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Structure-Function Relationship In Dual Function Hemoglobin/Dehaloperoxidase From Amphitrite Ornata

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STRUCTURE-FUNCTION RELATIONSHIP IN DUAL FUNCTION HEMOGLOBIN/DEHALOPEROXIDASE FROM AMPHITRITE ORNATA

by

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Submitted in Partial Fulfillment of the Requirements
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DEDICATION

This work is dedicated to my parents in China who always give me constant love, supports and encouragements. I would like also dedicate this to all my friends who inspire me to follow my heart and share life with me. Thank you all for everything you have done for me. Thank you!
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ABSTRACT

Hemoglobin/dehaloperoxidase (DHP) from Amphitrite ornata, is the first discovered globin which in addition to the oxygen binding has an additional physiological function as an enzyme. In the ferric state it is a dehaloperoxidase, which dehalogenates a wide range of halophenols. DHP evolved from an ancestral oxygen carrier and acquired the peroxidase function in response to environmental pressures. The peroxidase activity of DHP is much higher than that of vertebrate myoglobins (Mb) yet lower than that of typical peroxidases. Also, DHP exhibits about 10-fold lower oxygen affinity than Mb.

The first aim of this research was to identify the halophenolic substrates binding sites by determining the crystal structures of complexes of DHP with a substrate 2,4,6-trichlorophenol (TCP). Two mutually exclusive TCP binding modes were observed in the crystal structures of DHP mutants. They provided important implications for the DHP catalytic mechanism. The substrates binding order is the same as in classical peroxidases: hydrogen peroxide binds first leading to the formation of Compound I and only this intermediate binds halophenolic substrates in the productive manner. The binding of halophenols to DHP, prior to the formation of Compound I, prevents the approach of hydrogen peroxide to the heme and is inhibitory. This model was confirmed by our observation that higher substrate concentrations are inhibitory.
The second aim of this research was to study the functional and structural properties of two closely related DHP isoenzymes: DHP A and DHP B which differ in only five amino acids. The first approach was to investigate functional and structural differences, based on the analysis of transition mutants from DHP A to DHP B (Y34N DHP A and Y34NS/91G DHP A). The second approach is to study the “DHP A-like” K42Y Mb mutant and “DHP B-like” K42N Mb mutant, which mimic the heme environment at position 34 in DHPs. These studies suggest the roles of amino acids at positions 34 and 91 in the variation of functional properties between DHP A and DHP B.

The third part of this thesis describes the binding of phenol, a relatively big ligand, in the proximal cavity. The kinetic studies showed that phenol acts both as a competitive inhibitor likely interfering with the substrate binding at the heme edge and as a weak activator, likely through binding in the proximal cavity.
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LIST OF ABBREVIATIONS

2xYT ................................................................. 2x Yeast extract/tryptone
4-IP ........................................................................... 4-iodophenol
AS ............................................................................. ammonium sulfate
CM52 ................................................................. Carboxymethyl Cellulose
CO ............................................................................. carbon monoxide
CpI ................................................................. Compound I
CpII ................................................................. Compound II
DHP ................................................................. hemoglobin/dehaloperoxidase
DMSO ................................................................. dimethyl sulfoxide
DNase ................................................................. deoxyribonuclease
DTT ................................................................. dithiothreitol
EDTA ................................................................. ethylenediaminetetraacetic acid
H₂O₂ ................................................................. hydrogen peroxide
HPR ................................................................. horseradish peroxide
IPTG ................................................................. isopropyl-l-thio-3-D-galactoside
LB ................................................................. Lysogeny broth
Mb ................................................................. myoglobin
MbCO ............................................................... carbon monoxide myoglobin
N₂ ................................................................. nitrogen

xx
O₂ ................................................................. oxygen
PDB ................................................................. protein data bank
PEG ................................................................. polyethylene glycol
RNase .............................................................. Ribonuclease
SER-CAT ......................................................... South Eastern Region Collaborative Access Team
TCP ............................................................... Trichlorophenol
Tris ................................................................. Tris(hydroxymethyl)aminomethane
CHAPTER 1

MINI REVIEW OF DHP PROPERTIES
INTRODUCTION

Hemoglobin/Dehaloperoxidase, referred as DHP, was first isolated from *Amphitrite ornata*, which is a sea worm classified as a terebellid polychaete. The body of the worm is bright red due to the presence of its coelomic hemoglobin - DHP. This organism inhabits coastal mud flats contaminated by toxic bromoaromatic compounds produced by other polychaete worms, which secret these bromometabolites to defend against competing species. In order to survive in such toxic environment, hemoglobin in *A. ornata* has evolved to posses the dehaloperoxidase function as a mechanism for detoxifying these compounds. DHP has been shown to have the ability to dehalogenate various halophenols in the presence of hydrogen peroxide (1). DHP as a globin, also retains the original function of oxygen transport and storage (2). Since dioxygen carrier/storage function is more ancient than the emergence of polychaete, the peroxidase activity of DHP was proposed to evolve relatively recently (2, 3).

The structures of DHP and several of its mutants have been determined by X-ray crystallography (2, 4-6). Based on these crystal structures, DHP forms a dimer with two identical 15.5 kDa subunits, and has a typical globin fold with eight $\alpha$-helixes. The structure of DHP closely resembles that of myoglobin (Mb), despite little amino acid sequence identity (2). The least-square superposition between DHP and Mb based on $C_{\alpha}$ positions is shown in Figure 1-1a (7, 8). Similar with other heme-containing globins and peroxidases, each subunit in DHP contains a heme center, in which there is a proximal histidine serving as a heme iron ligand and a distal histidine responsible for dioxygen or hydrogen peroxide binding. (2, 9-14).
**Unique distal histidine flexibility in DHP**

The distal histidine is a significant factor in the peroxidase catalysis and oxygen-binding reactivity because it regulates ligand binding to the heme iron in the distal pocket (7, 15). Previous studies, including X-ray structure determination and spectroscopic investigation indicate that the unique distal histidine in DHP is highly mobile (6, 16). The different extent of conformation flexibility of the distal histidine in the crystal structures are shown in Figure 1.2. At room temperature, the conformations of the distal histidine are in equilibrium between a closed form and an open form, which corresponds to a six-coordination high spin state (6cHS) and a five-coordination high spin state (5cHS), respectively (Figure 1.2 a) (2). In the closed form, His55 is stabilized in the distal cavity by forming a hydrogen bond with the coordinated ligands. The single closed conformation of distal histidine was observed in both the metraheme and oxyferrous DHP, in which there are a water molecule and a dioxygen molecule, respectively, coordinated to the heme iron (Figure 1.2 c) (5). In contrast, in the open form His 55 swings out from the pocket through rotating around the Cα-Cβ bond and is exposed to the solvent. This conformation is clearly seen in the structure of deoxy DHP, in which the sixth heme iron ligand is absent and the hydrogen bonding interaction for stabilization of distal histidine is lost (Figure 1.2 d) (6). Also, The binding of halogenated phenols (4-XP, 2,4,6-TBP or 2,4,6-TCP) in the distal pocket pushes the distal histidine into the open conformation (Figure 1.2 b) (2, 17, 18). In sperm whale myoglobin (swMb) the open conformation of the distal histidine (His64) was only observed in the structure of CO-Mb upon His64 protonation at pH 4, whereas the closed conformation is favored at the higher pH (19). However, the structures of DHP crystallized at pH 6.5 show that the open form of the
distal histidine in DHP is present at about 50% occupancy. The unique distal histidine conformational flexibility of DHP has been postulated to be important for functional switching from O2-carrier function to peroxidase by means of regulating the oxygen or water displacement from the heme iron in the presence of tri-substrates (5, 20). The two confirmations of the distal histidine were also suggested to mediate the competition between 4-XP inhibitors in the internal binding site and substrates in the external binding site (15, 17, 21).

**STRUCTURE-FUNCTION RELATIONSHIP IN PEROXIDASE/GLOBIN**

Many studies have been devoted to investigating effects of distal and proximal sites on peroxidase activity (22-26). In the distal site, the proper position of the distal histidine, which is suitable for the heterolytic cleavage of hydrogen peroxide to form compound I, was indicated as a crucial structural factor for peroxidase activity (7, 22). The short distance between the atom Nε² of the distal histidine and the heme iron (4.3 Å) was proposed to be a cause that led to weak peroxidase activity in Mb. Several myoglobin variants (Mb T56G, T56I and F43H/H64L) with increased His-Fe distance have been engineered successfully and shown to exhibit enhanced catalytic activities. In DHP the distance of the Nε² of the distal histidine to heme iron is 5.4 Å, which is in between 4.3 Å for Mb and 6.0 Å for horseradish peroxidase, while the peroxidase activity of DHP is also in between (27-29). Besides the distal site, the proximal effects on catalysis could result from charge relay mechanism or rotational position of the proximal histidine. The charge relay mechanism is referred to as the “push” effect in peroxidase (23, 30). In this effect, the hydrogen bonding between proximal histidine and nearby residues affects the basicity of proximal histidine and therefore controls the electron
density on the heme iron, which regulates catalysis. In globins, poor activity is partially due to a weaker push in proximal site; in peroxidase, the stronger hydrogen bond between aspartate with the proximal histidine leads to a stronger push for peroxidase activation. In the proximal site, the most significant difference observed between DHP and globins is that the plane of the imidazole ring in DHP is rotated by about 60° relative to that in Mb (Figure 1.1 b) (11). This rotation in DHP results in the formation of a stronger hydrogen bond between the atom Nδ1 of proximal histidine and a carbonyl in main chain, and therefore pushes more electrons on the heme iron. Additionally, the proximal histidine was observed to be positioned on a loop in DHP rather than a α helix as in the globins due to a 2 amino acid shift of the proximal histidine in the sequence (Figure 1.1 b). The double mutant H93K/T95H Mb, which was engineered to mimic the spatial shift and rotation in DHP, displays ~5 fold increase in peroxidase activity relative to wt-Mb (7).

These factors regulating peroxidase catalysis in the distal and proximal cavities were also found to modulate the oxygen binding on heme proteins (31-34). In the distal site, H-bond interaction between the distal histidine and FeO2 could stabilize the bonded oxygen (31). Consistently, the O2 dissociation constant was found negatively correlated with the electrostatic interactions in the distal binding site (32). In the proximal site, the Fe-O bond is stabilized due to electron donation from iron based on charge relay mechanism (35). The rotational position of the proximal histidine was also proposed to be an important factor to modulate oxygen affinity (36, 37). For example, in leghemoglobins the distal histidine doesn’t significantly contribute to oxygen affinity like Mb, whereas the 45 degrees rotation of imidazole ring relative to Mb was suggested to play key role to stabilize the O2 binding (36, 38).
THE INHIBITION AND SUBSTRATE BINDING SITES IN DHP

The parahalogenated phenols (4-XP) binding in the distal pocket was clearly shown in the X-ray crystal structure, Resonance Roman and NMR studies (2, 13, 17, 39-41). From the crystal structure, the monohalogenated phenol is positioned in the distal cavity, forming contacts with surrounding hydrophobic amino acids (Phe21, Val59, Phe35). The 4-XP appears to be stabilized in the pocket through the hydrogen bond between the hydroxyl groups of Tyr38 and 4-XP. It is notable that the monohalogenated phenol molecule is located above the heme but does not bind to the heme iron, which is a unique feature of DHP (Figure 1.3). In other globins, the large organic molecules, which bind into the distal cavity, were always observed to be coordinated to the heme iron (42, 43). In addition, the 4-XP binding leads to the displacement of the coordinated water molecule in the distal cavity and the solvent-exposed position of distal histidine 55. Originally, the 4-XP binding site was suggested to be a substrate-binding site based on the first observed DHP-4-iodophenol complex (2). Under this postulation, the distal histidine undergoes a conformation change towards the open form and enables the substrate to enter into the distal pocket after formation of a high-valent iron-oxo intermediate (Compound I). However, the follow-up mutagenesis studies suggest a different trihalophenol-binding site exists besides the internal binding site (44). The mutant Y38F, which remove the hydrogen bonding between the halophenols and protein, surprisingly increase the catalysis efficiency about 11 fold with 2,4,6-TBP as the substrate relative to DHP (44). Another mutant V59W, which blocks the internal binding site due to the bulky side chain, shows comparable turnover with DHP.
Further investigation revealed that the internal 4-XP binding site is inhibitory rather than the substrate binding site (17, 45). Accordingly, 4-BP, is a poor substrate, an effect likely resulting from self-inhibition. Moreover, at the 2:1 ratio of 4-bromophenol to 2,4,6-tribromophenol, 4-BP completely inhibits peroxidase catalysis of DHP. Thompson et al. proposed an unusual two-site competitive inhibition mechanism in DHP (17). The internal binding of 4-XP prevents the binding of co-substrate peroxide and the open conformation of distal histidine 55 eliminates the acid-base catalytic ability of His55. In contrast, the binding of tri-halophenol substrates on the heme edge pushes the distal histidine 55 into the closed form in order to promote the catalysis.

Extensive data suggested the external substrate binding site is present near the heme β- or δ- edge and oxidation of tri-halophenol likely occurs on the surface of DHP-A, similar with typical peroxidases (14, 17, 40). A high pressure xenon derivation provides the evidence of the existence of binding site on the surface, although less well-defined (46). Most recently, a novel internal substrate-binding site in the distal cavity was observed, which is located deeper inside the distal cavity compared to the internal 4-XP binding site (Figure 1.4). The DHP-2, 4, 6-triboromphenol (TBP) complex mode suggested oxidation of TBP possibly occurs inside the protein by a two-electron sequential mechanism (18).

**MECHANISM OF DHP**

Regarding the unique dual functional nature of DHP, it is reasonable that its mechanism is more complicated than classic peroxidases and globins. The mechanistic pathways proposed by Franzen and coworkers are shown in Figure 1.5 (15). For dehaloperoxidase activity, DHP acquires a ferric resting state and catalyzes the reaction
via typical peroxidase chemistry (47). Generally, the ferric DHP is activated by addition of H$_2$O$_2$ and converted to the intermediate Compound I, which is a high valent Fe$^{IV}$=O oxidant containing a $\pi$-cation radical. The internal electron transfer from porphyrin to a nearby tyrosine occurs in the absence of substrate and leads to the formation of Compound ES (40, 48). The Compound ES subsequently oxidizes a substrate molecule by a one-electron oxidation step and converts to another intermediate Compound II, which is the Fe$^{IV}$=O state. The second one-electron oxidation step sequentially takes place with Compound II in a similar way and regenerates the resting ferric state of DHP. Meanwhile, the phenoxy radicals are released from DHP and generate the quinone product by reacting with water. In our recent studies, we further confirmed that the dahalogenation reaction mechanism catalyzed by DHP involved two consecutive one-electron steps via Compound I and Compound II, in which peroxide binds first and trihalophenol binds second. In addition, we proposed that the distal His55 in Compound I or Compound II swung out of the pocket to recruit the substrate trichlorophenol through a hydrogen bonding contact, rather than remaining in the closed form (49).

In the organism, oxyferrous DHP is more abundant and stable than its ferric form, indicating that transporting oxygen is its main function. However, to carry out the peroxidase function to detoxify the haloaromatics in the living environment, oxy-DHP must be enzymatically activated and enter into the catalytic cycle. Both Dawson and Franzen and their coworkers have demonstrated that the dehalogenation reaction can be initiated from the oxy ferrous form of DHP via the formation of intermediate Compound II in the presence of substrates TCP and co-substrate peroxide, although the exact conversion mechanism is still controversial (28, 50). Two alternative mechanisms for
activation of oxyferrous DHP were proposed. In one mechanism, suggested by Dawson and coworkers (28), the formation of Compound II was induced by a trace amount of ferric TCP and thus oxidizes TCP and generates TCP radicals, which in turn oxidize ferrous DHP to ferric DHP. Another mechanism, advanced by Franzen and coworkers (15), proposed that the conversion from oxyferrous to ferric states of DHP is mediated by distal histidine in the presence of substrate TCP. In this mechanism, the binding of TCP results in tautomerization of the distal histidine by forming a hydrogen bond between the atom N$\text{e}^2$ of His55 and the hydroxyl group of TCP. The tautomerization interrupts the hydrogen bonding interaction between the distal His55 and bonded oxygen and destabilizes the oxygen binding in oxyferrous DHP, and therefore deoxy-DHP is populated in the equilibrium. Upon the binding of H$_2$O$_2$, the distal histidine is positioned into closed position and acts as a general base to facilitate the formation of Compound II.
Figure 1.1. (a) Superposition of DHP (blue; PDB code 1EW6) and Mb (green; PDB code 1A6K). The overall folds of the heme-containing DHP and Mb are closely similar. (b) Difference of the proximal histidine positions between DHP and Mb. The histidine is located in a coil loop in DHP, whereas it is in a helical conformation in Mb. The imidazole ring in DHP is rotated by about 60° relative to that in Mb [adapted from reference (7)].
Figure 1.2. Conformation flexibility of the distal histidine 55 in DHP. (a) In the x-ray structure at room temperature, the open form and closed form are in equilibrium (PDB code 1EW6). (b) The distal histidine is in the open form due to the 4-XP molecule binding into the distal pocket (PDB code 1EWA). (c) His 55 only presents in the closed form in the metaquo-DHP at 100K (PDB code 2QFK). (d) In the deoxy DHP, His 55 is the open conformation (PDB code 3DR9).
Figure 1.3. Structure of the DHP•4-idophenol (4-IP) complex (PDB code 1EW6). The 4-IP molecule, shown in pink, is positioned in the distal hydrophobic pocket and forms a hydrogen bond with hydroxyl group of Try 38.
Figure 1.4. Superposition of DHP•4-bromophenol (4-BP) complex (PDB code 3LB2), shown in turquoise atom color, and DHP •2,4,6-tribromophenol (TBP) complex (PDB code 4ILZ), shown in yellow atom color. The 4-BP molecule is shown pink and the TBP molecule is shown in red.
Figure 1.5. The proposed catalytic mechanism for DHP. The change of distal histidine positions was shown.
CHAPTER 2

COMPLEXES OF DUAL FUNCTION HEMOGLOBIN/DEHALOPEROXIDASE WITH SUBSTRATE 2,4,6-TRICHLOROPHENOL ARE INHIBITORY AND INDICATE HALOPHENOL BINDING TO COMPOUND I 49

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ABSTRACT

The hemoglobin of a sea worm *Amphitrite ornata*, which for historical reasons is abbreviated as DHP for dehaloperoxidase, has two physiological functions: it binds dioxygen in the ferrous state while in the ferric state it dehalogenates halophenols, such as 2,4,6-trichlorophenol (TCP), using hydrogen peroxide as the oxidant. The crystal structures of three DHP variants (Y34N, Y34N/S91G and L100F) with bound TCP show two mutually exclusive modes of the substrate binding. One of them, the internal site, is deep inside the distal pocket with the phenolic OH moiety forming a hydrogen bond to the water molecule coordinated to the heme Fe. In this complex, the distal histidine is predominantly located in the closed position and also forms a hydrogen bond to the phenolic hydroxide. The second mode of TCP binding is external, at the heme edge, with the halophenol molecule forming a lid covering the entrance to the distal cavity. The distal histidine is in the open position and forms a hydrogen bond to the OH group of TCP, which also hydrogen bonds to the hydroxyl of Tyr38. The distance between the Cl4 atom of TCP and the heme Fe is 3.9 Å, non-bonding. In both complexes TCP molecules prevent the approach of hydrogen peroxide to the heme indicating that the complexes are inhibitory and implying that the substrates must bind in the ordered fashion: hydrogen peroxide first, TCP second. Kinetic studies confirmed DHP inhibition by high concentrations of TCP. The external binding mode may resemble the interaction of TCP with Compound I, the catalytic intermediate to which halophenols bind. The measured values of the apparent $K_m$ for TCP were in a 0.3-0.8 mM range, much lower than the concentrations required to observe TCP binding in crystals. This indicates that during
catalysis TCP binds to Compound I. Mutant F21W, which likely has the internal TCP binding site blocked, has about 7% of the wt-DHP activity.

