the difference spectrum.

3.3.3.3 DHP in the Presence of 2,4,6-TCP

In order to investigate if the substrate, 2,4,6-TCP, affects the structure of DHP, resonance Raman spectra were obtained for WT-DHP A and T56 mutants in the presence of 2,4,6-TCP. Freshly oxidized ferric DHP samples were prepared as discussed above at a final concentration ~100 μM. 1 mM 2,4,6-TCP was mixed with DHP in the Raman sample holder. The spectra of the mixture of DHP and 2,4,6-TCP for WT and T56 mutants are shown in Figure 3.6 (A-E). The red lines show the initial 540 seconds measurement and black lines show the subsequent 540 seconds measurement for the same sample.

During the first 540 seconds, for WT-DHP A and the four mutants, the spectra of the mixture of DHP and TCP have no large difference from the spectra of DHP itself. However, as time progresses, T56V has an obvious increase in the 6cLS component. This change may
come from the presence of the substrate 2,4,6-TCP, which may bind externally and push the distal histidine closer to the heme iron as a function of time. If the substrate binds internally, as recent data have suggested as an alternative possibility [Dr. Franzen, unpublished data], then the origin of the change in the H55 conformation may be dependent on the effect of substrate on H$_2$O ligation. This may be altered in the mutants because of the change in the precise conformation of H55. T56A and T56S also have a subtle increase on the 6cLS component, but still within the margin of error.
3.3.4 FTIR Spectroscopy

CO is an ideal probe for detecting the distal pocket environment of WT-DHP A and the T56 mutants studied here. The spectra of DHP-CO for WT and T56 mutants are shown in Figure 3.7. The CO stretching band ν_{CO} is observed in the FTIR spectrum in the range of
1900 cm\(^{-1}\) – 2000 cm\(^{-1}\). WT-DHP A and T56V appear to have a similar band shape. However, the predominant peak for T56V shifted to lower energy than WT-DHP by ~5 cm\(^{-1}\). The mutants T56G and T56A show a similar band shape, which has a pronounced two split peaks at ~1955 cm\(^{-1}\) and ~1942 cm\(^{-1}\). One is significantly red shifted and the other one is blue shifted relative to the band of WT-DHP A. T56S exhibits a broad band shape intermediate between the band shape of T56A and T56S.

![Absorption difference spectra of CO-ligated DHP for WT-DHP (black), T56A (orange), T56G (green), T56S (blue) and T56V (red).](image)

**Figure 3.7.** Absorption difference spectra of CO-ligated DHP for WT-DHP (black), T56A (orange), T56G (green), T56S (blue) and T56V (red). The final concentration of DHP is 2-3 mM in 100 mM KP buffer at pH 7. Peak positions and fractional areas are determined by Gaussian distribution and listed in Table 3.4.

In previous studies, the \(\nu_{\text{CO}}\) in the 'closed' conformation and the 'open' conformation were observed at ~1950 cm\(^{-1}\) and ~1965 cm\(^{-1}\), respectively. The band at ~1940 cm\(^{-1}\) appears to be analogous to A\(_3\) conformation in MbCO\(^{[10]}\). The A\(_3\) component has been interpreted to arise from a conformation of the distal histidine that is closer to CO than the A\(_1\) conformation.
Such a conformation gives rise to an even stronger interaction of the Ne-H of H55 with bound CO and hence further lowers its frequency. Table 3.4 gives the frequency and population of each band obtained from Gaussian fitting. T56A, T56G and T56S have an even lower frequency for the A3 component at ~1930 cm\(^{-1}\), which could mean that the distal histidine is located even closer to the heme iron than WT. The population at ~1944 cm\(^{-1}\) for WT and T56 mutants is approximately constant at ~40%. The ordering for the population of A1 conformation at ~1954 cm\(^{-1}\) is WT-DHP > T56V > T56A > T56G > T56S, and that of A0 conformation at ~1960 cm\(^{-1}\) is WT-DHP < T56V < T56A < T56S and T56G. These two trends are opposite, suggesting a trade-off between the ‘closed’ and ‘open’ conformation.

Table 3.4. Infrared stretching frequencies of the C-O bond in WT-DHP and T56 mutants. (Fractions of total population are given in parenthesis)

<table>
<thead>
<tr>
<th>T(K)</th>
<th>v(_{\text{co}}) (cm(^{-1}))</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>WTA[^{[10]}]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>1940 (15)</td>
<td>1949 (80)</td>
</tr>
<tr>
<td>WTA [Schkolnik et al. unpublished data]</td>
<td>298</td>
<td>1945 (42)</td>
</tr>
<tr>
<td>T56A</td>
<td>298</td>
<td>1932 (2.5)</td>
</tr>
<tr>
<td>T56G</td>
<td>298</td>
<td>1933 (6)</td>
</tr>
<tr>
<td>T56S</td>
<td>298</td>
<td>1931 (4)</td>
</tr>
<tr>
<td>T56V</td>
<td>298</td>
<td>1944 (35)</td>
</tr>
</tbody>
</table>

3.3.5 Molecular Dynamics Simulations

Analysis of the MD simulations includes the energies and fluctuations from the trajectory, with a special emphasis on the motion of H55 relative to the heme iron. H55 is a key determinant of both oxygen binding and enzymatic activation for peroxidases. Based on the kinetic assay results, mutation of threonine at position 56 may affect the conformation of
H55. Therefore, the position of distal histidine H55 relative to the heme iron is of great interest for understanding the experimental results. To quantitatively estimate the structure fluctuation, the distances between heme iron and N\(\delta/N\epsilon\) of H55 are plotted as a function of time.

The pKa of histidine is 6.04\(^{[18]}\), so at pH 7.0, the protonated nitrogen could be N\(\delta\) or N\(\epsilon\). These two tautomers in the ferrous form were simulated for an initial period of 10 ns. The average distance between N\(\delta/N\epsilon\) of the distal histidine and heme iron and the standard deviation of this distance for WT-DHP and each mutant are listed in Table 3.5. According to previous studies, the distance between N\(\epsilon\) of the distal histidine and heme iron is 4.1–4.6 Å for globins and 5.5–6.0 Å for peroxidases\(^{[2]}\). The corresponding distance for DHP is between these two ranges, which is in consistent with its bifunctional property.

Based on the results, because of the smaller fluctuation, the \(\epsilon\) tautomer appears significantly more stable than the \(\delta\) tautomer, except for T56G. This stability may arise from hydrogen bonding with the heme ligand. In the \(\delta\) tautomer, the distance from heme iron to N\(\delta/N\epsilon\) is similar between WT-DHP and each mutant. However, this distance in \(\epsilon\) tautomer is distinctive from each other. The average distance from heme iron to N\(\delta\) and N\(\epsilon\) in two tautomers for WT-DHP and each mutant is in the trend T56G > WT > T56A ≈ T56S > T56V. This trend demonstrates that the N\(\epsilon\) in T56V is the nearest to the heme iron, while in T56G the N\(\epsilon\) is the furthest to the heme iron. In T56A and T56S, this distance is similar and a little smaller than WT-DHP. In the T56G \(\epsilon\) tautomer, the fluctuations are considerably larger than the other mutants. This may be because of the hydrogen bond strength of the distal histidine
has decreased due to the increased distance from the heme iron. In contrast, the fluctuations in the T56V mutant are the smallest.

**Table 3.5.** Distance and its standard deviation between the heme iron and Nδ/ Nε of H55 in the initial 10ns for ferrous WT-DHP and T56 mutants δ/ε tautomers.

<table>
<thead>
<tr>
<th>Delta tautomer</th>
<th>Heme_Nδ (Å)</th>
<th>Standard Deviation</th>
<th>Heme_Nε (Å)</th>
<th>Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>6.30</td>
<td>0.64</td>
<td>5.46</td>
<td>0.52</td>
</tr>
<tr>
<td>T56A</td>
<td>6.68</td>
<td>0.64</td>
<td>5.69</td>
<td>0.70</td>
</tr>
<tr>
<td>T56G</td>
<td>6.52</td>
<td>0.72</td>
<td>5.60</td>
<td>0.68</td>
</tr>
<tr>
<td>T56S</td>
<td>6.59</td>
<td>0.76</td>
<td>5.43</td>
<td>0.77</td>
</tr>
<tr>
<td>T56V</td>
<td>6.67</td>
<td>0.62</td>
<td>5.50</td>
<td>0.60</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Epsilon tautomer</th>
<th>Heme_Nδ (Å)</th>
<th>Standard Deviation</th>
<th>Heme_Nε (Å)</th>
<th>Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>6.53</td>
<td>0.47</td>
<td>5.04</td>
<td>0.45</td>
</tr>
<tr>
<td>T56A</td>
<td>6.58</td>
<td>0.47</td>
<td>4.83</td>
<td>0.45</td>
</tr>
<tr>
<td>T56G</td>
<td>7.23</td>
<td>0.93</td>
<td>5.58</td>
<td>1.02</td>
</tr>
<tr>
<td>T56S</td>
<td>6.29</td>
<td>0.40</td>
<td>4.77</td>
<td>0.52</td>
</tr>
<tr>
<td>T56V</td>
<td>6.09</td>
<td>0.43</td>
<td>4.48</td>
<td>0.43</td>
</tr>
</tbody>
</table>