**INTRODUCTION**

*Amphitrite ornata* is a sea worm which due to its appearance was named after the Greek goddess Amphitrite, the gorgeous wife of Poseidon. Its main body buried in mud is red while white tentacles extent into the water to catch food. The red color is due to coelomic hemoglobin, which is its most abundant protein. *A. ornata* co-inhabits estuarine mud flats with other polychaete worms, such as *Notomatus lobatus*, and hemichordata, such as *Saccoglossus kowalewskii*, which produce and secrete bromophenols presumably to repel predators and competing species. It has been proposed that to survive in such a toxic environment, the ancestral coelomic hemoglobin of *A. ornata* evolved to have, in addition to its dioxygen binding function, a dehaloperoxidase (DHP) functionality that neutralizes the poisonous bromophenols (13). As a dehaloperoxidase, DHP has a relatively broad specificity and although its physiological substrates are mainly bromophenols, it can oxidize a number of other haloaromatics according to Scheme 1 (1).

![Scheme 2.1](image)

Scheme 2.1. The reaction catalyzed by DHP.
Other globins like hemoglobin or myoglobin from vertebrates, whose physiological role is limited to oxygen binding, also have some peroxidase activity in the ferric state but it is lower by a factor in a 10-20 range, depending on the substrate (27-29). On the other hand, specialized peroxidases, such as horseradish peroxidase, catalyze the dehaloperoxidase reaction faster than DHP by over one order of magnitude (51). Thus the DHP catalytic efficiency is in between those of classical globins and peroxidases with a His-ligated active site that is more globin-like than typical peroxidases (25, 52). The dioxygen binding function is universal and much older, 1.8 billion years (3), than the emergence of polychaete in the Middle Cambrian about 530 million years ago (53). Thus, the contemporary DHP evolved from an ancestral oxygen carrier as environmental pressures led to the evolution of its dehaloperoxidase activity and the emergence of its physiologically relevant dual function.

Two genes (dhpA and dhp B) present in A. ornata code for two isoproteins DHP A and DHP B, each 137 amino acids long, which are different in only 5 amino acids (54). Among those differences, Y34N is the closest to the heme, S91G is in the loop that contains the proximal histidine, His89, while other differences, I9L, R32K and N81S are more conservative and placed further from the heme. The studies reported herein of the Y34N variant of DHP and Y34N/S91G double mutant were undertaken to identify the contribution of these variations to the functional differences observed between DHP A and DHP B (55). The L100F was designed to partially block the interior of the distal cavity (7). While the physiological substrates of DHP are mostly bromophenols, the protein has been considered for bioremediation of sites polluted with anthropogenic 2,4,6-trichlorophenol (TCP). Also, TCP is more soluble than bromophenols and has been
often used in kinetic and spectroscopic studies although its apparent K_m value has not been reported. This report is the first crystallographic study of TCP complexes.

**MATERIALS AND METHODS**

*Construction of native DHP plasmid*

The native DHP plasmid was generated from the pET-16b plasmid containing the gene of 6×His tagged DHP A and a unique NcoI site at 5′-terminus. In order to remove the 6×His tag, the second NcoI site was introduced by using a mutagenesis primer of sequence (5′→3′) CCACCACCACCCCATGGGTT and its reverse compliment. The new plasmid incorporated two NcoI sites and was double digested with NcoI. Subsequently, it was ligated with T4 ligase followed by transformation into DH10β cells.

*Site-directed mutagenesis*

The DHP mutants were generated using the QuickChange method. The mutagenic primers were designed to be complementary to wt-DHP cDNA around the site where the mutation was introduced. The desired DHP mutations were confirmed by LiCor DNA sequence analysis of the entire DHP gene (Engencore Laboratory at the University of South Carolina).

*Expression of DHP in Rosetta(DE3)pLysS*

The pET-16b plasmid containing the gene encoding Y34N DHP mutant was transformed into the Rosetta(DE3)pLysS competent cells and subsequently plated on agar plates with 30 µg/mL chloramphenicol and 100 µg/mL ampicillin. The cell cultures were inoculated in 2XYT medium with the proper antibiotics at 37 °C. When OD_{600} achieved
0.6, the temperature was lowered to 30 °C and IPTG was added to a final concentration of 2 mM in order to induce the DHP expression. The cells were kept at 30 °C overnight, pelleted by centrifugation and stored at -80 °C.

**Purification of 6xHisDHP and Native DHP**

Purification of the his-tag DHP mutants was conducted with established protocols and all the kinetic studies were performed using his-tag DHPs (7, 20). Recombinant native DHP mutants were purified as previously described with minor modifications (5). A 25 g cell pellet was thawed and suspend in 100 mL lysis buffer (20 mM Tris pH 8.0, 1 mM EDTA). Lysozyme, DNase, RNase, and dithiothreitol were separately added to final concentrations of 1 mg/mL, 15 µg/mL, 50 µg/mL, and 0.5 mM, respectively. After incubation for 45 min at 4 °C, the mixture was mildly sonicated with duty cycle 90% and output 6 (7 cycles of 30 seconds bursts and 4 approximately 5 min intervals). Subsequently, cell debris was removed by centrifugation for 40 min at 14,000 rpm. The reddish brown supernatant was subjected to ammonium sulfate fractionations. DHP was precipitated from 55% to 95% ammonium sulfate then recovered by centrifugation. The precipitant was resuspended in 20 mM Tris pH 8.0. The solution was dialyzed against 4 L of the same buffer to remove ammonium sulfate and then dialyzed against 4 L of 20 mM Na phosphate pH 5.0. After removal of precipitated contaminants by centrifugation, the solution was applied to SP Sepharose fast flow cation exchange column, which was equilibrated with Na phosphate pH 5.0. The protein was eluted using NaCl gradient of 0-0.5 M NaCl in Na phosphate pH 5.0 buffer. Its purity was analyzed using UV-Vis absorption spectroscopy by calculating the ratio of the heme absorbance at the Soret peak, 406 nm, to the protein absorbance at 280 nm. The fractions with the ratios
greater than 2.5 were collected and concentrated to be further purified using a Sephacryl S-200 column with Na phosphate pH 5.0 buffer. The fractions with the ratio greater than 3.2 were considered to be pure and were pooled and concentrated. Subsequently, the buffer was exchanged to 20 mM sodium cacodylate pH 6.5.

DHP in the ferric state was prepared by treating the purified protein with a small amount of potassium ferricyanide. The excess ferricyanide was removed using a Bio-Gel P-6 DG Desalting Gel gel-filtration column in the same buffer (7, 56, 57).

**Crystallization and crystal soaking**

The Y34N variant of DHP was crystallized in both ferrous and ferric forms using the vapor diffusion method in hanging-drop set-up. The protein dissolved in 20 mM sodium cacodylate pH 6.5 was concentrated to 10 mg/mL. The crystals were grown from solutions containing 0.2 M ammonium sulfate and 26-34% polyethylene glycol 4000, as previously reported (5, 6, 46, 58, 59).

For the substrate binding experiments, solid TCP was dissolved in a 50% ethanol-water mixture. Subsequently, DHP crystals were soaked in an artificial 200 mM TCP mother liquor. The native and soaked crystals were briefly transferred to cryo-solutions, which additionally contained 20% ethylene glycol, and were flash frozen in liquid nitrogen.

**X-ray diffraction data collection and structure determination**

All data sets were collected at SERCAT 22ID beamline at the Advanced Photon Source (APS) in the Argonne National Laboratory. The data were indexed, integrated and scaled with the HKL2000 software package (60). Data collection and processing statistics are listed in Table 2.1. The structure of Y34N•TCP was determined using
molecular replacement with the Phaser program (61) from the CCP4 suite of program (62) using the wt-DHP structure (PDB entry 1EW6) as the initial model. For the subsequent analyses the Y34N•TCP structure was used in molecular replacement. The resolution of the data is on borderline for anisotropic treatment of B factors. R-free values are only slightly better but R values are much lower for anisotropic treatment. Statistics for both refinements are included in Table 2.1. The atomic coordinates resulting from both refinements are essentially the same. Structure rebuilding and refinements were performed using Turbo (63) and Refmac5 (64, 65) respectively. Coordinates were superposed using the LSQKAB program (66) from the CCP4 suite. Figures 2.1-2.5, 2.7 and 2.8 were prepared using Turbo.

**Dehaloperoxidase activity measurements**

The dehaloperoxidase activity assay was performed on a Cary 400 spectrophotometer at 4 °C using a UV-Vis absorption spectroscopy assay as described previously (7, 29, 56, 67). The 272 nm absorbance peak of the 2,4-dichloroquinone product was monitored versus time (68). The initial rate for each reaction was calculated from the linear initial portion of the trace automatically using Cary Win UV software set to kinetic mode. To measure the turnover number ((k_{cat})), 150 µM TCP cosubstrate was mixed with DHP ([enzyme]=1~3 µM) in 100 mM potassium phosphate buffer at pH 7, and then the reaction was initiated by varied concentrations of H₂O₂ (0 ~ 480 µM). To determine the K_{m} for TCP, varied concentrations of TCP (0.02 ~ 1.6 mM) were mixed with DHP, and then the reactions were initiated by addition of 160 µM H₂O₂. The initial rates as a function of H₂O₂ or TCP concentration were fit to the Michaelis-Menten equation using the Prism 5 software.
RESULTS

The crystal data and refinement parameters are listed in Table 2.1 while Table 2.2 presents a summary of ligands bound in the studied complexes.

*The structure of Y34N•TCP complex*

Upon soaking Y34N crystals with TCP, the substrate bound in both subunits at the entrance to the distal cavity creating a lid which closes the cavity entrance (Figure 2.1). We will refer below to this mode of binding as “external”. The TCP binding is accompanied with the distal histidine swinging out of the cavity and forming a strong hydrogen bond (2.5 Å) with the TCP hydroxyl which in turn forms another strong hydrogen bond (2.6 Å) with the hydroxyl of Tyr38. In addition to these hydrogen bonds, hydrophobic interactions formed with the heme and the side chains of Phe21, Phe35, Phe52 and Val59 stabilize the TCP binding. Native Y34N crystallizes in the oxy-ferrous state, but the O₂ molecule was displaced upon TCP binding. However, there is no direct contact between the heme iron and the TCP molecule; the closest Fe-neighbor is the Cl-atom at position 4 but the observed distance of 3.4 Å is approximately 1 Å longer than the bonding distance. When the position of the Fe-coordinated O atom in oxy-ferryl [Fe(IV)=O] complex is modeled, the distance between the ferryl O atom and the TCP Cl4 is 2.3 Å, about 0.8 Å shorter than the sum of their radii (Figure 2.2). Thus a TCP molecule, although not coordinating the heme iron, created a steric hindrance that prevents bonding of water or peroxide molecules as a six ligand. Previous spectroscopic evidence for ferric DHP showed the transition from a six-coordinate high-spin state to a five-coordinate high-spin state upon TCP binding and thus indicated that the water molecule coordinated as the six ligand in the ferric DHP is also displaced (55, 69).
TCP binding resulted in a tilting of the heme by 6° and the movement of its edge by 0.8 Å (Figure 2.3). In both subunits, one of the heme propionates became disordered and the electron density for the loop K87-S90, which contains the proximal histidine, His89, is poor when compared to other parts of the molecule and to the corresponding density in the Y34N•O₂ complex (to be published). Consistently, the B-factors for the proximal histidine and its neighboring residues are much higher in the Y34N•TCP complex than in the Y34N•O₂ complex; they are shown in figure 2.4. This indicates that upon TCP binding, loop Lys87-Ser90 becomes more mobile and other conformations are populated. It appears that the loop mobility is induced by the heme movement. The standard conformation of this loop observed in the absence of TCP is unique for DHP, in other globins the proximal histidine and its neighbors are a part of α-helix F.

**The structure of Y34N/S91G•TCP complex**

Soaking crystals of double mutant Y34N/S91G in TCP solution led to a more complicated outcome. In subunit B, the TCP binding geometry is essentially the same as in the Y34N•TCP complex. However in subunit A, two modes of TCP binding were observed with approximately equal occupancy (Figure 2.5 and Figure 2.6). One mode is “external”, the same as in subunit B (and in the Y34N•TC complex). The other binding mode is entirely different with the TCP deep in the distal cavity with the hydroxyl pointing towards the oxygen binding site of the heme. We will refer to this mode of binding as “internal”. The binding in the internal and external sites cannot take place simultaneously because of steric hindrance, the distance between O₁ of TCP in the internal site and Cl⁴ in the external site is 1.4 Å. Subunit B, which has a molecule of TCP bound in the external site, is similar to the analogous Y34N•TCP complex with a tilted
heme and a partially disordered proximal histidine loop. The half occupancy of the two TCP sites in subunit A makes a detailed analysis of the conformational changes induced by ligand binding difficult.

**The structures of L100F•TCP complex**

Modeling suggested that the replacement of Leu100 with phenylalanine should block the internal TCP binding site. To check how the elimination of the internal TCP binding site affects the dehaloperoxidase activity DHP, the L100F mutant was investigated. Previous kinetic studies showed that the rates of TCP dehalogenation by L100F are similar to those of wt-DHP (7, 70). The structure of L100F indeed confirmed the presence of a steric hindrance(70). However, the structure of L100F crystals soaked in TCP showed its binding, with about 50% occupancy, in the internal site of subunit A and a much lower occupancy in subunit B. The binding is enabled by conformational changes of Phe100, Phe60 and Phe21 shown in Figure 2.7. In the presence of TCP, there is a water molecule bound to the heme Fe, in its absence a dioxygen molecule. The distal oxygen of O2 is too close to the TCP hydroxyl to have both ligands present at the same time. The TCP binding in the internal site does not lead to the heme tilting and the disorder of the proximal histidine loop, which were observed upon TCP binding in the external site.

Subunit B has a water molecule coordinated to the heme iron; it is hydrogen bonded to a molecule of ethylene glycol present in the distal cavity, which originated from the cryo-solvent. The other hydroxyl of the ethylene glycol molecule forms a hydrogen bond to the hydroxyl of Tyr38. The distal His55 is outside the pocket and interacts with one of the heme propionates.
Dehaloperoxidase activity of DHP variants

DHP is unique among globins in having its proximal histidine located in a loop rather than being a part of a helix. It has been proposed that the increased flexibility and altered dynamics of this loop may contribute to its dehaloperoxidase activity (Lebioda, 2000). The TCP binding in the external site indeed induced poor electron density and high B-factors for this loop. To test if this increased loop mobility is indeed related to the dehaloperoxidase activity, we generated mutants of Lys87, the residue with the highest B-factors. Conservative mutations Y34N/K87R and Y34N/K87S have approximately the same activity as Y34N while Y34N/K87P, in which the presence of proline should restrict accessible conformations, had activity slightly higher (Table 2.3). This would indicate that the variants have similar dynamics as wt-DHP and that the loop dynamics do not influence dehaloperoxidase activity.

The side chain of Thr56 forms a contact with the TCP molecule bound in the external site (CG2 – Cl6 distance is 3.5 Å). Modeling suggested that mutations of Thr56 that introduce larger side chains may affect TCP binding. We have prepared such variants and T56M showed essentially the same activity as DHP A, the activity of T56I was about two-fold higher (Table 2.3).

Since L100F accommodated internal TCP binding, a less conservative F21W mutant was designed to block the internal TCP binding site. Modeling showed that all potential rotamers either block the TCP binding in the internal site or are disallowed because of a steric hindrance. The best model is shown in Figure 2.5 and Figure 2.8. Some adjustment in the distal cavity to accommodate its larger side chain must have
taken place and the mutant expressed with standard yields. The mutant activity was only moderately lower, 7% of wt-DHP A (Table 2.4).

Kinetic studies of DHP and its variants as a function of TCP concentration yielded values of the apparent $K_m$ which are listed in Table 4 (Figure 2.9). They are in a 0.3-0.8 mM TCP range with the wt enzyme in the middle of the range, except for F21W which has a $K_m$ of 2.3 mM. To summarize, none of the mutations studied had a strong effect on the DHP kinetic properties.

**DISCUSSION**

Two modes of TCP binding to DHP have been observed: one site is internal, deep in the proximal cavity, while the other is external, forming a lid closing the distal cavity entrance. The internal binding mode is similar to the one observed in the recently reported low occupancy, about 10%, complex of DHP A with an analogous substrate 2,4,6-tribromophenol, TBP (18). The question that emerges is whether this is the productive mode of substrate binding as postulated by Zhao et al. (18), or just an opportunistic binding in the hydrophobic cavity? The answer to this question is crucial for our understanding of the mechanism of dehaloperoxidase catalysis employed by DHP. Two alternative mechanisms have been recently advanced. The first one parallels the mechanism established for classical peroxidases (47, 71). In this mechanism, the reaction is initiated by $H_2O_2$ binding to form Compound I or Compound ES which then abstracts a hydrogen atom (one electron plus one protein) from the substrate generating a phenoxy radical, which dissociates from the enzyme. The phenoxy radical then either disproportionates to the product, or a second one-electron oxidation takes place with Compound II to yield the quinone product after reacting with water. Halophenol
substrates interact with the heme edge and their binding is transient (47). The second mechanism, mostly advanced by Franzen and co-workers (18), assumes that a halophenol molecule binds first in the distal cavity. This is followed by H_2O_2 binding and a single two-electron oxidation. Interaction of the oxidized intermediate with a water molecule leads to its dehalogenation and product release. The recently reported structure of DHP•TBP complex with the TBP molecule bound in the internal site, analogous to the reported here DHP•TCP complex, was used to support the later mechanism (18).

The observation of an internal binding site was used to boost the mechanism advanced by Zhao et al. (18), arguing that organic substrate binding is a necessary event. However, the Y34N/S91G•TCP and L100F•TCP structures reported herein, which are more accurate than that of DHP•TBP complex because the TCP occupancies are 50% not 10% observed for TBP, raise a number of issues. First, the TCP binding in the internal site is very capricious. The data present in Tables 2.2 and 2.3 show that for DHP variants there is no correlation between their activity and the TCP binding mode and/or affinity observed in crystals. For some of the variants, two subunits present in the asymmetric part of the unit cell show different affinities and even different modes of binding. More consistency is expected for productive substrate binding especially at concentrations two orders of magnitude higher than the K_m values. Importantly, the binding variability is not due to differences in diffusion through the crystals since in the isomorphic structures of Y34N•TCP and Y34NS91G•TCP, the substrate (TCP) is present in both subunits with full occupancy.

Second, it was proposed that a water molecule which is about 10 Å away from the C4 atom of TCP, the proposed site of nucleophilic attack, somehow migrates and
interacts with the oxidized intermediate bound in the same site as the TCP substrate (18). The migration of this water molecule is highly unlikely to take place as there is neither space at the C4 or Cl4 atoms of TCP or any indications suggesting the existence of a channel suitable for the water molecule movement. Direct nucleophilic attack of the oxygen atom from Fe(IV)=O is also unlikely because the O-C4 distance, 6.0 Å, is too long and the substrate position is unsuitable. Thus the oxidized intermediate would have to leave the distal cavity for the final step of the reaction.

Third, simultaneous binding of O2 and TCP in the distal cavity cannot take place because of steric hindrance; the positions of the phenolic O atom and the distal atom of O2 are only 1.1 Å apart. Thus in the presence of TCP in the distal cavity, a peroxide molecule cannot bind with a geometry similar to that of the dioxygen molecule. Neither is there an alternative, suitable binding geometry for peroxide, especially with the distal histidine present in the cavity. It is not likely that TCP bound in the internal site can shift in such fashion that would allow hydrogen peroxide binding at the heme because of the size of the distal cavity. Also, the distal histidine forms a hydrogen bond with the TCP hydroxyl (Fig. 4) and this interaction would have to be broken to allow H2O2 binding.

Finally, the F21W mutant, in which the internal TCP binding site must be entirely blocked, still has about 7% of the wt-DHP A activity. This is too much activity for a mechanism requiring occupancy of the internal binding site. These four entirely independent observations argue against internal TCP binding as a productive step in catalysis.

The structures of the Y34N and Y34N/S91G complexes with TCP revealed a novel TCP binding site: an external one. This binding mode must also be inhibitory
because the TCP molecule blocks the entrance to the distal cavity and stabilizes the distal histidine in the catalytically inactive out position, thus disassembling the catalytic machinery for peroxide cleavage. Also the Cl4-atom of TCP prevents the binding of even a water molecule to the heme iron let alone an H2O2 molecule. Spectroscopic data positively show that TCP binding leads to a five-coordinate Fe indicating that in solution TCP binds in the external site (55, 69).

For classical peroxidases, the order of substrate binding and many aspects of their dehaloperoxidase mechanism, such as catalysis at the heme edge, are well established and likely similar to those of DHP (47). The external binding mode observed in DHP complexes with TCP may be related, though not closely similar, to the interaction between TCP and Compound I. In the absence of ligands, the distal histidine of DHP is observed in both the “in position” hydrogen bonded to the heme-iron ligand and in the “out position” outside the pocket (2); its mobility is much higher than in other globins (6). The oxygen atom bound to Fe(IV) in Compound I or Compound II is strongly polarized and thus a poor hydrogen bond acceptor; it is also further from the distal histidine than a coordinated water molecule since the Fe-O bond is shorter. Thus it appears that upon Compound I formation the distal histidine loses its hydrogen bonding partner in the cavity, swings out into solvent and is free to bind TCP and recruit it to the heme edge through the hydrogen bond observed in the complexes. Consequently, the affinity of DHP for TCP is higher upon the enzyme activation by hydrogen peroxide. Previous studies of myoglobin (Mb) dehaloperoxidative activity inhibition by phenol led to a similar conclusion: phenol, which acts as a competitive inhibitor, binds only after Mb activation by hydrogen peroxide (56).
In addition to the hydrogen bond with the distal histidine (His55), the hydroxyl of Tyr38 forms a strong hydrogen bond to the hydroxyl of TCP; this appears to be an important contribution to the observed external binding mode. However, the replacement of Tyr38 with phenylalanine or asparagine, and thus elimination of this hydrogen bond, led to increases in $k_{\text{cat}}$ by 4 and 13 fold respectively (44). Our studies confirm the better catalytic efficiency of the Y38N variant. A priori, it is not expected for less strong substrate binding to cause increased activity and, especially a lower $K_m$. One possible explanation of this observation is that the observed complex with TCP in the external site is, as we argue, inhibitory not the catalytic one. If one assumes that upon binding to Compound I the TCP molecule forms the hydrogen bonds observed in the inhibitory complex, it would approach the active oxygen with its Cl4 atom rather than C4. It may be speculated that the shorter side chain of Asn38 also forms a hydrogen bond with TCP which leads to a better geometry for the approach. We expressed non-His-tagged Y38N but the yield was low and the protein did not crystallize.

The structural analyses and kinetics studies reported here strongly disagree with the model in which TCP binding takes place before hydrogen peroxide (18). On the other hand, the data do not distinguish whether the reaction proceeds through two one-electron oxidation steps with the release of phenoxy radical intermediates or through one two-electron step producing benzoquinone. Interestingly, the establishment of the DHP mechanism will also help clarify the DHP physiological role. So far, the DHP dehaloperoxidase activity has been considered to be crucial for halophenol detoxification. However classical peroxidases producing dissociable radicals tend to be secretory and compartmentalized while DHP functions in the worm coelom where radicals may be
harmful. In plants, peroxidases are thought to produce phenoxy radicals to control bacterial population (72). A similar role of DHP as an activator of halophenols to augment their antibacterial properties should therefore also be considered.

**Conclusion**

Two alternative modes of TCP substrate binding to DHP have been observed. An internal TCP binding site is seen deep in the distal cavity partially blocking the dioxygen and, presumably, hydrogen peroxide binding side. An external TCP binding site has been found that overlaps with the position of the reactive oxygen atom in the DHP Compound I intermediate. Thus both binding modes are inhibitory. Binding in a productive fashion must be to the Compound I state of DHP, implying ordered substrate binding: hydrogen peroxide first followed by halophenol. Several considerations lead us to project that the productive DHP binding mode may be similar to the external site reported herein.
Table 2.1. Crystallographic data and refinement statistics.

<table>
<thead>
<tr>
<th>DHP complex</th>
<th>Y34N•TCP 4kmw</th>
<th>Y34N/S91G•TCP n3</th>
<th>100F•TCP cmv</th>
</tr>
</thead>
<tbody>
<tr>
<td>X-ray source</td>
<td>APS SER-CAT ID</td>
<td>APS SER-CAT ID</td>
<td>APS SER-CAT ID</td>
</tr>
<tr>
<td>Wavelength(Å)</td>
<td>1.0000</td>
<td>1.000</td>
<td>1.000</td>
</tr>
<tr>
<td>Number of Frames</td>
<td>140</td>
<td>75</td>
<td>180</td>
</tr>
<tr>
<td>Oscillation Range (degree)</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Temperature(K)</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Space group</td>
<td>$P2_12_12_1$</td>
<td>$P2_12_12_1$</td>
<td>$P2_12_12_1$</td>
</tr>
<tr>
<td>Unit cell dimensions</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>a (Å)</td>
<td>58.553</td>
<td>58.982</td>
<td>58.539</td>
</tr>
<tr>
<td>b (Å)</td>
<td>67.259</td>
<td>67.657</td>
<td>67.804</td>
</tr>
<tr>
<td>c (Å)</td>
<td>67.890</td>
<td>67.870</td>
<td>68.368</td>
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<tr>
<td>Volume (Å$^3$)</td>
<td>267365</td>
<td>270840</td>
<td>271360</td>
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<tr>
<td>Mosaicity (deg.)</td>
<td>0.80</td>
<td>0.40</td>
<td>0.49</td>
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<td>Resolution range (Å) (highest shell)$^a$</td>
<td>47.8-1.79</td>
<td>44.5-1.78</td>
<td>4.78-1.44</td>
</tr>
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<td></td>
<td>(1.82-1.79)</td>
<td>(1.81-1.78)</td>
<td>(1.46-1.44)</td>
</tr>
<tr>
<td>Redundancy (highest shell)</td>
<td>5.4 (4.0)</td>
<td>3.0 (2.7)</td>
<td>3.6 (1.9)</td>
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<tr>
<td>Average I/σ(I)</td>
<td>13.4</td>
<td>20.2</td>
<td>14.4</td>
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<tr>
<td>Total number of reflections</td>
<td>140011</td>
<td>140586</td>
<td>332066</td>
</tr>
<tr>
<td>Number of unique reflections</td>
<td>25737</td>
<td>26181</td>
<td>49456</td>
</tr>
<tr>
<td></td>
<td>h. shell</td>
<td>h.s.</td>
<td>h.s.</td>
</tr>
<tr>
<td>---------------------</td>
<td>----------------</td>
<td>-----------</td>
<td>-----------</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>98.9 (91.8)</td>
<td>98.8 (97.9)</td>
<td>98.3 (72.0)</td>
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<tr>
<td>Total linear R-merge</td>
<td>7.9 (49.0)</td>
<td>4.0 (11.1)</td>
<td>5.4 (54.0)</td>
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<td>R-value (%)</td>
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<td></td>
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<tr>
<td>aniso (h.s.)</td>
<td>14.6 (23.8)</td>
<td>13.3 (14.0)</td>
<td>13.3 (25.9)</td>
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<tr>
<td>iso (highest shell)</td>
<td>19.0 (29.6)</td>
<td>18.3 (22.2)</td>
<td>18.1 (32.9)</td>
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<td>R_{free} -value (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>aniso (h. shell)</td>
<td>23.8 (34.7)</td>
<td>21.4 (25.6)</td>
<td>20.1 (35.5)</td>
</tr>
<tr>
<td>iso (highest shell)</td>
<td>23.6 (32.9)</td>
<td>23.7 (30.1)</td>
<td>22.7 (37.0)</td>
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<tr>
<td>Ramachandran statistics</td>
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<tr>
<td>% of residues</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>In most favored regions</td>
<td>94.4</td>
<td>94.0</td>
<td>92.9</td>
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<tr>
<td>In additional allowed</td>
<td>5.6</td>
<td>6.0</td>
<td>6.3</td>
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<tr>
<td>In generously allowed</td>
<td>0</td>
<td>0</td>
<td>0.8</td>
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<tr>
<td>Average B factor for protein</td>
<td>27.7</td>
<td>24.3</td>
<td>22.2</td>
</tr>
<tr>
<td>Average B factor for solvent</td>
<td>35.7</td>
<td>31.0</td>
<td>36.6</td>
</tr>
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Table 2.2. Ligands bound in or at the distal cavity of DHP mutants

<table>
<thead>
<tr>
<th>DHP</th>
<th>PDB code</th>
<th>Subunit A</th>
<th>Subunit B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y34N•TCP</td>
<td>4kmw</td>
<td>TCP external</td>
<td>TCP external</td>
</tr>
<tr>
<td>Y34N/S91G•TCP</td>
<td>4kn3</td>
<td>½ (TCP int. + H₂O)</td>
<td>TCP external</td>
</tr>
<tr>
<td>L100F•TCP</td>
<td>4kmv</td>
<td>½ TCP external</td>
<td>H₂O + ethylene glycol</td>
</tr>
</tbody>
</table>

TCP external ½ (TCP int. + H₂O) ½ O₂
Table 2.3. Activity of DHP and its variants. Data were measured at 150 μM TCP and varying H₂O₂ concentrations in 100 mM phosphate buffer at pH 7 in 4 °C.

<table>
<thead>
<tr>
<th>DHP Mutants</th>
<th>$k_{cat}$ (min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DHP A</td>
<td>26.1±0.9</td>
</tr>
<tr>
<td>DHP Y34N/K87S</td>
<td>22.0±1.0</td>
</tr>
<tr>
<td>DHP Y34N/K87R</td>
<td>22.3±0.7</td>
</tr>
<tr>
<td>DHP Y34N/K87P</td>
<td>36.9±0.9</td>
</tr>
<tr>
<td>DHP T56M</td>
<td>22.1±1.0</td>
</tr>
<tr>
<td>DHP T56I</td>
<td>42.6±1.1</td>
</tr>
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</table>
### Table 2.4. Kinetic Studies of DHP mutants

<table>
<thead>
<tr>
<th>DHP mutant</th>
<th>$K_m^{TCP}$ (µM)</th>
<th>$k_{cat}^{TCP}$ (min$^{-1}$)</th>
<th>$k_{cat}^{H2O2}$ (min$^{-1}$)</th>
<th>$k_{cat}/K_m^{TCP}$ (mM$^{-1}$ min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DHPA</td>
<td>495±62</td>
<td>63±4</td>
<td>26.1±0.9</td>
<td>127</td>
</tr>
<tr>
<td>Y34N</td>
<td>591±76</td>
<td>85±11</td>
<td>20.0±0.6</td>
<td>143</td>
</tr>
<tr>
<td>T56M</td>
<td>828±179</td>
<td>111±14</td>
<td>22.1±0.5</td>
<td>134</td>
</tr>
<tr>
<td>T56I</td>
<td>416±72</td>
<td>111±10</td>
<td>42.6±1.2</td>
<td>267</td>
</tr>
<tr>
<td>Y38N</td>
<td>280±94</td>
<td>119±20</td>
<td>229±8</td>
<td>425</td>
</tr>
<tr>
<td>F21W</td>
<td>2260±512</td>
<td>19±3</td>
<td>ND</td>
<td>8.3</td>
</tr>
</tbody>
</table>
Figure 2.1. Stereoview of the electron density for TCP in Y34N complex. The final FoFc omit map was contoured at a 2 σ level. The hydroxyl of TCP forms two hydrogen bonds, one with Tyr38 and the other with the distal histidine, His55, which is swung out of the cavity.
Figure 2.2. The distal cavity of Y34N•TCP complex viewed from the entrance side. In red is the TCP molecule bound in the external site. In green is shown the position of a 4-iodophenol (4-IP) molecule observed in the DHP•4-IP complex and superposed based on Ca positions. 4-IP penetrates the cavity much deeper. In violet is the modeled position of the active oxygen atom in Compound I or Compound II. Its distance to Cl4, 2.3 Å, is much shorter than the sum of their radii, 3.2 Å.
Figure 2.3. Stereoview of the least squares superposition of Y34N complexes with oxygen (in blue) and TCP (in atom colors). The TCP molecule pushes the heme down leading to its tilting. The Cl atom at position 4 is not in contact with the heme; its position is very close to that of the distal atom of the dioxygen molecule.
Figure 2.4. A comparison of average temperature factors for main chain atoms for Y34N•TCP and Y34N•O2 complexes. A and B refer to the two symmetry independent molecules present in crystals.
Figure 2.5. The distal cavity of subunit A in the structure of Y34N/S91G•TCP complex and a modeled position for Trp21. DHP is in atom colors, TCP bound in the external position is in green, in the internal position in red. The best modeled position of a tryptophan in F21W is in violet.
Figure 2.6. The electron density for TCP in subunit A of the Y34N/S91S•TCP complex. Omit FoFc map is contoured at a 2σ level. The molecule bound in the internal site is in red, the one in the external site in green; each was refined with 0.5 occupancies.
Figure 2.7. The distal cavity of L100F•TCP complex in subunit A. The TCP molecule bound in the internal site is in red; in cyan are Phe21, Phe60 and Phe100 in the absence of bound TCP; in atom colors is the structure in the presence of TCP.
Figure 2.8. Stereoview of the Y34NS91G•TCP complex with an alternative modeled position of Trp21 shown in violet. DHP is in atom colors, TCP bound in the external position is in green, in the internal position in red.
Figure 2.9. The kinetics of DHP A and its mutants with TCP as the substrate. The enzyme activity is inhibited by high concentrations of TCP.
CHAPTER 3
STRUCTURE AND FUNCTIONAL DIVERGENCE OF
HEMOGLOBIN/DEHALOPOEROXIDASE ISOPROTEINS DHP A AND DHP B FROM

AMPHITRITE ORNATA

This work was supported by NSF Grant 0820456. Data were collected at the Southeast Regional Collaborative Access Team (SER-CAT) and Structural Biology Center (SBC) 22-ID and 22-BM beamlines at the Advanced Photon Source, Argonne National Laboratory. Use of the Advanced Photon Source was supported by the U.S. Department of Energy, Office of Basic Energy Sciences, under Contract No. W-31-109-Eng-38.

Mutagenesis, Protein purification of all the DHPs (Y34N DHP A, Y34N/S91G DHP A, DHP B), including non his tag and his tag proteins, were performed by Chunxue Wang.

Crystallization and structural determination of Y34N and Y34N/S91G DHP A reported here were performed by Chunxue Wang.