**3.4 Discussion**

Based on the spectroscopic (UV-vis, resonance Raman, FTIR), kinetic assays, and MD simulations data, we have formulated the hypothesis that a smaller amino acid in position 56 may permit the distal histidine H55 to rotate with greater flexibility, and to move to a position closer to the heme iron than accessible in WT-DHP. On the other hand, a larger amino acid in position 56 restricts H55 rotation to only a small range due to steric hindrance. The evidence for this hypothesis consists of the following five points. First, according to the FTIR data, the smaller amino acids in position 56, A, G and S, cause multiple νco bands which may be explained by a greater number of conformations of H55. This hypothesis is further confirmed by an increase in the A3 substate of νco band, which is much smaller in WT-
DHP and T56V. The FTIR spectra of WT-DHP and T56V are the most similar, with no obvious multiple bands, which is consistent with the fact that T and V are isosteric and may be large enough to limit the rotation of H55. Second, the RR spectra of DHP-CO samples indicate T56A, T56G and T56S have multiple νFe-c stretching bands, which further confirm the multiple conformations of H55 in these three mutants. Third, the RR spectra of ferric T56A and T56S exhibit a 6cLS component, which may result from the ligation of H55 to the heme iron to form hemichrome. The ability to form hemichrome is consistent with the greater flexibility of H55, since significant multiple conformations and even the A3 substate of distal histidibe are observed in RR and FTIR spectra. Fourth, MD simulations provide the data on the fluctuation of H55. The standard deviation for H55 motion decreases in the order T56G > T56A > T56S. While H55 has a relatively small fluctuation in both WT-DHP and T56V, which both have larger neighboring amino acids. Finally, the kinetic data suggest at high 2,4,6-TCP concentration, the initial turnover velocity for T56G is unexpectedly decreased. This may result from the fact that glycine is small enough to allow H55 to have greater flexibility, which permits even 2,4,6-TCP to enter the distal pocket in a manner that inhibits the enzyme. This is not entirely unexpected since inhibitors such as 4-BP, 4-CP, etc. can enter the distal pocket under all conditions \cite{15}. A mutant that permits greater fluctuation of H55 may result in a greater ability for a larger molecule to reside in the pocket.

Kinetic data were obtained in order to explore the correlation between the structure and the enzyme catalytic efficiency. The rate scheme for DHP is shown in Scheme 3.1.
**SCHEME 3.1. Rate Scheme for DHP Catalysis**[^19]

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

H\textsubscript{2}O\textsubscript{2} binds the heme iron as the co-substrate leading to the formation of Compound ES, which acts as the active intermediate for DHP. In the presence of the substrate, Compound ES will oxidize the substrate via two sequential one-electron oxidations and regenerate the ferric state. Based on this peroxidase modification, the Michaelis-Menten model for the DHP rate scheme is shown below[^19],

\[
V_0 = \frac{V_\text{max}[\text{XAOH}]}{K_m + [\text{XAOH}]}
\]

\[
K_m = \left(\frac{1}{k_2} + \frac{1}{k_3}\right)k_1[H_2O_2] \quad V_\text{max} = k_1[H_2O_2][E]_0
\]

The Michaelis-Menten fitting parameters are listed in Table 3.2. The catalytic rate constant, \(k_{\text{cat}}\) is in the trend T56G < WT < T56A/ T56S < T56V. \(k_{\text{cat}}\) is proportional to \(k_1\), which is the rate constant in the formation of Compound ES[^19]. The distal histidine acts as an acid-base catalyst to form Compound ES according to the Poulos-Kraut mechanism[^20]. The trend of \(k_{\text{cat}}\) is totally consistent with the trend of average distance from the heme iron to the distal histidine as shown in the MD simulation. Mutants with the distal histidine closer to

[^19]: Reference to the source of the rate scheme.
[^20]: Reference to the Poulos-Kraut mechanism.
the heme iron also have higher k\textsubscript{cat} values. This is in agreement with the previous study that if the distal histidine lies too far from the heme iron, it may not be an efficient acid-base catalyst to activate H\textsubscript{2}O\textsubscript{2} and form Compound ES\textsuperscript{[7]}. The catalytic efficiency, k\textsubscript{cat}/K\textsubscript{m} is observed in the order T56V < WT < T56S < T56A < T56G, which is opposite to the trend in the size of amino acid 56, V > T > S > A > G.

The most restricted motion appears in the T56V mutant. In the presence of substrate 2,4,6-TCP, the T56V mutant has an unexpected increase in the component of 6cLS as the time increases as shown in Figure 3.6. Therefore, we hypothesized that 2,4,6-TCP may bind externally and push the distal histidine of T56V to form hemichrome as the function of time. This may explain the kinetic result of T56V that k\textsubscript{cat} value is the highest for this mutant. However, the formation of hemichrome may compete with formation of a ferryl intermediate, which could explain why the catalytic efficiency is the lowest for the T56V mutant.