Kinetics assays and functional switching studies were performed by Chunxue Wang and Shengfang Sun. Oxygen affinities were measured by Shengfang Sun.
ABSTRACT

The two isoenzymes of dehaloperoxidase from *Amphitrite ornata* (DHP A and DHP B), evolved from an ancestral globin. During recent evolution they acquired the new dehaloperoxidase function while preserving the oxygen carrier capabilities. In order to obtain further insight into divergence between isoproteins DHP A and DHP B during evolution we investigated their three physiological functions: oxygen binding, peroxidase activity and conversion from the ferrous to the ferric state which is necessary for the peroxidase activity. In addition to DHP A and DHP B two mutants Y34N and Y34N/S91G of DHP A, which were designed to mimic the transition from DHP A to DHP B, were investigated. Our functional analysis indicates that in DHP B all three functions are enhanced relative to DHP A. In addition, we found that the oxygen binding function was enhanced in Y34N DHP A and further improved in double mutant Y34N/S91G DHP A, suggesting an important role of amino acids 34 and 91 in the functional divergence between DHP A and DHP B. Furthermore, to clarify the structural factors responsible for the enhanced oxygen affinity in DHP B, we determined the crystal structures of Y34N DHP A•O₂, Y34N/S91G DHP A•O₂ and metaquo-Y34N/S91G DHP A. Our structural analysis shows that the enhanced oxygen binding capabilities in DHP B is caused by the subtle change of distal hydrogen bonding network due to the substitution of Tyr34 with Asn and the increased mobility of proximal loop Gln88-Ser92 which contains the proximal histidine.
INTRODUCTION

Hemoglobin/Dehaloperoxidase (DHP) from Amphitrite ornata is the first discovered heme-containing globin functioning as an enzyme (1, 4, 13). In the ferrous state, it functions as an oxygen carrier, but in the ferric state it is a peroxidase which dehalogenates haloaromatic compounds. Like classic globins, DHP can reversibly bind dioxygen to the heme iron to carry it and release where needed. As a peroxidase, DHP can catalyze the oxidative dehalogenation of various halophenols to the corresponding quinone products according to Scheme 1 (1, 20, 52, 67). The reaction mechanism is similar to that of typical peroxidases and involves two sequential one-electron oxidation steps with the formation of high-valent oxidants Compound I (Fe^{IV}=O porphyrin π-cation radical) and Compound II (Fe^{IV}=O) (47-49).

[Chemical diagram]

Scheme 3.1. The reaction catalyzed by DHP

_A. ornata_ DHP was initially discovered as coelomic hemoglobin which stores and transports dioxygen, the function similar to that of mammalian hemoglobins (73). In the 1990’s, _A. ornata_ DHP was found to be a dehalogenating peroxidase which enables _A. ornata_ to detoxify biogenic halophenols often present in its living surroundings (1). Usually, _A. ornata_ cohabits coastal sediments with other polychaete worms such as Notomatus lobatus, which produces halogenated secondary metabolites. The
dehalogenation function of DHP provides a defending mechanism for *A. ornata* to survive in such a toxic environment.

DHP has been proposed to evolve from an ancient oxygen carrier and its peroxidase activity arose under conditions of environmental stress ((2, 11, 13, 28)). From the phylogenetic perspective, dioxygen carrier/storage function of globins is more universal and ancient (1.8 billion years ago) than appearance of terrebellid polychaetes (530 million years ago) (3, 53, 74). In fact, the stable oxyferrous DHP is most abundant protein purified from the organism, implying oxygen carrying is its major function (1, 52). In addition, the amino acid sequence of DHP has 30% identity to the 28-amino acid sequence in the N-terminal of hemoglobin isolated from *Enoplobranchus sanguineus*, which is also a polychaete worm (2, 73). Most importantly, the first X-ray structure of DHP determined by LaCount et al reveals DHP has a typical globin fold and with the proximal histidine ligated to the heme iron and a distal histidine that are essential for ligands binding in the distal pocket (2, 4, 11). Despite the relatively low sequence similarity between DHP and sperm whale myoglobin (Mb), the overall protein fold of DHP highly resembles that of Mb, while it is entirely distinct from the protein fold of classic peroxidases, such as horseradish peroxidase (HRP) or cytochrome c peroxidase (CcP) (2, 54, 71, 75, 76). Several Mb mutants, such as Mb G65T, G65I and H93K/T95H, were engineered to mimic the heme environment in DHP and had indeed enhanced peroxidase activity (7). The most recent studies clearly showed that DHP in the presence of substrates, halophenols and hydrogen peroxide switches from the ferrous state to the enzymatic ferric, although the exact mechanism is still controversial (28, 50).

Separate genes (*dhpa* and *dphb*) encoding two isoenzymes, named DHP A and
DHP B were identified (54). The DHP expressed in *A. ornata* appears to be predominantly DHP A (11) but that may depend on the presence of halophenols in the environment. Both DHP A and DHP B contain 137 amino acids and are very closely related; they differ from each other only in 5 amino acids: I9L, R32K, Y34N, N81S, and S91G (54). DHP B exhibited 2-4 fold higher peroxidase activities than DHP A for different substrates (55). The overall structure of DHP B is almost identical to that of DHP A, however, the substitution of active site residues in DHP B subtly change the heme environment by altering the hydrogen-bond network at the distal and proximal sites, which may contribute to the difference in the enzymes activity (58). Since the two isoenzymes are highly homologous with each other, the gene duplication and divergence must have occurred quite recently. Since the oxygen transport is likely an essential function while the dehaloperoxidase is not and since DHP A is the dominant isozyme, we assumed that the ancestral DHP, that is before the gene duplication, was more similar to DHP A than to DHP B or to put this in simplistic way that DHP B evolved from DHP A and not vice versa. Thus it is interesting to examine the structural and functional differences between the two isoproteins, DHP A and DHP B and the likely intermediates in the process of evolution.

The change from tyrosine in DHP A to asparagine in DHP B at position 34 (Y34N) is perhaps the most important difference because it is located in the active site and close to the heme. Another significant variation is S91G which is located in the loop containing the proximal histidine 89 (11). In order to identify the contributions of the variations at positions 34 and 91 to functional differences between DHP A and DHP B, we assessed and compared functional properties of DHP A, DHP B, and two
intermediates: DHP A mutant Y34N and double mutant Y34N/S91G. We studied peroxidase activity, oxygen affinity and the heme oxidation rates in the presence of the substrates, the event that converts the protein from dioxygen binder to peroxidase. In addition, the structural analysis of Y34N DHP A•O₂, Y34N/S91G DHP A•O₂ and metaquo-Y34N/S91G DHP A complexes was carried out to probe structural factors that are associated with distinct properties of DHP A and DHP B.

**MATERIALS AND METHODS**

*Site-directed mutagenesis*

All of the DHP mutants were generated using the Quick Change Method. The sequences of mutagenic primers are listed in Table 3.1. The PET 16B plasmid encoding DHP (6Xhis tag and non-his-tag) was used as a template to produce DHP mutants. The plasmid of DHP B was generated from the DHP A plasmid in a stepwise fashion. Briefly, a single mutant pDHP Y34N was generated and subsequently employed for the double mutant Y34N/S91G DHP A. In a similar way, the plasmids pDHP A Y34N/S91G/R32K, Y34N/S91G/R32K/I9L, and Y34N/S91G/R32K/I9L/N81S (DHP B sequence) were then sequentially produced. DNA with desired mutations were verified by LiCor DNA sequencing (Engencore at the University of South Carolina) and then transformed into *Escherichia coli* BL21 (DE3) cells.

*Protein expression and purification of Native DHP*

Recombinant DHP mutants without his-tag were overexpressed in Rosetta *Escherichia coli* BL21 (DE3) cells and then purified with the established procedure for DHP A as previously described (49).
**Protein expression and purification of 6xHisDHP**

The his-tag DHP plasmids were transformed into *Escherichia coli* BL21 (DE3) cells, and then screened by LB agar plates containing ampicillin. His-tag DHP proteins were expressed and purified as previously described (20, 41). Generally, the *E. coli* cells expressing DHP were grown at 37 °C to an OD value of 0.6, and then induced by adding IPTG with a final concentration of 200 µM. The temperature was switched to 30 °C followed by incubation overnight. The cells were collected and then lysed with lysis buffer (50 mM sodium phosphate pH 8, 300 mM NaCl, 10 mM imidazole). Appropriate amounts of RNase A, DNase I, lysozyme and dithiothreitol were added, and the lysate was incubated at 4 °C for 30 min. After mild sonication the cell debris was removed by centrifugation. Subsequently, the reddish supernatant was applied to a Ni-NTA agarose column followed by adding 5 column volumes of wash buffer (50 mM Na Phosphate PH 8, 300mM NaCl, 20mM imidazole). 6xHisDHP was eluted with elution buffer (50 mM sodium phosphate pH 8, 300 mM NaCl, 250 mM imidazole) and collected in 2 mL fractions. The purity of DHP was evaluated by UV-vis and SDS-PAGE. The fractions with an A406/A280 ratio greater than 3.0 were pooled and concentrated.

**Preparation of ferrous and ferric DHP**

Homogenous DHP in the ferric state were prepared by adding potassium ferricyanide (57). The excess ferricyandie was removed using a Bio-Gel P-6 DG desalting column. In order to obtain oxyferrous DHP, the ferric proteins were treated with excess sodium dithionite followed by running through a P-6 DG desalting column (56).
**Crystallization**

All of the DHP crystals were grown using a hanging drop method at 277 K. Native proteins without his-tag were buffer exchanged into 20 mM sodium cacodylate pH 6.5 and concentrated to 8 mg/mL. The best crystals were obtained in conditions of 0.2 \(M\) ammonium sulfate and PEG 4K with a concentration of 30% - 36% (4-6). The crystals were transferred into a cryoprotectant, which was mother liquor with additional 10% ethylene glycol, and subsequently flash-frozen in nitrogen at 100 K.

**Data collection and structure determination**

All X-ray diffraction data were collected at SERCAT 22ID or 22BM beamline at the Advanced Photon Source (APS) in the Argonne National Laboratory and then processed using the HKL2000 suit (60). The structures were determined by molecular replacement with the CCP4 software (CCP4, 1994). The wt-DHP structure (PDB entry 1EW6) and Mb G65T structure (PDB entry 3OCK) were utilized as the starting models for solving the structures of DHP and Mb mutants, respectively. Model rebuilding was carried out using the Coot (77) and Turbo-Frodo software (63). The refinements and map calculations were performed using *Refmac5* (64) from the CCP4 suit. The parameters and statistics for data collection and processing are summarized in Table 3.2. The superpositions were conducted using *Lsqkab* (78) from the CCP4 suit. Figures 3.1-3.2, 3.4 and 3.6 were generated using PyMOL, and Figures 3.3 and 3.5 were produced by Turbo-Frodo.

**Dehaloperoxidase activity assay**

The dehaloperoxidase activity measurements were carried out with a Cary 400 spectrophotometer at 4 °C using a UV-vis absorption spectroscopy assay as described previously (7, 49). The 272 nm absorbance peak of the 2,4-dichloroquinone product was
monitored versus time (68). The initial rate for each reaction was calculated from the linear initial portion of the trace automatically using Cary Win UV software set to the kinetics mode. To measure the turnover number of DHP mutants, 500 µM TCP was mixed with DHP at concentrations 1~3 µM in 100 mM potassium phosphate buffer at pH 7 and then the reaction was initiated by the addition of H2O2 at varied concentrations (0 ~ 480 mM). To determine the K_m for TCP, the substrate at concentrations 0.02 ~ 1.6 mM was mixed with DHP, and then the reaction was initiated by the addition of 160 mM H2O2. The initial rates as a function of H2O2 or TCP concentration were fit to the Michaelis-Menten equation using the Prism 5 software.

**Stopped-flow UV-vis spectrophotometrics studies**

Stopped-flow spectrophotometry (1.0 cm path length, model SF-61 DX2; Hi-Tech Scientific, Salisbury, U.K.) was used to monitor the DHP heme oxidation from ferrous to ferric. The experiment was performed in a single-mixing mode at 4 °C in 100 mM KP_i buffer, pH 7. Briefly, 5 µM oxyferrous DHP was premixed with 150 µM TCP and then mixed with 1 equiv (5 µM) of H2O2. Data were collected over time and evaluated using the KinetAayst3 software package (Hi-Tech Scientific, Salisbury, U.K.).

**RESULTS AND DISCUSSION**

**Functional studies**

As motioned before, we examined three different physiological properties (peroxidase activity, oxygen affinity and switching from the oxyferrous to the ferric state) for DHP A, DHP B and the mutants in the putative evolution pathway (Y34N DHP A and Y34N/S91G DHP A).
The oxygen binding abilities of these DHP mutants, as oxygen carriers, were investigated by determining the oxygen dissociation constant. A progressive increase of oxygen affinity was observed along the pathway from isoenzyme A to B (Table 3.5). According to the $K_{O2}$ values, these DHP variants were ranked as follows: DHP B $>$ Y34N/S91G $>$ Y34N $>$ DHP A. In particular, DHP B shows a 2.1-fold increase of oxygen affinity relative to DHP A. It is noted that oxygen affinity of Y34N/S91G is comparable to that of DHP B, indicating the remaining three mutations are quite neutral in the process of evolution from DHP A to DHP B.

In order to probe the differences on dehalogenation activity among DHP A, Y34N, Y34N/S91G and DHP B, the Michaelis-Menten parameters were determined for TCP as well as H$_2$O$_2$ for these DHP variants (Table 3.4 and 3.5). By comparing enzymatic efficiency ($K_m/k_{cat}$) for TCP, DHP B exhibits around 2-fold higher peroxidase activity than DHP A, in agreement with the result obtained by the Ghiladi group (55). The increased effect on $K_m/k_{cat}$ in DHP B primarily results from the increase in $k_{cat}$ since the $K_m$ values are comparable for these two isoenzymes. The enzymatic efficiencies in the DHP mutants Y34N and Y34N/S91G are similar to that in DHP A, indicating that the variation at position 34 and 91 do not contribute to the peroxidase activity differences between DHP A and DHP B. With respect to H$_2$O$_2$, only small differences between the $K_m/k_{cat}$ values were observed among these DHP variants, although the enzymatic efficiencies were gradually increased following the transition from DHP A to DHP B (DHP B $>$ Y34N/S91G $>$ Y34N $>$ DHP A). It is notable the turnover number of DHP B increases ~ 5 fold compared to that of DHP A, however, the 3-fold increase in $K_m$ for DHP B leads to the comparable enzymatic efficiencies between DHP A and DHP B. The
difference in \( K_m \) for hydrogen peroxide shows that DHP B has lower binding affinity for the co-substrate than DHP A.

Purified recombinant DHP is isolated mainly in the ferrous state. However, DHP possess its dehalogenation peroxidase activity only in the ferric state. To tolerate the haloaromatic- contaminated environment, original ferrous DHP must be efficiently converted in the worms to active ferric DHP when needed (1). Previous studies showed that the simultaneous presence of both substrate TCP and co-substrate \( \text{H}_2\text{O}_2 \) can induce the conversion from oxyferrous DHP to enzymatic ferric DHP. This process is probably mediated by radicals produced from TCP by minute amounts of ferric DHP present in the organism (28). To follow the evolutionary trend for this process, the oxidation rates for proteins from DHP A to DHP B were measured using the single-mixing rapid stop flow spectroscopy. The starting Soret peak is at 417 nm corresponding to oxyferrous DHP. Injection of \( \text{H}_2\text{O}_2 \) led to a Soret peak shift to 407nm, resulting from formation of ferric DHP. The absorbance increases at 407nm were plotted as a function of time (0 ~ 300s) (Figure 3.7) and the oxidation rate constant was estimated by fitting its exponential part to an exponential rise to max equation. Using this method, the fist order oxidation rate constant from ferrous to ferric state in DHP B is about 3.5 fold faster than that in DHP A while the rate constants for this conversion in DHP A, Y34N and Y34N/S91G are comparable (Table 3.4). These data suggest the mutations Y34N and S91G should be not responsible for the difference of oxidation rate between DHP A and DHP B.

**The structure of the Y34N DHP A•O2 complex**

In general, the structure of Y34N DHP A•O\(_2\) is very similar to that of oxyferrous C73S DHP A•O\(_2\) with r.m.s. difference 0.42 Å between C\(\alpha\) positions. The only
significant structural difference in the main chain conformation is at Lys36 (3.0 Å), which is close to the mutation site. This local conformational difference is smaller at the mutation site (0.48 Å). The difference is likely due to the mutation as the main chain conformation in Y34N is virtually the same as in DHP B (r.m.s. difference is 0.17 Å, maximum difference is 0.39 Å). The heme does not form direct contacts with the side chain of either variant, although the mutation site is close. Other structural differences resulting from the mutation are local. A new hydrogen bond was introduced between the ND2 atom of Asn34 and the carbonyl of Glu31 ((3.1 Å), while in DHP A (PDB code 2QFN) the hydroxyl of Tyr34 is the acceptor of hydrogen bonds from Arg33 (3.1 Å) and Asn96 (3.7 Å). Perhaps more importantly for the heme environment, the aromatic ring of Tyr34 forms a hydrophobic contact (3.7 Å) with the side chain of Leu92, which in turn interacts (3.6 Å) with the imidazole of the proximal histidine.

The coordinated dioxygen in the Y34N DHP A•O₂ complex was observed in a similar geometry with that in the first determined structure of oxy-DHP (5). The difference between the Y34N DHP A•O₂ and C73S DHP A•O₂ complexes is shown in Figure 3.1 and 3.3. The Fe-O1-O2 angle is 144° in subunit A of oxyferrous Y34N protein (150° in subunit B), which is about 20° smaller than oxyferrous DHP A. The oxygen molecule is positioned closer to the heme iron in the case of oxyferrous Y34N, with the Fe-O1 distance of 2.1 Å in both A and B subunits (Table 3.3). In oxyferrous DHP A, the O1 atom lies from the heme iron at a distance of 2.4 Å and 2.2 Å, respectively. In subunit A of oxyferrous Y34N, the hydrogen-bonding distance between the atom O2 and Nε2 of His55 is 3.1 Å versus 3.4 Å for the O1-His55Nε2 distance (Figure 3.3). Only the closed conformation of the distal histidine was observed in subunit A and
its $N^2$ atom is located at a distance of 5.2 Å to heme iron. In subunit B of oxyferrous Y34N, the Iron-O1-O2 angle is 146 degrees. The distances of $N^2$ of His55 to O1 and O2 are 2.4 Å and 3.1 Å respectively. The distal histidine exhibits two confirmations in subunit B, with 75% occupancy in closed conformation and 25% occupancy in open conformation. Due to bonded oxygen instead of water, the distal histidine in closed conformation moved a little further from heme iron with Fe-His55 $N^2$ distance 5.2 Å (4.9 Å in subunit B) compared to that in metaquo ferric DHP (5) (Figure 3.4).