The potential for formation of hemichrome is always present in globins, particularly in the ferric state of the heme iron. All of the proteins studied here have the heme iron in a ferric state, which is a typical resting state for peroxidases. Hemichrome is formed in wild type SWMb subsequent to heme oxidation to ferric iron\textsuperscript{[21]}. Studies of mutants of Scapharca clam hemoglobin show low pH can also favor hemichrome\textsuperscript{[22]}. While previous studies were conducted at lower pH\textsuperscript{[23]}, we have conducted studies at pH 7 – 7.5 once we determined that this pH is optimum for product formation\textsuperscript{[24]}. Obviously, pH 7.4 is also the best model for physiological conditions. Nonetheless, the pervasive tendency for ferric hemoglobins to form hemichrome makes this possibility in DHP an entirely reasonable concern for the function. We have previously observed that proximal mutants that increase reactivity of the
heme iron by forming a charge relay can also increase the extent of hemichrome formation as well \[25\]. The present study makes a parallel finding for the distal side of the protein. All of the T56 mutations had an increase in catalytic rate, but correspondingly an increase in the tendency for hemichrome formation. Thus, we find that there is a tuning of reactivity on both the proximal and distal sides of the heme such that increased catalytic rate must be balanced with protection against hemichrome formation. As we systematically investigated the T56 mutations, this trade-off turned out to be the major issue.

In distinction to T56V, H55 in the T56G mutant appears to have the greatest flexibility to rotate. closed to the heme iron despite the fact that it is located on average further from the heme iron than WT-DHP and the other mutants in the initial 10 ns in the MD simulation. This model based on the simulation is also consistent with the available spectroscopic data. The higher catalytic efficiency of T56G cannot depend on the distance of H55 to the heme Fe, but rather must be related to the greater flexibility of the H55 evident in the r.m.s. motion of H55 in the MD simulations, which is ca. 1 Å compared to ca. 0.5 Å for the other mutants. Such a role for the flexibility of the distal amino acid has been suggested as a reason for the residual activity of aspartate in the H55D mutation \[26\]. In that case, the aspartate in the distal position appears to have a distribution of ca. 10% closed (internal) and 90% open (external).

The unexpected 6cLS component of T56A and T56S in the RR spectra and red-shifted Q band of T56A, T56G and T56S in the UV-vis spectra may suggest the formation of hemichrome. The hemichrome is formed when the distal histidine rotates to a position so close to the heme iron that it can form a covalent bond to the heme iron. The formation of hemichrome further inhibits the enzyme activity. However, these three mutants have higher
catalytic efficiency than WT-DHP. There are two possible explanations for this result. First, the distal histidine of T56A, T56G and T56S is able to move closer to the heme iron, which may promote a higher enzymatic activity than in WT-DHP. The formation of hemichrome in small fraction of the molecules may be offset by this greater enzymatic activity in the remaining fraction. It is difficult to estimate this fraction precisely by Gaussian fitting, but from the area of the band at 1505 cm$^{-1}$ we can estimate that ranges from 10-30% of the sample for the T56A and T56G mutants. However, another possibility is that the hemichrome is not formed during the time frame of kinetic assay, but rather as an artifact of laser excitation in the RR experiment. The Raman spectra were obtained under laser irradiation for circa 9 minutes for each measurement. This condition may also lead to more rapid degradation of the protein with the distal histidine closer to the heme iron. If hemichrome is dependent on laser irradiation it still must mean that these mutants have a conformation that permits the formation to a greater extent than in WT-DHP. Thus, the same factor that increases enzymatic activity also can result in inhibition under certain conditions. There appears to be a balance of factors that leads to optimum reactivity over the life of the enzyme.

3.5 Conclusion

Although the structural changes among threonine, alanine, glycine, serine and valine in position 56 are very subtle, there is a large effect on the peroxidase activity of these mutants compared to WT-DHP. These results suggest that the surrounding residues do have an effect on the flexibility of the distal histidine. Flexibility refers to the ability of H55 to move closer
to the heme iron and even to ligate to it. The experiments and simulations agree that the neighboring residue at position 56 plays a key role on controlling the conformation of H55 poised in a conformation that can function both for the globin and peroxidase function. Greater flexibility has been observed in WT-DHP relative to globins such as SWMb. However, that flexibility apparently is tailored to permit activation of bound H₂O₂ without permitting rapid formation of hemichrome. The results indicate that the small amino acids (A, G and S) in position 56 may remove the steric hindrance and allow the distal histidine to rotate with greater flexibility. The greater range of motion may accelerate the cleavage of O-O bond of H₂O₂ and increase the catalytic efficiency. Consistent with this hypothesis, T56A, T56G and T56S also permit the distal histidine to locate so close to the heme iron that they form a stable six-coordinate ferric adduct involving H55 itself (hemichrome). Hemichrome appears with the highest yield in the T56V mutant in the presence of 2,4,6-TCP on a longer time scale following laser irradiation in the rR experiment. While hemichrome appears to be accelerated by laser irradiation, the tendency for formation of this inactive form is clearly a disadvantage for the function of DHP. Only the WT-DHP keeps the heme iron stable and active in all situations. The balance of factors involving flexibility without hemichrome formation and access to inhibitors 4-BP could explain why WT-DHP has threonine at position 56, rather than any of the amino acids we have chosen to replace it in this study.

Further work will be done on the crystallography for each mutant to obtain the accurate conformation of the distal histidine. Moreover, other residues surrounding H55 can be focused on to insight the role of ‘spectator’ residues in order to understand a network
mechanism on DHP peroxidase activity. On the other hand, the mutants with greater activity could be chosen for bioremediation application benefit to the environment.
REFERENCES

1. Dunford, H., Heme peroxidases. 1999: WILEY-VCH.


CHAPTER 4
Study of the Effect of Electrostatic Surface Mutants on the Enzymatic Rate of DHP A

4.1 Introduction

DHP is the first peroxidase found with a globin structure. Therefore, DHP is a bifunctional protein which can transport and/or store oxygen as a globin as well as oxidize a substrate as a peroxidase [1-4]. 2,4,6-TBP is the natural substrate for DHP, which can be oxidized to 2,6-DBQ in the natural environment [4]. However, due to the limited solubility of 2,4,6-TBP, its analog 2,4,6-TCP has been used as the substrate in the most of the studies that have been conducted.

DHP has a unique two-site competitive inhibition mechanism [5]. Crystal structures, resonance Raman spectra and kinetic assays result indicate that DHP has a unique binding site at the distal pocket [5]. Para-halophenols, 4-XP, can enter the distal pocket and inhibit enzyme activity. Although resonance Raman and NMR spectroscopic data suggest 2,4,6-TCP binds externally [5-7], the exact location of the substrate binding site has not been determined. Recent evidence suggests that there is an internal binding site for the natural substrate 2,4,6-TBP [Serrano et al. unpublished data]. The significance of this observation is still not known.

Understanding the exact binding site for substrates 2,4,6-TCP and 2,4,6-TBP is very important to the study of DHP. In order to obtain this information, X-ray crystallography [Serrano et al. unpublished data], MD simulations [Franzen et al. unpublished data] and mutagenesis studies [8] have been done. Docking studies provide evidence that 2,4,6-TCP may bind outside of the distal
pocket near Tyr34\cite{Franzen et al. unpublished data}. This is not surprising because for the active intermediate Compound ES, the free radical may locate on Tyr34 or Tyr38 based on EPR data\cite{9}. Moreover, mutagenesis studies on the surface of DHP have been recently published\cite{8}. Since the pKa of 2,4,6-TCP is 6.2\cite{8}, at pH 7.0 which is the pH value for this study, TCP is negatively charged under physiological conditions. It was hypothesized that 2,4,6-TCP has an electrostatic interaction with surface charged amino acids and that the catalytic efficiency is reduced by an increase in the negative surface charge. This hypothesis has been tested by the kinetic results of a series of DHP surface mutants as shown in Figure 4.1\cite{8}. Mutations on the surface residues from positively charged to neutral or from neutral to negatively charged introduced negative charge on the protein surface. For negatively charged mutants, the repulsion between DHP and 2,4,6-TCP leads to a decrease on the DHP catalytic efficiency ($k_{cat}/K_m$ value) due to an electrostatic effect. On the other hand, the positively charged DHP mutant, like N61K, causes a slight increase on the catalytic efficiency. However, the change on the catalytic efficiency is still too small to determine the exact binding position for 2,4,6-TCP.

In order to further confirm the electrostatic effect between DHP and 2,4,6-TCP as well as test the exact binding position for TCP, a series of double mutants and positively charged mutants were designed. Our hypothesis is that if the mutation is near the binding site, there will be a large effect reflected on the catalytic efficiency.
Figure 4.1. Single wavelength kinetics at 273 nm (2,6-DCQ) for the oxidation of 2,4,6-TCP catalyzed by DHP. Enzymatic reaction initial velocities were plotted as a function of 2,4,6-TCP concentration. Assay conditions were [DHP] ~ 2.4 μM, [TCP] = 0.2 mM-1.5 mM, [H₂O₂] =1.2 mM, 150 mM KP buffer at pH 7 at 293 K\(^{[8]}\).