**Structure of the Y34N/S91G•$O_2$ complex**

The overall structure of the Y34N/S91G DHP A•$O_2$ is analogous to those of native DHPs (DHP A and DHP B) and Y34N DHP mutant. The geometry of dioxygen in Y34NS91G DHP is very similar as that observed in oxyferrous Y34N DHP (Figure 3.2 and Table 3.3). In subunit A, the distance of $N^2$-O2 is 3.1 Å versus 3.7 Å for $N^2$-O1 (Table 3.3). The atom O1 of dioxygen as the sixth ligand is coordinated to heme iron with a distance of 2.3 Å. Only the internal conformation of distal histidine was observed. In subunit B, the oxygen molecule is positioned closer to heme iron than that in subunit B, with an O1-Fe coordination distance of 2.1 Å. Accordingly, dioxygen is located from the distal histidine with a distance of 2.8 Å for $N^2$-O2 versus 3.1 Å for $N^2$-O1. Both closed and open confirmations of distal histidine were found in subunit B, with occupancy of 70% and 30% respectively. Notably, the replacement of Ser91 with glycine likely increases the flexibility of helix around the proximal histidine and further affects the state of heme iron. Consistent with this hypothesis, the election density of the loop 89-93 is relatively poor, indicating the increased mobility of the loop (Figure 3.5). Also, the
covalent bond between Iron and N°² of proximal Histidine is enlarged to 2.20 Å in Y34NS91G from 2.10 Å in DHP A.

Structure of aquomet-Y34NS91G DHP A

The overall structure of DHP Y34NS91 is remarkably similar with that of DHP A and DHP B. The least square superposition’s of aquomet-Y34NS91G DHP A on DHP A and DHP B is shown in the Figure 3.6. In subunit B, the closed of distal histidine is observed with 60% occupancy, whereas the open conformation is observed with 40% occupancy. The distances of Fe-N°² are 4.4 Å and 10.1 Å in the closed and open confirmations, respectively. Accordingly, the coordinated water molecule is located at a distance of 2.23 Å from the heme iron with 60% occupancy as the sixth coordination ligand. However in subunit A, approximate 50% occupancy is observed in the closed confirmation, but there is no election density corresponding to the open confirmation. There is an oxygen molecule instead of water bound to heme iron with approximate 50% occupancy. The distal histidine appears to be stabilized into the closed conformation by the sixth ligand and forms hydrogen bonds of 3.1 Å with the O (2) atom and 3.2 Å with O(1) of oxygen molecule. The imidazole ring of histidine 55 is slightly further from the heme iron in subunit A (Fe-N°² distance of 5.16 Å) than in subunit B due to the oxygen molecule bound into the distal active site. The partial occupancy of the sixth ligand is likely the consequence of photoreduction of the heme iron upon X-ray radiation when crystallographic data was collected. The photoreduction leading to partial occupy was also observed in the subunit A of DHP B previously. The presence of oxygen rather than water bound to the heme iron in subunit A appears to be due to complete reduction upon
X-ray exposure. The complete Fe reduction was also previously observed in the subunit B of DHP B and the DHP mutant C73S.

**DISCUSSION**

*Correlation between structures and functional roles*

To find the mechanism how mutations Y34N and S91G enhance the oxygen affinity in DHP B, the crystal structures of Y34N•O₂ and Y34N/S91G•O₂ complexes were determined. Their overall structures are quite similar to that for DHP A and DHP B. In the subunit A of oxyferrous DHPs, the distal histidine is in the closed conformation and hydrogen-bonded with a dioxygen molecule. In subunit B, the electron density for the dioxygen is not as convincing as in subunit A, since the distal histidine is disordered between two positions: one is the same as in subunit A, the other is out of the distal cavity. The disorder in subunit B makes structural interpretation less reliable and therefore the discussion below is based on observations from subunit A.

In the crystal structure of Y34N•O₂, the replacement of tyrosine with asparagine leads to the loss of a weak hydrogen bond between the hydroxyl group of Tyr34 and the side chain of Asn96, but instead introduces a new hydrogen bond between Asn34 the carbonyl of Glu31. This subtle change of hydrogen-bonding networks in the vicinity of the heme probably is an important factor that affects the oxygen affinity of this mutant versus DHPA.

In the other hand, perhaps more importantly for the heme environment, in DHP A the aromatic ring of Tyr34 forms a hydrophobic contact (3.7 Å) with the side chain of Leu92, which in turn interacts (3.6 Å) with the imidazole of the proximal
histidine. The altering of heme environment in Y34N DHP due to loss of the hydrophobic contact may also plays an important role on the oxygen binding abilities.

The most striking difference between the Y34N•O$_2$ and Y34NS91G•O$_2$ structures is the quality of the electron density for loop Gln88-His91 that includes the proximal histidine, His89. In Y34NS91G•O$_2$ the density is relatively poor (Figure 3.5) while in Y34N•O$_2$ is excellent (Figure 3.3), reflecting increased mobility of the loop; it likely results from the presence of a Gly91 that adds conformational flexibility. The elevated B factors for the loop in Y34N/S91G•O$_2$ are consistent with the increased loop mobility. On the basis of the structure analysis, a chain of interactions was proposed to be responsible for the observed differences in binding affinity in Y34N•O$_2$ and Y34N/S91G•O$_2$. The side chain of Glu88 on this mobile loop interacts with heme propionate D through solvent because propionate D is disordered in this region. The propionate D forms a hydrogen bonding contact with the ND1 atom of distal histidine, which in turn interacts with distal oxygen atom of dioxygen positioned within a hydrogen bonding distance. Perhaps through this chain of interactions, the loop mobility slightly affects the geometry of dioxygen binding in Y34N/S91G and therefore results in the increased binding affinity (Figure 3.2). Interestingly, the phenomenon of poor electron density around the Gly91 and the elongation of His89-Fe bond, was observed in the structure of Y34N/S91G•O$_2$, whereas the structure of ferric Y34N/S91G does not show such effects. As mentioned above, our studies indicate the loop dynamics caused by the amino acid at position 91 likely plays more important role on peroxidase function than oxygen carrier function. Thus, distinct contribution of S91G to oxygen affinity and
peroxidase activity would be interpreted by the difference of the loop mobility between oxyferrous and ferric Y34N/S91G.

A number of experiments have identified the important distal and proximal factors that influence the reactivity in globins/peroxidase. For these transition variants from DHP A to DHP B, the specific structural factors of interest, such as His55-ligand distance, are less easy to infer directly from their X-ray crystal structures for three reasons. First, functional and structural differences of these DHP proteins are too subtle to distinguish the small structural changes, which are inside the error limit. Second, X-ray resolution of structures limits accuracy of the measured parameters. All the DHP structures were refined at the range of 1.4 Å to 1.8 Å resolution. Third, X-ray photo-reduction affects the heme iron state and ligand occupancy in different magnitude, which in turn impacts the position of corresponding distal histidine.

**Evolution implication**

*Amphitrite ornata* co-habitats estuarine mud flats with other sea worms (capitellids) which secret a number of brominated metabolites, such as mono-, di-, tri-bromophenols and bromoinodolenes (1, 54). In order to survive in such a toxic environment *A. ornata* evolved its hemoglobin so that it also functions as dehaloperoxidase. Two isoforms of this protein (DHP A and DHP B) that detoxify various haloaromatics are present, both with wide substrate specificities. The question that we try to answer is if we observe a case of early events in gene duplication and divergence. The present study indicates DHP B is clearly a better dehaloperoxidase than DHP A; it is also a stronger oxygen-binder when compared to DHP A. Given that both peroxidase and globin functions are carried out utilizing the heme as an active center, it is not surprising that
DHP B has lower $K_{O_2}$ for dioxygen binding but higher $K_m$ for hydrogen peroxide binding. The increased catalytic efficiency of DHP B for dehaloperoxidation of TCP was driven by the higher catalytic rate ($k_{cat}$). As determined by this study, the oxidation rate in DHP B from oxyferrous state to ferric state is also faster than that in DHP A. It is assumed that the expression level of DHP B in *A. ornata* would be expected to be higher when this organism was exposed to the environment that severely contaminated by halophenols.

Looking at the evolution of dual function DHP in *A. ornata*, the interesting question is: Why DHP B evolved to improve its peroxidase activity as well as oxygen affinity concurrently? One possible answer is that the increased oxygen binding capability in DHP B may not really benefit *A. ornata* in term of oxygen transport, because high oxygen affinity would make oxygen binding too tight to release it. Mammalian myoglobins must have stronger oxygen binding abilities to obtain oxygen released from hemoglobin during relaxation, whereas it must off-load O2 efficiently to support metabolism during contraction (31), accordingly, myoglobin shows about 10 fold higher oxygen affinity than hemoglobin. It may be speculated that in *A. ornata*, divergent evolution of DHP may reflect emergence of myoglobin (DHP B) to perform O$_2$ storage function, whereas DHP A is maintained to transport O$_2$, like hemoglobin.

**CONCLUSION**

In the present study, we have demonstrated DHP B not only showed enhanced peroxidase function and conversion capabilities from ferrous to ferric state, but also improved oxygen-binding abilities. Our functional studies demonstrated that the variations at positions 34 and 91 affect more oxygen binding affinity than peroxidase
function and functional switching properties of DHP A and DHP B. From the evolutionary perspective, the two functions in DHP are competing. Although DHP B possesses relatively high oxygen binding affinities, it is presumably not an advanced oxygen carrier in *A. ornata* because of the attenuated oxygen release abilities. In addition, we have prepared two mutants DHP A Y34N and Y34N/S91G, which partially transits DHP A to DHP B, to further clarify the structural and functional difference observed between DHP A and DHP B. The studies present herein strongly indicates the amino acid variations at position 34 and 91 in DHP mainly contributes the functional difference in oxygen affinity between DHP A and DHP B, rather than peroxidase function and oxidation conversion abilities. In the distal site, the substitution at position 34 in the vicinity of the heme undergoes a subtle change in hydrogen bonding and therefore alters the heme environment, which may play an important role on the enhanced oxygen affinity. In the proximal pocket, the substitution of S91G increases the mobility of the loop including the proximal histidine and likely affects the geometry of bound oxygen, thereby resulting in the increased oxygen binding affinity in DHP B. A detail of structural and functional analysis in this work is essential to understand how *A. ornata* acquires two different isoforms of bifunctional DHP in the nature and has given some insight that how the divergence of DHP into DHP A and DHP B occurs in the process of evolution.
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Table 3.2. Crystallographic data and refinement statistics for DHP mutants.

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<td>Average B factor for solvent</td>
<td>30.5</td>
<td>40.0</td>
<td>33.2</td>
<td></td>
</tr>
</tbody>
</table>
Table 3.3. Comparison of selected parameters in the heme center for oxy ferrous DHP variants. Only the parameters in subunit A are list for all the structures.

<table>
<thead>
<tr>
<th></th>
<th>C73S•O₂  (PDB code: 2qkn)</th>
<th>Y34N•O₂</th>
<th>Y34N/S91G•O₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe-His89Nε² (Å)</td>
<td>2.15</td>
<td>2.15</td>
<td>2.26</td>
</tr>
<tr>
<td>Fe-His55Nε² (Å)</td>
<td>5.13</td>
<td>5.19</td>
<td>5.54</td>
</tr>
<tr>
<td>Fe-Cα (Å)</td>
<td>8.43</td>
<td>8.43</td>
<td>8.45</td>
</tr>
<tr>
<td>O1-His55Nε² (Å)</td>
<td>2.82</td>
<td>3.06</td>
<td>3.12</td>
</tr>
<tr>
<td>O2-His55Nε² (Å)</td>
<td>3.22</td>
<td>3.38</td>
<td>3.66</td>
</tr>
<tr>
<td>Fe-O1 (Å)</td>
<td>2.42</td>
<td>2.11</td>
<td>2.25</td>
</tr>
<tr>
<td>Fe out of porphyrin plane (Å)</td>
<td>0.08</td>
<td>0.13</td>
<td>0.08</td>
</tr>
</tbody>
</table>
Table 3.4. Michaelis-Menten parameters of DHP variants for TCP, oxygen affinity and first-order oxidation rate from ferrous to ferric state of DHP variants in 100 mM potassium phosphate buffer (pH 7) at 4 °C.

<table>
<thead>
<tr>
<th>Protein (4 °C, pH 7.0)</th>
<th>$k_{cat}$ (min$^{-1}$)</th>
<th>$K_m$ (µM)</th>
<th>$k_{cat}/K_m$ (mM$^{-1}$min$^{-1}$)</th>
<th>$K_{O2}$ (µM)</th>
<th>$k$ (s$^{-1}$) Oxidation Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>DHP A</td>
<td>63 ± 4.0</td>
<td>495 ± 62</td>
<td>127</td>
<td>3.23 ± 0.37</td>
<td>0.022 ± 0.001</td>
</tr>
<tr>
<td>Y34N DHP A</td>
<td>85 ± 12</td>
<td>591 ± 76</td>
<td>143</td>
<td>2.50 ± 0.22</td>
<td>0.029 ± 0.003</td>
</tr>
<tr>
<td>Y34N/S91G DHPA</td>
<td>51 ± 4.1</td>
<td>349 ± 58</td>
<td>146</td>
<td>1.71 ± 0.01</td>
<td>0.023 ± 0.001</td>
</tr>
<tr>
<td>DHP B</td>
<td>123 ± 4.5</td>
<td>430 ± 33</td>
<td>285</td>
<td>1.56 ± 0.06</td>
<td>0.076 ± 0.003</td>
</tr>
</tbody>
</table>
Table 3.5. Michaelis-Menten parameters of DHP variants for H$_2$O$_2$

<table>
<thead>
<tr>
<th>Protein</th>
<th>$k_{\text{cat}}$ (min$^{-1}$)</th>
<th>$K_m$ (µM)</th>
<th>$k_{\text{cat}}/K_m$ (mM$^{-1}$min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DHP A</td>
<td>25 ± 1.0</td>
<td>62±7.4</td>
<td>411</td>
</tr>
<tr>
<td>Y34N DHP A</td>
<td>48 ± 0.9</td>
<td>112±10</td>
<td>424</td>
</tr>
<tr>
<td>Y34N/S91G DHPA</td>
<td>31± 0.1</td>
<td>65±1.9</td>
<td>480</td>
</tr>
<tr>
<td>DHP B</td>
<td>116 ± 5.2</td>
<td>172 ±18</td>
<td>679</td>
</tr>
</tbody>
</table>
Figure 3.1. Superposition of the Y34N•O₂ complex, shown in pink, and the C73S•O₂ complex (PDB code: 2qfn), shown in grey. The substitution of tyrosine with asparagine at position 34 introduces a new hydrogen bond between Asn34 and Glu31 in Y34N and breaks the hydrogen bonds between Tyr34 and the surrounding amino acids (Arg33 and Asn96) in C73S.
Figure 3.2. Superposition of Y34N•O$_2$ (pink), Y34N/S91G•O$_2$ (blue) and C73S•O$_2$ complexes (grey; PDB code: 2qfn) showing the geometries of the dioxygen ligand in the different oxyferrous DHPs. The distal histidine 55 forms hydrogen bonding contacts with O$_2$ and the heme propionate D.
Figure 3.3. Stereoview of the electron-density map of the Y34N-O$_2$ complex contoured at a 1.0σ level. The hydrogen bonds formed between distal histidine 55 and the dioxygen molecule are represented by dotted lines.
Figure 3.4. Superposition of the Y34N•O₂ complex (pink) and aquomet-DHP A (yellow; PDB code: 2qfk) displaying the difference of distal histidine 55 positions in the different oxidation states of DHP.
Figure 3.5. Stereoview of the electron-density map of the Y34N/S91G•O₂ complex. Final 2Fo - Fc electron density is contoured at a 1.2 σ level. The lack of density of loop 88-91 reflecting the increased loop mobility is displayed.
Figure 3.6. Superposition of aquomet-Y34N/S91G (cyan), aquomet-DHP A (green; PDB code: 2qfk) and aquomet-DHP B (orange; PDB code 3IXF). The water molecules are represented as spheres.
Figure 3.7. Conversion of oxyferrous-DHPs to ferric DHPs in the presence of excess of TCP and 1 equiv of H$_2$O$_2$. The inset shows the absorption change as a function of time, after the reaction of the premixture oxyferrous-DHPs and TCP was initiated with H$_2$O$_2$. 
CHAPTER 4

COMPLEX OF MYOGLOBIN WITH PHENOL BOUND IN PROXIMAL CAVITY

This work has been published in *Acta Crystallogr Sect F Struct Biol Cryst Commun* 2012, 68, Pt 12, 1465-71.

This work was supported by NSF Grant 0820456. Data were collected at the Southeast Regional Collaborative Access Team (SER-CAT) and Structural Biology Center (SBC) 22-ID beamlines at the Advanced Photon Source, Argonne National Laboratory. Use of the Advanced Photon Source was supported by the U.S. Department of Energy, Office of Basic Energy Sciences, under Contract No. W-31-109-Eng-38.

The PDB files with atomic coordinates of Sperm Whale Myoglobin (Mb) have been deposited in the Protein Data Bank as entries (3U3E) wtMb•phenol complex, (4H07) G65T•phenol complex and (4H0B) G65T•DMSO complex.

Protein purification, crystallization of wtMb and kinetic analysis were performed by Chunxue Wang.

Protein purification, crystallization and structural determination of G65T were performed by Xiao Huang.

Structural determination of wt-Mb were performed by Lesa R. Celeste.