4.2 Experimental

4.2.1 Materials

All chemicals were purchased from Fisher Scientific and Sigma-Aldrich without further purification. Deionized water was purified by a Barnstead system. The oligo-nucleotide primers were synthesized by IDT DNA Technologies, Inc.

4.2.2 DHP Expression and Purification

Primers for each mutant are listed in Table 4.1. To prepare the plasmids containing two mutations, the first one was introduced using the plasmid of 6XHis-tagged WT-DHP A as the template, extracted and sequenced as discussed in Chapter 3. The second mutation was then
synthesized using the plasmid from first mutation as the template. The transformation, expression and purification are described in Chapter 3.

**Table 4.1.** The primers used in the construction of the surface mutants.

<table>
<thead>
<tr>
<th></th>
<th>Primer Sequence</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>E31Q</td>
<td>5’-G AAT AAG TAT CCG GAC <strong>CAG</strong> CGC CGC TAC TTC AA-3’</td>
<td>5’-TT GAA GTA GCG GCG <strong>CTG</strong> GTC CGG ATA CTT ATT C-3’</td>
</tr>
<tr>
<td>E65Q</td>
<td>5’-G TTC AAC CTG ATG ATG <strong>CAG</strong> GTT GCG GAC CCA GCC A-3’</td>
<td>5’-T GGC TCG GTC CGC AAC <strong>CTG</strong> CAT CAT CAG GTT GAA C-3’</td>
</tr>
<tr>
<td>G40K</td>
<td>5’-GC CGC TAC TTC AAA AAC TAT GTC <strong>AAG</strong> AAA TCT GAC CAA GAG CTC AAG TC-3’</td>
<td>5’-GA CTT GAG CTC TTG GTC AGA TTT <strong>CTT</strong> GAC ATA GTT TTT GAA GTA GCG GC-3’</td>
</tr>
<tr>
<td>K51G</td>
<td>5’-GAG CTC AAG TCA ATG GCC <strong>GGG</strong> TTC GGT GAT CAC ACT GA-3’</td>
<td>5’-TC AGT GTG ATC ACC GAA <strong>CCC</strong> GGC CAT TGA CTT GAG GTC-3’</td>
</tr>
<tr>
<td>N61K</td>
<td>5’-CAC ACT GAG AAA GTG TTC <strong>AAA</strong> CTG ATG ATG GAA GTT GCG G-3’</td>
<td>5’-C CGC AAG TTC CAT CAT CAG <strong>TTT</strong> GAA CAC TTT CTC AGT GTG-3’</td>
</tr>
<tr>
<td>N96D</td>
<td>5’-CC AGC CTG ACG ACT GGA <strong>GAC</strong> TTC GAG AAA CTG TTC GTG GC-3’</td>
<td>5’-GC CAC GAA CAG TTT CTC GAA <strong>GTC</strong> TCC AGT CGT CAG GCT GG-3’</td>
</tr>
<tr>
<td>Q88K</td>
<td>5’-C AAC ACA CTC GTC CAG ATG AAA <strong>AAG</strong> CAT TCC AGC C-3’</td>
<td>5’-G GCT GGA ATG <strong>CTT</strong> TTT CAT CTG GAC GAG TGT GTT G-3’</td>
</tr>
<tr>
<td>R33Q</td>
<td>5’-CCG GAC GAG CGC <strong>CAG</strong> TAC TTC AAA AAC TAT GTC GGC-3’</td>
<td>5’-GCC GAC ATA GTT TTT GAA GTA <strong>CTG</strong> GCG CTC GTC CGG-3’</td>
</tr>
</tbody>
</table>

**4.2.3 Kinetic Assay for Enzymatic Activity**

The kinetic assays were performed on a UV-vis photodiode array spectrometer (Agilent 8453) equipped with a Peltier temperature controller. All DHP samples were freshly oxidized to the ferric form at a final concentration of 2.4 μM in 150 mM potassium phosphate buffer
(150 mM KH$_2$PO$_4$, 100 mM K$_2$HPO$_4$, pH 7). 2,4,6-TCP concentrations were varied from 100 to 1400 μM. DHP and 2,4,6-TCP were mixed and incubated at 20 °C for 3 minutes in the thermal cells prior to the reaction to reach thermal equilibrium. H$_2$O$_2$ at a final concentration of 1200 μM was added to the cuvette to initiate the assay. The spectra were collected every 0.5 second for 60 seconds, monitoring the formation of the 2,6-DCQ at $\lambda_{\text{max}} = 273$ nm ($\varepsilon_{273} = 13200 \text{ M}^{-1} \text{cm}^{-1}$). The initial velocity $v_0$ was plotted as function of each 2,4,6-TCP concentration for the Michaelis-Menten fitting. The data were analyzed in Igor 5.0.