ABSTRACT

Sperm whale myoglobin (Mb) has weak dehaloperoxidase activity and catalyzes the peroxidative dehalogenation of 2,4,6-trichlorophenol (TCP) to 2,6-dichloroquinone. Crystals of Mb and its more active G65T variant were used to study the binding of TCP, 4-iodophenol (4-IP) and phenol. The structures of crystals soaked overnight in a 10 mM solution of phenol revealed that a phenol molecule binds in the proximal cavity forming a hydrogen bond to the hydroxyl of Tyr146 and hydrophobic contacts which include interactions with C-beta and C-gamma of the proximal histidine, His93. The phenol position corresponds to the strongest xenon binding sites, Xe1. It appears that the ligand enters the proximal cavity through a gate formed by flexible loops 79-86 and 93-103. TCP and 4-IP do not bind to Mb in this manner under similar conditions, however, it appears likely that dimethyl sulfoxide (DMSO) used to facilitate 4-IP dissolution, at 0.8 M concentration, bound in the phenol/Xe1 binding site. In this structure, a water molecule coordinated to the heme iron is replaced by an oxygen molecule reflecting the reduction of the heme. Crystals of Mb and G65T Mb soaked for 5-10 min did not show bound phenol. Kinetic studies of TCP dechlorination showed that phenol has dual effect: it acts as a competitive inhibitor likely interfering with TCP binding at the heme edge and as a weak activator likely through binding in the proximal cavity. The lack of phenol bound at the heme edge, in the crystal structures, suggests that its inhibitory binding takes place only when the heme is activated by hydrogen peroxide.
INTRODUCTION

Myoglobin (Mb) is one of the most thoroughly studied proteins. Its principal function is as an oxygen carrier, but recently it was found that in some organisms it has acquired an additional function as a dehaloperoxidase, utilizing its heme as the catalytic center to generate active oxygen species. The first dual-function myoglobin/dehaloperoxidase (DHP) was discovered in *Amphitrite ornata*, a member of the *Terebellidae* family (sea worm). This enzyme catalyzes the oxidative dehalogenation reaction of polyhalogenated phenols utilizing $\text{H}_2\text{O}_2$ (1). Ferric myoglobin from species such as sperm whale or horse has a low-level peroxidase activity, but their physiological role appears to be solely as an oxygen binding protein (29). Dual function globin/peroxidase proteins in the ferric state bind peroxide in the distal cavity and their catalytic mechanism is similar to that well established for classical peroxidases (47).

Peroxidases typically do not have a well-defined binding site for organic substrates; rather their oxidation takes place at the heme edge (71). Surprisingly, crystal-binding studies revealed that DHP binds 4-iodophenol (4-IP), an organic substrate, in the distal cavity (2). Binding of 4-IP in this mode requires the distal histidine to swing out of the cavity, which consequently leads to the disassembly of the catalytic machinery. This observation generated controversy: is the 4-IP binding a part of the catalytic cycle or is it simply opportunistic? Recent results indicate that the latter is true (17). Other small ligands such as $\text{O}_2$, $\text{CN}^-$, $\text{CO}$, and imidazole, which bind in the distal cavity, tend to coordinate to the heme iron. Phenylhydrazine reacts with myoglobin and produces a covalent bond between one of the carbon atoms of the phenyl group and the heme iron (79).
Ligand binding in the unaltered proximal cavity was limited to the observation that exposure of Mb crystals to xenon gas at 7 atm resulted in the presence of one Xe atom in the cavity; its position is referred to as “Xe1 site” (PDB 1J52). Interestingly, the xenon binding sites in hemoglobin are different than in myoglobin and no Xe binding in the hemoglobin proximal cavities was observed, likely due to different protein dynamics (80). In general it was thought that the presence of the proximal histidine in its cavity prevents binding of larger molecules. In order to study the effects of the proximal histidine replacement by other ligands the “cavity mutant” H93G was engineered (81). This mutant was able to accommodate ligands such as 4-methylimidazole (81), β-mercaptoethanol, acetate (57), or phenol (82).

Mb ligands like O₂ or CO bind to the heme iron, producing the A state; the binding is stabilized by electrostatic interactions with His64 (83, 84). The binding can be disrupted upon photon absorption (85). After photo-dissociation, the ligand migrates into other internal cavities of the protein and then may diffuse back to the heme iron or escape into solution (86-88). After photodissociation, the CO molecule moved to the primary docking site yielding the B state; from there, the CO molecule migrates into internal cavities named after their xenon binding ability. Subsequently, the ligands may rebind to the heme iron or leave through the His64 gate (89, 90).

Our studies of other Mb variants that mimic DHP and have increased dehaloperoxidase activity (7) led us to investigate whether crystals of one such mutant, G65T, would bind 4-IP, as was observed for DHP. Since 4-IP is poorly soluble in water, we complemented this study by soaking the crystals in solutions containing 10 mM phenol. Unexpectedly, the crystal structure of G65T revealed a phenol molecule bound in
the proximal cavity. A follow up experiment with wild type Mb showed the formation of virtually the same complex, indicating that this mode of phenol binding is not a result of the mutation but a property of Mb. Soaking G65T crystals in 4-IP solution and DMSO yielded an entirely different complex: an oxyferrous protein with a DMSO molecule bound in the Xe1/phenol site.

**MATERIALS AND METHODS**

**Site-directed mutagenesis**

The pUC19 plasmid with wild type sperm whale Mb gene was a gift from Prof. Yoshihito Watanabe (Nagoya University). The mutagenic primers were designed to be complementary to the sense strand of sperm whale Mb cDNA. Primers were synthesized and purified by Integrated DNA Technology (IDT, Coralville, IA). Site-directed mutagenesis was performed using the Quick Change method and results were confirmed by LiCor DNA sequence analysis (USC Engencore DNA server, Columbia, SC) of the entire Mb gene.

**Protein expression and purification**

Plasmids containing either the Mb gene or the G65T variant gene were transformed into the *E. coli* BL21(DE3) cell line and screened successively on LB plates containing 100 µg/mL ampicillin. Colonies were picked from ampicillin plates, grown overnight in 5 mL of LB containing 100 µg/mL ampicillin and used to make cell stocks for protein expression and purification. The recombinant protein was purified as described previously (91) with modifications. *E. coli* BL21(DE3) harboring pUC19 was grown at 37°C in 2XYT (5 g of NaCl, 10 g of yeast extract, 16 g of tryptone per liter) in presence of ampicillin (100 mg/L). The dark-brown cells were collected and frozen in -
80°C until used. 20 g of cells were thawed at 4°C for 16 hours in 100 mL lysis buffer (50 mM Tris-HCl (pH 8.0), 1.0 mM EDTA, 0.5 mM dithiothreitol, 20 units of DNase per mL, 3 units of RNase per mL, 2 mg of lysozyme per mL). The cells were sonicated for 30 min on ice and cell debris was removed by centrifugation. The blood-red supernatant was collected and subjected to ammonium sulfate fractionation. Mb was precipitated from 55-95% ammonium sulfate. The precipitate was recovered by centrifugation, resuspended in a minimum volume of 20 mM Tris-HCl pH 8.0, 1 mM EDTA. The solution was applied to a Bio-Gel P-100 (Bio-Rad) gel-filtration column (3.0 x 30 cm). The appropriate fractions were pooled, concentrated, and applied to CM52 pre-swollen microgranular carboxymethyl cellulose (Whatman) cation exchange column (5 x 15 cm) equilibrated in 25 mM potassium phosphate pH 6.0. Protein was eluted by a linear pH gradient of 25 mM potassium phosphate buffer, pH 6.0 - pH 9.0, containing 1 mM EDTA. The protein purity was assessed by using the ratio of the Soret absorbance at 409 nm to the protein absorbance (mostly tryptophan) at 280 nm. Fractions of Mb with A409/A280 greater than 3.5 were considered to be pure and were pooled. To ensure that only the ferric form was isolated, the Mb proteins were treated with 1.7 molar excess of potassium ferriyanide on ice (Belyea et al., 2005). Excess ferriyanide was removed by allowing the sample to flow through a Bio-Gel P-6 DG Desalting Gel (Bio-Rad) gel-filtration column by gravity. The Mb proteins were concentrated to 10 mg/mL for crystallization.

**Crystallization**

Crystals of G65T Mb and wt-Mb were grown by the vapor diffusion method using the hanging-drop setup at conditions similar to those reported previously (22) with a well volume of 500 microliters and a drop volume of 2.5 microliters protein and 2.5
microliters well solution. The conditions used for all crystals consisted of 50 mM Tris-HCl pH 8.5, between 2.6 and 2.8 M ammonium sulfate, and 1.0 mM EDTA. For the phenol binding experiments, crystals of either Mb or G65T were soaked for approximately 16 hours in 10 mM phenol, 50 mM Tris-HCl pH 7.4, 1.0 mM EDTA, 3.2 M ammonium sulfate solution. For the 4-IP binding experiment, solid was dissolved in a DMSO solution (60% DMSO, 40% Tris-HCl pH 7.4 with 1.0 mM EDTA) to get 0.1 M 4-IP stock solution. The stock solution was then diluted to 10 mM soaking solution (in 50 mM Tris-HCl pH 8.5, 3.2 M ammonium sulfate, 1.0 mM EDTA) with a final concentration of DMSO at 0.8 M (6%). No precipitation was observed upon dilution. The crystals were soaked for approximately 16 hours. All crystals were cryo-conditioned by soaking in 20% v/v ethylene glycol enriched mother liquor for a few seconds and flash-frozen in N₂ vapor at -178°C.

**X-ray diffraction data collection and structure determination**

Data were collected at the SER-CAT 22-ID and LS CAT 21-ID-G beamlines at the Advanced Photon Source (APS), Argonne National Laboratory (92), Argonne, IL. The data were indexed and processed with the HKL2000 software package (60). Data collection and processing statistics are listed in Table 4.1. The structures were determined using molecular replacement with AMoRe (93) or Phaser (94) from the CCP4 suite of program (62) using the G65T structure (PDB entry 3OCK; (7)) as the search model. Structure rebuilding and subsequent refinements were performed using Turbo-Frodo (63) and Refmac5 (64). Coordinates were superposed using the LSQKAB program (95) from the CCP4 suite. Figures 4.1-4.6 were prepared using Turbo-Frodo. Figure 4.7 was made using MOLSCRIPT (96) and Raster3D (97).
**Dehaloperoxidase assay**

The effects of phenol binding on peroxidase activity of Mb were measured using a UV absorption spectroscopy based assay as described previously (7). Briefly, assays were conducted on a Cary 400 spectrophotometer at 4 °C and the absorbance peak at 272 nm was monitored to detect the concentration of the product, 2,4-dichloroquinone, versus time. Generally, Mb (5 µM) and variable concentrations of TCP were mixed in 100 mM potassium phosphate buffer at pH 7 in the absence or presence of phenol (0, 250 µM, 500 µM). The reactions were then initiated by addition of 2 mM H$_2$O$_2$. The time between phenol addition and readout was 3-4 minutes, or in the annealing experiment 3 days. The initial velocity was calculated by Cary WinUV software based on the linear initial portion of each reaction. The initial rates as a function of TCP concentration were fit to the Michaelis-Menten equation using the Prism 5 software.

**RESULTS AND DISCUSSION**

**Structures of Mb•phenol and G65T• phenol complexes**

The structures of Mb•phenol and the G65T•phenol complexes are very similar; their superposition yielded a rms distance between the positions of C$_\alpha$ of 0.12 Å. Below, where differences are observed, data for the G65T•phenol complex are given in parentheses.

The crystals had excellent scattering power and the electron density for the complex covered the whole molecule with the exception of a few side chains. The electron density for the heme and the proximal cavity for the Mb•phenol complex is shown in Figure 4.1 while for the G65T•phenol complex in Figure 4.2. Unlike in the DHP•4-IP complex (2), the phenol molecule binds in the proximal cavity. The binding
site is not saturated; the occupancy factors are approximately 0.6 and 0.8 in G65T and Mb complexes, respectively. The phenol snugly fits into a hydrophobic part of proximal cavity (Figure 4.3); its hydroxyl forms a 2.8 Å (2.7 Å) hydrogen bond to the phenolic hydroxyl of Tyr146, which in turn forms a 2.7 Å hydrogen bond to the carbonyl of Ile99 (Figure 4.4). The planes of the phenol molecule and the imidazole ring of His93 are not parallel; they interact through contacts between the C1 and O atoms of the phenol molecule and the Cγ and Cβ atoms of His93, 3.3 Å and 3.0 Å respectively (Figure 4.5). The phenol binding site corresponds to the Xe1 binding site, the main binding site for xenon (PDB 1J52). Also in this site a bound CO molecule was observed in the structure of the D state (photolyzed) Mb•CO complex (PDB code 1DO3, (98)). These complexes are remarkably similar as the rms. deviation between the positions of Cα is 0.15 Å (0.19 Å). In both complexes there is a water molecule bound to the heme iron, as expected for its ferric state.

To analyze the effects of phenol binding the structure of the aquomet Mb (PDB code 1A6K, (8)) was selected because it was determined at a comparable resolution, 1.1 Å, and crystals were also obtained from ammonium sulfate at pH 7.0. A superposition of the structures of the phenol complex and native Mb yielded a rms. distance between the positions of Cα of 0.48 Å. Comparison of these two structures reveals a change of the Leu89 rotamer, shown in Figure 4.4, which results in a 1.9 Å shift of Cγ. It is apparent that the side chain movement is due to phenol binding, as the same rotamer change is also observed for G65T and the G65T•phenol complex. The occupancy of the rotamers of Leu89 correlates with the phenol occupancy. Additional comparisons show that the rms. deviation between the positions of Cα of G65T•phenol complex and native G65T Mb is
0.07 Å while the rms deviation between the positions of Cα of native G65T and aquomet wt-Mb is 0.49 Å. This relatively high rms. deviation is likely due to different packing, space groups are P6 and P21, and the most affected region is the C-terminus, residues 148-152 where the positions of Cα shift by up to 1.9 Å.

The visible spectrum of the phenol complex is not significantly different from that of unliganded Mb (not shown). Isothermal titration calorimetric measurements did not allow us to determine the phenol binding constant. No peaks were observed upon titrant injections but rather a drift in the baseline. The most likely interpretation is that the binding process is too slow to be measured using this technique.

**G65T•dimethyl sulfoxide complex**

In the structure of G65T crystals soaked in 4-IP solution, there is no electron density that may correspond to a 4-IP molecule. However, in the distal cavity, there is a density feature that corresponds very well to an oxygen molecule coordinated to the Fe atom. It appears that 4-IP, reduced the heme to the ferrous state; this reaction enabled the binding of oxygen present in the soaking solution.

In the proximal cavity, there was a strong electron density peak (15σ in an omit Fo - Fc map) at the Xe1/phenol binding site. In the omit 2Fo - Fc map the corresponding peak also appeared too big for a water molecule (Figure 4.6); in other Mb structures no bound water was observed at this site. The hydrophobic environment of the peak makes for a plausible interpretation that a molecule of DMSO, present in the soaking solution, bound in this site. The density is not structured well and it appears that the DMSO molecule, if indeed present, is rotationally disordered. When a molecule of DMSO was inserted into the model and refined, the values of B-factors were 15 Å² for the S-atom
and approximately twice that for the terminal atoms. Those observations indicate that the ligand is heavier than a water molecule but disordered. We had no alternative hypothesis so we refer to the structure as G65T•DMSO complex.

The G65T•DMSO structure was superposed with that of Mb•CO complex in A state (CO bound to Fe) (PDB code 2MGK; (99)). The r.m.s deviation between the positions of $C_\alpha$ was 0.34 Å. It was also superposed with D state structure of Mb•CO (PDB code 1DO3; (98)) which has a CO molecule bound at the Xe1 site. The rms. deviation between the positions of $C_\alpha$ was 0.18 Å. The electron density for the heme and its environment in the G65T•DMSO complex is shown in Figure 4.6.

**Putative gate to the proximal cavity**

Based on CO photodissociation studies, it is assumed that small ligands such as CO and Xe enter the proximal cavity from the distal cavity through a transient channel between Ile107 and the heme (83). However it appears unlikely that larger ligands, such as phenol or imidazole, can enter the proximal cavity through the His64 gate and the same path because the heme makes a formidable hindrance. H93G Mb often referred to as “the cavity mutant” has been extensively used to study the effects of proximal ligands on myoglobin properties. Successes in binding ligands such as 4-methylimidazole, β-mercaptoethanol, phenol or acetate in the proximal cavity in the absence of the proximal histidine side chain preceded our observation (57, 81). Since the observed phenol binding took place in crystals, loops movement likely is responsible for phenol entry into the cavity rather than some major protein unfolding. NMR studies provide more information about the molecular dynamics; Mb NMR structures (PDB entry 1MYF, (100)) reveal four flexible loops that are residues 15-23, 48-59, 79-86 and 93-103. The
The effects of phenol binding on peroxidase activity of Mb

Short, 5-10 min, soaking of Mb crystals did not show phenol binding indicating that in crystals the process is relatively slow. Long soaks of Mb crystals in saturated solutions of the substrate, TCP, did not show TCP binding, which is consistent with our modeling studies indicating that there is not enough room for TCP binding in the proximal cavity (not shown). However, our kinetic studies of peroxidative TCP dechlorination in the presence of phenol showed that phenol is mainly a competitive inhibitor for this reaction, Figure 4.8, Table 4.2. If the deviations from Michaelis-Menten kinetics, apparent in Figure 4.8, are disregarded, the $K_i$ value is about 0.5 mM. One explanation of these observations is that the peroxidase reaction catalyzed by Mb takes place at the edge of the heme, like established for classical peroxidases (71). The absence of TCP or phenol bound at the heme edge in crystals soaked in concentrated solutions (about 20x$K_i$ for phenol) suggests that the binding takes place only when Mb is activated by hydrogen peroxide. We soaked crystals with both hydrogen peroxide and phenol but as in previous experiments no bound ligand was observed, probably due to the low stability of active intermediates. The observed slight increase of $V_{\text{max}}$ as a function of phenol concentration (Table 4.2 and Figure 4.9) can perhaps relate to phenol binding in the proximal cavity and increased “electron push” or altered protein dynamics.
The crystal soaking experiments resulted in partial occupancies of phenol suggesting that the rate of diffusion is a significant factor in crystals. On the other hand, the kinetic experiments showed only modest differences between dehaloperoxidase activity measurements after the protein was annealed for three days in phenol or not (Table 4.2). We estimate that in the absence of annealing the protein was exposed to phenol for 2-3 min. Thus in contrast to the situation in crystals, where soaks of about 10 min. did not show phenol binding, in solution time of several minutes appears to be sufficient for phenol entry into the proximal cavity.

**CONCLUSIONS**

The proximal cavity of Mb even in the presence of its Fe-coordinating histidine has enough room to bind modest size molecules such as phenol or DMSO in the Xe1 binding site. Phenol binding somewhat alters the electronic state of the proximal histidine as judged from increased $V_{\text{max}}$ for peroxidative dechlorination of TCP. It was previously noticed that myoglobin, although a binding protein, does not have a channel leading to its binding site but rather depends on the molecular dynamics to create a path, through the distal histidine swinging out of the pocket and creating the “gate”. The same is true at the opposite side of the heme, there is no channel leading to the proximal cavity. The ligand entry into the cavity likely takes place through a “side path” not through the distal cavity.

The affinity for the substrate – halophenol - binding at the heme edge, which is necessary for the dehalogenase activity, appears to be small in the absence of co-
substrate, hydrogen peroxide. The same is true for phenol functioning as competitive inhibitor and likely also binding at the heme edge.
Table 4.1. Crystallographic data and refinement statistics.

<table>
<thead>
<tr>
<th>Complex</th>
<th>wtMb•phenol</th>
<th>G65T•phenol</th>
<th>G65T•DMSO</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDB code</td>
<td>3U3E</td>
<td>4H07</td>
<td>4H0B</td>
</tr>
<tr>
<td>X-ray source</td>
<td>APS SERCAT ID</td>
<td>APS LS-CAT ID-G</td>
<td>APS LS-CAT ID-G</td>
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<td>Wavelength(Å)</td>
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<td>0.9800</td>
<td>0.9800</td>
</tr>
<tr>
<td>Number of Frames high pass (low pass)</td>
<td>128</td>
<td>100 (100)</td>
<td>200</td>
</tr>
<tr>
<td>Oscillation Range (degree)</td>
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<td>1.0</td>
<td>0.5</td>
</tr>
<tr>
<td>Crystal to detector distance (mm) (low pass)</td>
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<td>125 (250)</td>
<td>150</td>
</tr>
<tr>
<td>Temperature(K)</td>
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<td>100</td>
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<tr>
<td>Space group</td>
<td>P6</td>
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</tr>
<tr>
<td>Unit cell dimensions</td>
<td></td>
<td></td>
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</tr>
<tr>
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<td>90.309</td>
<td>90.531</td>
<td>90.499</td>
</tr>
<tr>
<td>b (Å)</td>
<td>90.309</td>
<td>90.531</td>
<td>90.499</td>
</tr>
<tr>
<td>c (Å)</td>
<td>45.204</td>
<td>45.128</td>
<td>45.319</td>
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<tr>
<td>Volume (Å³)</td>
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<td>320310</td>
<td>321438</td>
</tr>
<tr>
<td>% Solvent, Matthews coefficient</td>
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Table 4.2. Effects of phenol concentration on dehaloperoxidation of TCP by Mb. The data were fitted to the Michaelis-Menten model.

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<th>( K_M ) (mM)</th>
<th>( V_{\text{max}} ) (( \mu \text{M/s} ))</th>
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<tr>
<td>No inhibitor</td>
<td>0.77 ± 0.19</td>
<td>0.64 ± 0.08</td>
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<tr>
<td>250 ( \mu \text{M Phenol} )</td>
<td>1.49 ± 0.34</td>
<td>0.84 ± 0.12</td>
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<td>250 ( \mu \text{M Phenol} ) After 3 days annealing</td>
<td>1.07 ± 0.16</td>
<td>0.90 ± 0.08</td>
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<td>500 ( \mu \text{M Phenol} )</td>
<td>2.25 ± 0.32</td>
<td>0.94 ± 0.10</td>
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<td>500 ( \mu \text{M Phenol} ) After 3 days annealing</td>
<td>1.28 ± 0.13</td>
<td>1.05 ± 0.06</td>
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Figure 4.1. Stereoview of the heme environment in the Mb•phenol complex. The final 2Fo-Fc omit electron density is contoured at a 1.3 σ level.
Figure 4.2. Stereoview of the phenol binding site in the G65T•phenol complex. 2Fo-Fc omit electron density is contoured at a 1.0 $\sigma$ level.
Figure 4.3. Proximal cavity in the Mb\textbullet{phenol complex. The phenol molecule, shown in yellow, fits snugly within its hydrophobic part.
Figure 4.4. Superposition of Mb-phenol complex, shown in atom type with aquomet-Mb (PDB: 1A6K), shown in blue. The presence of the phenol ligand in the proximal binding site displaces the Leu89 side chain approximately 1.9 Å.
Figure 4.5. Structure of the Mb•phenol complex. The phenol hydroxyl hydrogen bonds with the hydroxyl of Tyr146 and forms contacts with the proximal histidine.
Figure 4.6. Stereoview of the heme environment in the 
G65T•DMSO complex in subunit A. Final 2Fo-Fc electron 
density is contoured at a 1.3 σ level. The electron feature 
assigned to a partially disordered DMSO molecule corresponds 
to the phenol binding site and also Xe1 binding site.
Figure 4.7. The structure of G65T•phenol complex superposed on variable loops observed in the Mb NMR structures. The peptide chain in the G65T•phenol complex is shown in blue; the variable loops are in other colors.
Figure 4.8. Lineweaver-Burk plot for peroxidative dechlorination of TCP by Mb.
Figure 4.9. Phenol binding on WT-Mb slightly the dehaloperoxidase activity after three days incubation. The initial velocities were plotted versus the increasing amounts of 2,4,6-TCP in absence and presences of variable concentrations of phenol (0, 250 µM, 500 µM).
CHAPTER 5

TAUTOMERISM OF DISTAL HISTIDINE IN HEMOGLOBIN/DEHALOPEROXIDASE

FROM AMPHITRITE ORNATA

This work was supported by NSF Grant 0820456. Data were collected at the Southeast Regional Collaborative Access Team (SER-CAT) and Structural Biology Center (SBC) 22-BM beamlines at the Advanced Photon Source, Argonne National Laboratory. Use of the Advanced Photon Source was supported by the U.S. Department of Energy, Office of Basic Energy Sciences, under Contract No. W-31-109-Eng-38.
ABSTRACT

Hemoglobin/dehaloperoxidase A (DHP A) is a dual function protein. In the ferrous state it functions as an oxygen carrier, but upon oxidation to the ferric state catalyzes the dehalogenation of halophenols in the presence of H₂O₂. Studies of a crystal of DHP A, variant Y34N, showed pentacoordinated iron atom with the heme geometry indicating the ferric state. The structure shows the distal histidine in the distal cavity in a novel location, hydrogen-bonded through the Nδ1 atom to a water molecule which also interacts with the hydroxyl of Tyr38. This structure may represent the state in which DHP binds hydrogen peroxide.

INTRODUCTION

Hemoglobin/Dehaloperoxidase (DHP) from *Amphitrite ornata* is the first discovered dual-function globin (1, 2, 4, 11, 13). Its principle role, common with other globins, is oxygen transport and storage. The second physiological function is H₂O₂-dependent oxidative dehalogenation of bromophenols i.e. peroxidase activity. Although globins from other organisms have been known to have intrinsic low level peroxidase activity in the ferric state there is no evidence for physiological function of this activity. Rather peroxidase functions are carried out by specialized enzymes (71). Although the folds of globins and classical peroxidase are distinct, both protein superfamilies utilize heme as an active center to either bind dioxygen to the ferrous heme or to bind hydrogen peroxide to the ferric heme respectively. It has been a subject of extensive investigation to explore DHP and its variants due to their unique nature of combining the two competing functions in a single protein (7, 15, 28, 50).
Dehalogenation reaction mechanism catalyzed by DHP is similar to that in typical peroxidases. Initially, hydrogen peroxide binds to ferric heme iron to yield high-valent oxidants known as Compound I (Fe$^{IV}$=O porphyrin $\pi$-cation radical) or Compound ES (Fe$^{IV}$ =O tyrosyl radical). The Compound I (or ES) oxidizes a halophenol substrate molecule by one electron to form a phenoxy radical and Compound II (Fe$^{IV}$ =O). The later subsequently oxidizes another substrate molecule to return to the ferric state (47-49). For peroxidase activity, the distal histidine is essential. It functions as an acid to facilitate the peroxide binding and then as a base to assist the heterolysis of the O-O bond in peroxide (101-104). As expected, the substitution of distal histidine causes striking effects on the peroxidase activity (44). In addition, the effects of the distance between the distal histidine and heme iron on peroxidase activity were probed by site-directed mutagenesis (7, 22). Substituting the amino acid Gly56 adjacent to distal His55 with Leu or Ile places the distal histidine in a position similar to that observed in classical peroxidases and leads to increased peroxidase function (7).

It is remarkable that the distal histidine in DHP exhibits much higher conformational flexibility compared to those in typical globins and peroxidases (2, 6, 15, 16, 50, 105). The crystal structures and extensive spectroscopic studies of DHP clearly show that distal histidine may occupy two positions at room temperature: The “closed” conformation that forms a hydrogen bond with the heme-bound ligand and an “open” conformation that is out the distal pocket (2, 5, 6, 15, 16, 50). In the structure of deoxy DHP, only the “open” conformation was observed likely due to the absence of heme iron ligand (6). The mobility of distal histidine in DHP has been proposed to be responsible for the functional switching between globin and peroxidase roles (15, 17).
Since the heme iron is redox sensitive and capable of absorbing free electron liberated by X-ray beam, the oxidation state of heme-containing proteins is easily impacted by synchrotron X-ray radiation (106-110). Its change may further affect the heme coordination and the conformation of amino acids in its vicinity. Conversely, mutations in the heme neighborhood may affect its redox potential and X-ray sensitivity (106). Some variants of DHP showed such behavior. The heme iron in DHP C73S crystals was completely reduced upon X-ray exposure and a ferrous heme with bound dioxygen was observed (5).

DHP has two isoforms, DHP A and DHP B, which differs in five amino acids (54). Because the difference between tyrosine (DHP A) and asparagine (DHP B) at position 34 was predicted to be an important variation, we crystallized and determined the structure of DHP A Y34N mutant. This structure shows a five-coordinate form likely generated by photoreduction during the data collection. Most interestingly, in this structure a novel conformation of the distal histidine different from those observed in other globins and peroxidases is observed.

**MATERIALS AND METHODS**

**Mutagenesis and Protein purification**

The PET 16B plasmid encoding DHP (non-his-tag) was used as a template to produce DHP mutants. The Y34N mutant was generated using the Quick Change Method as our previous study. DNA with Y34N mutation was verified by LiCor DNA sequencing (Engencore at the University of South Carolina). Recombinant DHP Y34N without his-tag was overexpressed in Rosetta *Escherichia coli* (DE3) cells and then purified with the established procedure as previously developed (49). DHP in the ferric
state was prepared by treating the purified protein with a small amount of potassium ferricyanide. The excess ferricyanide was removed using a Bio-Gel P-6 DG Desalting Gel gel-filtration column in 100 mM potassium phosphate (pH 7) at 4 °C. The spectra were consistent with the presence of aquo-met DHP.

**Crystallization**

The Y34N variant of DHP was crystallized in the ferric form using the vapor diffusion method in hanging-drop set-up at 277 K. Native proteins without his-tag were buffer exchanged into 20 mM sodium cacodylate pH 6.5 and concentrated to 10 mg/ml. The crystals were grown from solutions containing 0.2 M ammonium sulfate and 26-34% polyethylene glycol 4000, as previously reported (2, 49). The crystals were transferred into a cryoprotectant that was mother liquor with additional 10% ethylene glycol, and subsequently flash-frozen in liquid nitrogen at 100 K.

**Data collection and structure determination**

X-ray diffraction data set was collected at SERCAT 22BM beamline at the Advanced Photon Source (APS) in the Argonne National Laboratory. The data were indexed, integrated and scaled with the HKL2000 software package (60). Initially all data, 100 frames, were processed together, later the first 15 frames and last 85 were processed separately and the structures refined independently. Data collection and processing statistics are listed in Table 5.1. The structure of Y34N was solved using molecular replacement with the Phaser program (61) from the CCP4 suite of program (62) using the wt-DHP structure (PDB entry 1EW6) as the initial model. Structure rebuilding was carried out using Coot (111) and Turbo (63). Structure refinement and map calculation were performed using Refmac5 from the CCP4 (64, 65). Coordinates
were superposed using the LSQKAB program (66) from the CCP4 suite. Figures 5.1 were prepared using Turbo and Figures 5.2-5.4 were prepared using Pymol. The porphyrin mean planes were calculated based on the atoms of CHA, CHB, CHC and CHD from the porphyrin ring using Mercury (112) and the distances between Fe and the plane were then measured.

**RESULTS AND DISCUSSION**

*The overall structure of Y34N*

Purified recombinant DHP is a mixture of the oxyferrous (major fraction) and the aquomet protein (minor fraction). For the crystallization experiment the protein was oxidized with ferricyanide; its spectrum was consistent with the aquomet state. However, the structure of Y34N was not consistent with the aquomet state, in which a six-coordinated aquo-complex for the ferric heme is expected (5). There was no ligand bound to the heme iron in the distal cavity in both subunits, as evidenced by the lack of corresponding electron density at the heme iron in Fourier maps (Figure 5.1). This is likely due to the heme iron reduction by electrons generated upon X-ray exposure. The photoreduction of heme upon X-ray exposure leading to pentacoordinated Fe was observed previously in DHP B (PDB code: 3ixf, (58)) and C73S (5). The distal histidine, His55, in Y34N is positioned differently than in the aquomet DHP structure (PDB 2QFK), as shown in Figure 5.2. In the structure of aquomet DHP A, the N°1 atom of distal histidine forms a hydrogen bond (3.0 Å) with the hydroxyl of Tyr38 and the N°1 atom forms a hydrogen bond (3.1 Å) with the Fe coordinated water. In Y34N the side chain of His55 remained in the cavity but is rotated along Cβ-Cγ bond to form a hydrogen bond with a water molecule present at the Tyr38 hydroxyl. In this
conformation, the direct hydrogen bond between the distal histidine and Tyr38 is no longer present rather their interaction is mediated by the water molecule. The water molecule is located at a hydrogen bonding distance of 2.5 Å (2.2 Å in subunit B) from the atom N81 of His 55 and also forms a hydrogen bond with hydroxyl group of Tyr 38 at a distance of 2.8 Å (2.8 Å in subunit B) (Table 5.2 and Figure 5.1). The distal histidine position in Y34N is entirely different from that in oxyferrous Y34N (to be published), as shown in Figure 5.3. Our manual inspection of other globin structures deposited in the PDB did not show a similar histidine orientation. The Y34N structure also differs from deoxyferrous DHP A (PDB code: 3dr9), as shown in Figure 5.4. The crystal of deoxyferrous DHP A used for structure determination was generated by exposure to a reducing reagent, dithionite, at room temperature prior to crystal freezing. (PDB code 3dr9) (6). In both structures the heme iron is pentacoordinated, yet the unusual “closed” conformation of distal histidine in Y34N is entirely different than the “out of the pocket” conformation observed in deoxyferrous DHP A. It is possible that the water molecule observed at Tyr38/His55 is actually the one previously coordinated to the heme iron because in aquomet DHPA its position is blocked by His55 hydrogen-bonded directly to Tyr38.

The oxidation state of heme iron in Y34N

It was observed in globins that a major difference between the structures of oxyferrous state and deoxyferrous state is the position of the metal atom relative to the porphyrin plane (113-115). In the oxyferrous Mb, the heme iron is in the six-coordination low spin state (S=0), whereas in the deoxyferrous Mb the iron is in the five-coordination high spin state (S=2). The increase in spin state from S=0 to S=2 leads to the movement
of the iron away from the porphyrin ring \((116)\). In Mb, the heme iron in oxyferrous myoglobin is at a distance of 0.19 Å from the heme plane. Upon dioxygen dissociation the iron atom moves further out of the plane of porphyrin ring to 0.38 Å \((117-119)\). This effect was also observed for DHP. In oxyferrous DHP and deoxy DHP, the heme iron is at distances of 0.09 Å and 0.25 Å below the heme plane, respectively \((5, 6)\). In our structure of Y34N, the heme iron is positioned at a distance of 0.10 Å below the heme plane compared to that corresponding distance of 0.17 Å in the structure of oxyferrous Y34N \((5, 6)\). This closer distance between the iron atom and the porphyrin plane in the unliganded Y34N relative to oxyferrous Y34N is not consistent with the pentacoordinated deoxy ferrous state of the iron. Rather, this displacement of 0.10 Å is comparable to the corresponding distance of 0.09 Å observed in metaquo ferric DHP A, indicating that the heme iron in the structure of Y34N is likely in the high-spin ferric form with the unoccupied position for a sixth ligand. This high spin ferric resting state was predominantly observed in heme-containing peroxidases, such as lignin peroxidase, horseradish peroxidase and cytochrome c peroxidase \((71, 109, 120)\). For the ferric heme the Fe atom displacement from the porphyrin plane is less sensitive to the presence of the six ligand, In pentacoordinated horseradish peroxidase and ascorbate peroxidase the 0.17 Å displacement was found \((121, 122)\) not far from the 0.10 Å observed in Y34N. The five-coordinated resting state horseradish peroxidase is able to react with \(\text{H}_2\text{O}_2\) to yield oxidizing equivalents Compound I and Compound II via two sequential one-electron oxidation steps. We thus conclude that this structure is of unliganded Y34N, likely in the ferric state, with the side chain of His55 in an unusual closed conformation.
It remains an open question why the pentacoordinated iron is observed in Y34N crystal structure while spectral data indicate metaquo as the initial state. The simplest explanation is the heme reduction by X-ray generated electrons followed by the dissociation of water coordinated to the heme and the conformational change of His55. This is, however, not consistent with the observed lack of out-of-heme plane displacement of the iron atom which is pointing to the ferric heme. A more complex hypothesis that can be proposed is that the heme is indeed transiently reduced, the coordinated water lost and the conformation of His55 changed but eventually electrons return to their original sites leaving the heme oxidized while His55 and water do not return to their original positions at 100 K temperature but remain in the metastable state.

**Mechanistic implication for peroxidase reactivity**

The Y34N pentacoordinated structure is similar to the high spin resting state of classic peroxidases and may be related to the state in which hydrogen peroxide binds to the heme iron of DHP. The competition between water and hydrogen peroxide for the ferric heme distal binding site is likely a major factor limiting the peroxidase activity of globins. Indeed, the longer distance between the heme iron and the distal histidine, which weakens water binding to the ferric heme, correlates with higher peroxidase activity (7). The unusual conformation of the distal histidine observed in Y34N does not enable hydrogen bonding to the water molecule at the heme and the lack of this interaction results in the peroxidase-like pentacoordinated iron. Binding of a hydrogen peroxide molecule must induce the imidazole flipping and return to the standard conformation necessary for peroxide activation.
**CONCLUSION**

The structure of Y34N's shows a new conformation of the distal histidine which does not stabilize the water molecule at the heme. As a result, the heme iron is pentacoordinated and ferric as indicated by its small displacement from the porphyrin plane. The lack of a sixth ligand likely facilitates the binding of hydrogen peroxide, thus the structure may represent the DHP state in which the co-substrate $\text{H}_2\text{O}_2$ approaches the heme.
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<th>Y34N</th>
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Table 5.2. Comparison of selected parameters in the heme center for Y34N, oxyferrous Y34N, metaquo-DHP A, deoxy DHP A and oxyferrous C73S.