4.3 Results

E31Q, E65Q and G40K were designed to be single mutants with an increase of positive charge on the surface of the protein. Q88K/N61K was designed as a double mutant carrying even more positive charge by mutating both residues from neutral to positively charged. R33Q/N61K, N96D/E31Q and G40K/K51G were designed as double mutants in which one residue was mutated to more positively charged, and the other one to more negatively charged, but the net charge in the total charge is zero.

The initial velocities of the formation of the product 2,6-DCQ ($\lambda_{\text{max}} = 273$ nm, $\varepsilon_{273} = 13200 \text{ M}^{-1} \text{cm}^{-1}$) versus different 2,4,6-TCP concentrations are shown in Figure 4.2 for each mutant compared to WT-DHP. The Michaelis-Menten fitting parameters are given in Table 4.2 for WT-DHP and each surface mutant. The higher $k_{\text{cat}}/K_m$ value indicates a higher catalytic efficiency. The positively charged single mutants, E31Q and E65Q have a slightly higher $k_{\text{cat}}/K_m$ value than WT-DHP, as predicted in the previous study$^{[8]}$. G40K has a relatively distinct higher $k_{\text{cat}}/K_m$ value than WT enzyme. Moreover, the positively charged
double mutant Q88K/N61K has an expected even higher $k_{cat}/K_m$ value. For the neutral double mutants, the $k_{cat}/K_m$ value for R33Q/N61K has no significant difference from the WT enzyme. However, the $k_{cat}/K_m$ value for N96D/E31Q and G40K/K51G are far greater than WT-DHP, which indicates that these two neutral double mutants have 3-4 fold higher catalytic activity than WT-DHP.

![Figure 4.2](image)

**Figure 4.2.** Michaelis-Menten analysis of WT-DHP A (black), G40K (red), E65Q (green), E31Q (purple), Q88K/N61K (blue), R33Q/N61K (aqua), N96D/E31Q (teal) and G40K/K51G (olive), showing the initial reaction velocity versus substrate (2,4,6-TCP) concentrations. The assay conditions were [DHP] ~ 2.4 μM, [TCP] = 0.1 mM-1.4 mM and [H$_2$O$_2$] = 1.2 mM in 150 μM pH 7 KP buffer at 293 K. The Michaelis-Menten fit parameters are given in Table 4.2.
Table 4.2. Michaelis-Menten fit parameters for WT-DHP A and surface mutants.

<table>
<thead>
<tr>
<th></th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$K_m$ (mM)</th>
<th>$k_{cat}/K_m$ (mM$^{-1}$s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT$^{[8]}$</td>
<td>5.62±0.31</td>
<td>1.73±0.15</td>
<td>3.25±0.33</td>
</tr>
<tr>
<td>R33Q/N61K</td>
<td>5.82±0.54</td>
<td>1.58±0.24</td>
<td>3.68±0.66</td>
</tr>
<tr>
<td>N96D/E31Q</td>
<td>10.52±0.56</td>
<td>1.05±0.11</td>
<td>9.98±1.18</td>
</tr>
<tr>
<td>G40K/K51G</td>
<td>11.54±0.90</td>
<td>0.96±0.15</td>
<td>12.06±2.10</td>
</tr>
<tr>
<td>Q88K/N61K</td>
<td>6.27±0.49</td>
<td>1.05±0.16</td>
<td>5.94±1.02</td>
</tr>
<tr>
<td>E31Q</td>
<td>9.17±2.25</td>
<td>2.75±0.93</td>
<td>3.33±1.39</td>
</tr>
<tr>
<td>E65Q</td>
<td>6.87±0.63</td>
<td>1.72±0.25</td>
<td>3.99±0.69</td>
</tr>
<tr>
<td>G40K</td>
<td>7.33±0.84</td>
<td>1.47±0.28</td>
<td>4.97±1.11</td>
</tr>
</tbody>
</table>

4.4 Discussion

The previous study confirmed the electrostatic interaction affect the substrate binding$^{[8]}$. This effect is a common feature for peroxidases, such as for cytochrome c6 and cytochrome P450scc$^{[8, 10, 11]}$. For DHP, changing the charge of a surface residue appears to be a global effect, which means the catalytic efficiency will be affected regardless of the position of the mutation on the surface. Nonetheless, it is believed that if the mutated residue is near the 2,4,6-TCP binding site, there should be a large effect on the enzymatic catalysis. This rationale assumes that the substrate binding site is on the surface of the DHP protein, which has been called into question by a recent X-ray crystal structure that revealed an internal binding site for the native substrate, 2,4,6-TBP. The rationale for the design of neutral double mutants in this work is that they do not change the global charge of DHP, but may change the local electrostatic field near the mutation site. If these mutations happen to coincide with the substrate binding site, the local effect could be large, despite the fact that the global effect should be nearly zero for these neutral charged double mutants.
According to the electrostatic effect, there should be no major effect on the catalytic efficiency for the neutral charged mutants, which is indeed the case for the R33Q/N61K mutant. However, the high $k_{cat}/K_m$ value for N96D/E31Q and G40K/K51G suggests that the binding site may be near these residues. The positions of these residues are shown in Figure 4.3. The radical of the active intermediate Compound ES is located on Y38 or Y34 [9]. Therefore, Y34 or Y38 may be included in the electron transfer pathway to the 2,4,6-TCP binding site. Figure 4.3 demonstrates that N96 and E31 are located on one side of Y34 while G40 and K51 are located on another side. When the substrate binds, the electron could be transferred between N96 and Q31 or G51 and K40 for each double mutant, respectively.

**Figure 4.3.** Key residues related to the substrate binding shown in the WT-DHP A X-ray crystal structure (PDB 2QFK).
Overall, the considerable effect on the catalytic efficiency for the neutral double mutants N96D/E31Q and G40K/K51G supports the hypothesis that the substrate binding site is outside the distal pocket and perhaps located near Y34. However, a recent X-ray crystal structure [Serrano et al. unpublished data] indicates that the natural substrate 2,4,6-TBP has an internal binding site in the distal pocket rather than the previously proposed external site. This internal binding site is proposed to be different from the other internal binding site of the inhibitor 4-XP. An internal binding site may also be consistent with observed kinetic effects since the substrate must enter the protein at a defined location, and the rate of binding may be affected by the local electrostatic effect in the double mutants.

The internal binding of 2,4,6-TBP may require a new two-electron oxidation mechanism, which occurs inside the protein and is followed by \( \text{H}_2\text{O} \) attack. In a previous study \([12, 13]\), flow-EPR data indicated a transient free radical in solution, which suggested 2,4,6-TCP phenoxy radical diffuses away from the DHP surface following oxidation and subsequently reacts with another radical by disproportionation to form a phenoxy cation and a substrate molecule. This is consistent with the one-electron oxidation mechanism and the proposed external binding of substrate \([13, 14]\). NMR data also indicated that 2,4,6-TCP binding does not affect the internal residues \([6, 7]\). Combined with the results in this study, we believe that 2,4,6-TCP binds externally. 2,4,6-TCP and 2,4,6-TBP may bind in different sites because of their different properties, such as different molecular shape and hydrophobicity. Crystallography, NMR and further mutagenesis studies are needed in the future to determine the exact position of 2,4,6-TCP binding site.
4.5 Conclusion

The previous study confirmed the electrostatic effect between substrate 2,4,6-TCP and the surface residues of DHP\textsuperscript{[8]}. Since at the physiological condition, pH 7, substrate 2,4,6-TCP is most likely in the anionic form\textsuperscript{[8]}, mutants with more positive charge on the surface has higher catalytic efficiency than WT-DHP. Three single positively charged mutants (E31Q, E65Q and G40K) demonstrate higher catalytic efficiency than WT-DHP and the double positively charged mutant (Q88K/N61K) has even higher catalytic efficiency. This result further confirm the electrostatic effect between substrate 2,4,6-TCP and DHP. We also designed three neutral double mutants. R33Q/N61K shows similar catalytic efficiency as WT-DHP as expected. However, N96D/E31Q and G40K/K51G show unexpected much higher catalytic efficiency, which indicate the substrate 2,4,6-TCP may bind closed to these residues, since the mutation changed the local electric field. N96 and Q31 or G51 and K40 locate on two different sites of heme, but both sites locate outside the distal pocket and next to Y34, which is hypothesized the key residue carrying the free radical in the intermediate Cmp ES. Even though the X-ray crystal structure gave some evidence that the natural substrate 2,4,6-TBP binds inside the distal pocket, combined our studies with previous flow EPR\textsuperscript{[12, 13]} and NMR data\textsuperscript{[6, 7]}, the substrate 2,4,6-TCP is believed to bind externally. This difference may come from different properties between 2,4,6-TBP and 2,4,6-TCP, such as different molecular shape, electronegativity, etc. Due to the limited solubility of 2,4,6-TCP, the crystal structure of DHP soaked with TCP has not been obtained. Further crystallography,
NMR and mutagenesis studies are needed in the future to determine the binding site of substrates.
REFERENCES