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<th>Oxyferrous Y34N</th>
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<th>Deoxyferrous DHP A (PDB:3dr9)</th>
<th>Oxyferrous C73S (PDB:2qfn)</th>
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<th>Deoxyferrous DHP A (PDB:3dr9)</th>
<th>Oxyferrous C73S (PDB:2qfn)</th>
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<th>Oxyferrous Y34N</th>
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<th>Deoxyferrous DHP A (PDB:3dr9)</th>
<th>Oxyferrous C73S (PDB:2qfn)</th>
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<tr>
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<td>8.43</td>
<td>8.51</td>
<td>9.07</td>
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<td>8.73/8.74</td>
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<th>Fe out of porphyrin plane (Å)</th>
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<th>Oxyferrous Y34N</th>
<th>Metaquo DHP A (PDB:2qfk)</th>
<th>Deoxyferrous DHP A (PDB:3dr9)</th>
<th>Oxyferrous C73S (PDB:2qfn)</th>
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<tr>
<td>Subunit A</td>
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<td>0.17</td>
<td>0.09</td>
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<tr>
<td>Subunit B</td>
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<td>0.18</td>
<td>0.12</td>
<td>0.31</td>
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Figure 5.1. Electron-density map of Y34N contoured at a $1.2\sigma$ level. There is no electron density observed for a ligand bound to heme iron in the distal cavity. The water molecule located in a new position is represented as red sphere and forms two hydrogen bonds, one with Tyr38 and the other with the distal histidine, His55.
Figure 5.2. Stereoview of the positions of the distal His55 in the Y34N, shown in cyan and ferric DHP A (PDB code: 2qfk), shown in green. The water molecules are represented as spheres.
Figure 5.3. Stereoview of the positions of the distal His55 in Y34N shown in cyan and the oxyferrous-Y34N shown in yellow. The positions differ although both are located in the distal pocket.
Figure 5.4. Stereoview of the distal His55 in Y34N shown in cyan and deoxy DHP shown in magenta. In contrast with the solvent-exposed open conformation of the distal His55 in DHP (partially disordered between two positions), the His55 in Y34N is inside the distal pocket. The water molecules near His 55 are represented as spheres.
CHAPTER 6

INFLUENCE OF HEME ENVIRONMENT STRUCTURE ON DIOXYGEN AFFINITY FOR THE DUAL FUNCTION *AMPHITRITE ORNATA* HEMOGLOBIN/DEHALOPOREDOXIDASE.

INSIGHTS INTO THE EVOLUTIONAL STRUCTURE-FUNCTION ADAPTATIONS

The work reported here, combing with other data, has been submitted to *Inorg. Chem*.

Mutagenesis, Protein purification, Crystallization and Structural determination of L100F•O$_2$ were performed by Chunxue Wang.

Preliminary results were reported by Shengfang Sun.

The PDB file with atomic coordinates of L100F•O$_2$ have been deposited in the Protein Data Bank with PDB code 4KJT.

ABSTRACT

Sea worm, *Amphitrite ornata*, has evolved its globin [an O$_2$ (dioxygen) carrier] so that it also serves as a dehaloperoxidase (DHP) to detoxify haloaromatic pollutants generated by competing species. A previous mutagenesis study by our group on both DHP and sperm whale myoglobin (SW Mb) revealed some structural factors that influence the dehaloperoxidase activities (significantly lower for Mb) of both proteins. Using a method based on isocyanide/O$_2$ partition constant measurements, the effects of these structural factors on the O$_2$ equilibrium constants (K$_{O2}$) of DHP, SW Mb, and their mutants have been examined in this study. For Mb, the results showed a trend of decreasing O$_2$ affinity and increasing catalytic activity along with the increase in the distal His N$^{ε2}$– heme iron distance. An H93K/T95H Mb double mutant mimicking the DHP proximal His positioning exhibited markedly enhanced O$_2$ affinity, confirming the essential effect of proximal His rotation on the globin function of DHP. For DHP, the L100F and M86E variants showed the effects of distal volume and proximal electronic push, respectively, on the O$_2$ affinity (moderate enhancement). The O$_2$ affinity of the ferrous T56G DHP mutant that likely has the distal His-55 with increased mobility was more responsive to the binding of the substrate 2,4,6-trichlorophenol and the inhibitor 4-bromophenol than the wild-type DHP, on which these halophenols have little effect. This study provides insights into how DHP has evolved its heme environment to gain significantly enhanced peroxidase capability for self-protection without compromising its primary function as an O$_2$ carrier.
INTRODUCTION

When the coelomic hemoglobin from the terebellid polychaete *Amphitrite ornata* was discovered in the 1970’s (73) its peroxidase activity was unknown. In the 1990’s, a dehaloperoxidase (DHP) enzyme was discovered in the same sea worm that detoxifies halophenols in the tidal sediments where it grows. It is the coelomic hemoglobin that carries out the DHP activity. Traditional heme-containing peroxidases (123) and globins [hemoglobins (Hbs) and myoglobins (Mbs)] (124) have distinct physiological functions: catalyzing the H₂O₂–dependent oxidation of substrates versus the reversible binding of oxygen (O₂, dioxygen), respectively. These protein superfamilies have entirely different folds and are not evolutionally related. However, both contain a heme unit bound to the protein scaffold through a proximal histidine that serves as a coordinating ligand to the heme iron, leaving the distal side of the heme capable of binding peroxide (peroxidase) or oxygen (globin). DHP, which is the most abundant protein in *Amphitrite ornata*, carries out both functions as a globin that transports and stores O₂ and as a peroxidase that detoxifies phenolic substrates (Scheme 6.1) (125). Its structure (with very similar folds to Mb (11), Figure 6.2) and amino acid sequence indicate that DHP have evolved from an ancient oxygen carrier globin (2). It has been a subject of intense investigations to probe what structural factors enable a globin to obtain peroxidase activity while still maintaining the O₂ carrier function (15, 28, 50). DHP has two isozyme forms, DHP A and DHP B encoded by two separate genes.
Scheme 6.1. The reaction catalyzed by DHP

They have very similar structures while only differing in five amino acids. (125, 126) This study will focus on DHP A as an extension of our previous investigations on DHP A and its mutants that we have prepared (7). In a recent study by our group, a mutagenesis approach was utilized to investigate several structural factors that influence the peroxidase activity of DHP (7). For example, the proper position of the distal His has been found crucial for the peroxidase activity of DHP (7). An O\textsubscript{2} binding property ($K_{O2}$ value) was reported by Weber et al. in 1977 for a coelomic hemoglobin isolated from the terebellid polychaetes *Amphitrite ornate* that was likely a mixture of two isoproteins (isozymes), DHP A and DHP B (see Results) (73). In the exogenous ligand-free ferric states (Figure 6.1) as well as in the ferrous O\textsubscript{2}-bound form (see Table of Content illustration, where PDB codes used for the structures of oxy-Mb, -DHP and HRP are 1a6m, 4kjt and 1h5d, respectively), DHP has an intermediate distal His-Fe distance (5.4 – 5.5 Å) compared to that in Mb (4.3 – 4.5 Å) and horseradish peroxidase (HRP) (~6.0 Å) and its dehaloperoxidase activity is also the intermediate of the three (7, 51, 127). This position of the distal His in DHP appears to be convenient for carrying out both O\textsubscript{2} binding in the ferrous protein and acid-base catalysis for O-O bond cleavage of the heme iron-bound peroxide by ferric DHP. Several Mb mutants with an elongated distal His-Fe distance (G65T, G65I and F43H/H64L Mb) showed enhanced dehalogenation abilities
The acquisition of peroxidase activity by DHP during evolution seems to have been achieved by the shift of the distal histidine position further from the heme iron relative to the usual location in O₂ carrying globins. As a consequence, the other function of DHP, reversible O₂ binding, might be affected since it has been shown that the bound O₂ in Mb is stabilized by H-bonding to the distal His (128-130). Another distal side factor that could regulate O₂ binding in Mb is the steric effect of Ile107. Replacing I107 in both human and sperm whale (SW) Mbs with a bulky Phe has been shown to increase the O₂ affinity (131, 132). In our previous mutagenesis study, a L100F DHP variant has been prepared and studied for its dehaloperoxidase activity (which is similar to that of the wild type) (7). DHP L100 is in the same position as the I107 in Mb (Figure 6.2). Thus, how the O₂ affinity of L100F DHP, as an analogue to the I107 Mb, is altered compared with that of wt-DHP has also been examined in the present study.

Besides the distal effect, a charge relay mechanism from the proximal side may also affect the dual functions of DHP. In classic peroxidases, there is a push-pull concept in the mechanism for the formation of the high-valent ferryl intermediate. The “push” effect comes from the partially deprotonated proximal His generated by formation of a strong hydrogen bond with a negatively charged adjacent residue (e.g., Asp in HRP, Figure 6.1) (133, 134). In DHP, there is no such “push” effect because the proximal His is neutral as in other globins (135, 136), which helps explain its relatively low enzymatic activity compared to typical peroxidases (67). The M86E mutation in DHP introduces a negatively charged residue nearby the originally neutral proximal His and expectedly enhances the peroxidase activity of DHP (7, 137). This mutation could also modify the
O$_2$ affinity since the charge relay system could stabilize ligand binding by enhancing the π-back-bonding from the iron to the ligand (138, 139).

Although DHP lacks the “push-pull” effect found in typical peroxidases, its peroxidase activity is still much higher than that of Mb. Comparing the heme coordination structures of DHP and Mb, a roughly 60° rotation of the proximal His imidazole ring has been found and is believed to play an essential role in the peroxidase function of DHP (Figure 6.2) (7, 11). This factor might also influence the O$_2$ binding ability of DHP as the rotation of the proximal His (Figure 6.3) has also been seen to affect O$_2$ affinity in heme proteins (36, 140). One example is soybean leghemoglobin-a (Lba) which exhibits ~20-fold higher O$_2$ affinity than SW Mb. The proximal His of Lba is in the “staggered” conformation (Figure 6.3B) while that of SW Mb is in “eclipsed” conformation (Figure 6.3A). The “staggered” conformation favors ligand binding with higher affinity than the eclipsed one via an electronic effect (141, 142). Therefore this 45° rotation of the proximal His in Lba compared to that in Mb is mainly responsible for its much higher O$_2$ affinity (140, 143-145). In our previous mutagenesis study, a proximal side Mb double mutant (H93K/T95H) has been prepared to mimic the rotational position of the proximal His in DHP (Figure 6.3C) that is close to the staggered conformation. This mutant is a much better peroxidase than wild type Mb (7). In this study the O$_2$ affinity for this mutant has been measured to probe the effect of proximal His rotation on O$_2$ affinity.

The distal His in DHP has two conformations, open and closed, and thus is thought to provide a regulation mechanism for both the peroxidase activation or inhibition as well as O$_2$ affinity (15, 17). Trihalophenol substrates such as 2,4,6-TXP (X =
F, Cl, Br) and parahalophenol inhibitors such as 4-XP bind to the distal side of DHP externally on the heme edge (2,4,6-TXP) or internally in the distal pocket (4-XP) and are believed to mediate the closed and open conformational switching of the distal His (15, 17, 45).

In this study, both heme distal and proximal side structural and electronic factors that influence the O₂-binding function of DHP have been examined to elucidate how DHP has altered its heme environment to evolutionally gain significantly enhanced peroxidase capability for self-protection without compromising its primary function as an O₂ carrier. The effects of substrate (2,4,6-TCP) and inhibitor (4-BP) binding on the O₂ affinities have also been evaluated for both wild type and mutant DHP (T56G) to probe their possible role(s) in functional switching of DHP from an O₂ carrier (ferrous state) to a peroxidase (ferric state).

**MATERIALS AND METHODS**

**Materials.**

All reagents and biochemicals were purchased from Aldrich, ACROS, or Fisher and used without further purification except for potassium ferricyanide, which was recrystallized from water. H₂O₂ stock (10 mM) in deionized water was prepared daily from a 30% commercial stock solution and the concentrations were routinely verified by UV-visible (UV-Vis) spectroscopic analysis at 240 nm (ε₂₄₀ = 39.4 M⁻¹cm⁻¹) (146). Trichlorophenol (TCP) and 4-bromophenol (4-BP) stock solutions (100 mM) were freshly prepared in a 50/50 (v/v) ethanol/deionized water mixture.
**Mutagenesis and Sample Preparation.**

The expression vectors (pUC 19) for wild-type and F43H/H64L sperm whale Mb were gifts from Prof. Yoshihito Watanabe (Nagoya University). The procedures of expression and purification for sperm whale Mb mutants (pUC 19-G65T, -G65l and –H93K/T95H) and 6×His-tagged DHP A mutants (pET 16b-T56G and M86E) can be found in our previous mutagenesis study (7). The isolated Mb and DHP proteins were completely oxidized to the ferric state by addition of a few crystals of potassium ferricyanide (Fluka) followed by gel-filtration column chromatography in 100 mM potassium phosphate (pH 5) at 4 °C. Protein concentrations were determined by the pyridine hemochromogen method ($\varepsilon_{555} = 34.4 \text{ M}^{-1} \text{ cm}^{-1}$) (147). The oxyferrous protein was formed by addition of a slight excess of sodium dithionite to the ferric protein, followed by aerobic Bio-Gel P6DG (Bio-Rad) desalting column with 100 mM potassium phosphate buffer (pH 7) at 4 °C. Deoxyferrous protein samples were prepared in a rubber septum-sealed cuvette by addition of a small amount of solid sodium dithionite to a thoroughly degassed ferric sample in 100 mM potassium phosphate buffer (pH 7) at 4 °C.

**O$_2$ dissociation equilibrium constant ($K_{O2}$) determination.**

O$_2$ dissociation equilibrium constants ($K_{O2}$) of DHP (or Mb) were determined by the method used by Makino and Yamazaki (148) by first measuring a partition constants ($K_p$) for the replacement of the bound O$_2$ in oxyferrous protein by isocyanide ligand (L) (eq. 1) and then a dissociation constant ($K_L$) for an isocyanide bound deoxyferrous protein (eq. 2).

\[
\text{DHP-O}_2 + L \xrightleftharpoons{\text{eq. 1}} K_p \text{ DHP-L} + O_2
\]

\[
K_p = \frac{[\text{DHP-L}] [O_2]}{[\text{DHP-O}_2] [L]}
\]

\[
\text{DHP-O}_2 + L \xrightleftharpoons{\text{eq. 2}} K_L \text{ DHP-L} + O_2
\]

\[
K_L = \frac{[\text{DHP-L}] [O_2]}{[\text{DHP-O}_2] [L]}
\]
\[
= \frac{[\text{DHP}][\text{O}_2]}{[\text{DHP-O}_2]} \times \frac{[\text{DHP}][\text{L}]}{[\text{DHP-L}]}
\]

\[
= K_{\text{O}_2} / K_{\text{L}}
\]

\(K_{\text{O}_2}\) and \(K_{\text{L}}\) are defined as follows:

\[
\text{DHP} + \text{L} \xrightleftharpoons{K_{\text{L}}} \text{DHP-L}
\]

(2)

\[
K_{\text{L}} = \frac{[\text{DHP}][\text{L}]}{[\text{DHP-L}]}
\]

(3)

\[
\text{DHP} + \text{O}_2 \xrightleftharpoons{K_{\text{O}_2}} \text{DHP-O}_2
\]

Thus, from Eq. (2)

\[
K_{\text{O}_2} = K_{\text{L}} \times K_{\text{p}}
\]

(5)

\(K_{\text{p}}\) and \(K_{\text{L(O}_2)}\) were determined by step-wise isocyanide titration of oxyferrous (~10 \(\mu\)M) and deoxyferrous (~3 \(\mu\)M) DHP/Mb, respectively, in 100 mM potassium phosphate buffer (pH 7.0 or 9.0 for one particular case of a Mb mutant) at 4 °C (or ~22 °C for a few cases). Two kinds of isocyanide ligands were used in the titration experiments depending on proper affinity ranges between the values \(K_d\) for the \(\text{O}_2\)-bound \((K_d = 10^{-5} \text{–} 10^{-3}\) M) and deoxyferrous states bound \((K_d = \sim10^{-6}\) M) of titrated proteins (if the isocyanide affinity is too high or too low, its accurate determination would be difficult or practically impossible because of large errors or ligand solubility limits, respectively): Isopropyl isocyanide (IPIC) was used for titrations of wt-Mb and H93K/T95H Mb; \(t\)-butyl isocyanide (TBIC) was used for titrations of other Mb mutants and all DHPs. For the titration of oxyferrous proteins, 4 mM ascorbate (pH adjusted to a neutral value) and bovine liver catalase (0.1 – 0.5 \(\mu\)M based on heme) were added to the sample to prevent autoxidation of the heme and ascorbate-caused undesirable reactions (149). To help maintain the \(\text{O}_2\) concentration constant throughout the titration (<3 h), the
sample were bubbled with air through a Pasteur pipette after each addition of ligand. The titration of deoxyferrous proteins were carried out anaerobically in the presence of slight excess of sodium dithionite in a rubber septum-sealed cuvette and the isocyanide ligands were added in a step-wise manner using a gas-tight microliter syringe.

Titration spectra were analyzed using a simple biomolecular association scheme and the plots of absorbance change vs. ligand concentration were analyzed by a hyperbolic regression fit of a saturation plot (absorbance change vs. ligand concentration), giving the values of $K_p$ and $K_L$. Three or more sets of $K_p$ and $K_L$ were obtained for each titration to calculate the final $K_{O2}$ using eq. 3. The O$_2$ concentrations were considered as 400 µM at 4 °C and 270 µM at room temperature (~22 °C) (150, 151).

**Peroxidase Activity Assay**

UV-Vis absorption spectroscopy was used to measure the peroxidase activity of DHP. TCP were added to the ferric enzyme (~3 µM) before addition of hydrogen peroxide to initiate the reactions in a 0.5-cm cuvette. The turnover number ($k_{cat}$) can be measured by monitoring the change in absorbance at 272 nm for the appearance of quinone products ($\varepsilon_{272} = 14 \text{ mM}^{-1}\text{cm}^{-1}$) (68). The $k_{cat}$ values were determined by calculating the velocity ($v_o$) from the initial linear portion of the trace at 272 nm as a function of H$_2$O$_2$ concentration with a fixed [TCP], followed by a graphical calculation (hyperbolic curve fitting) of a $v_o$ value at infinite [H$_2$O$_2$] to obtain a $v_{max}$ value and then by dividing the $v_{max}$ by the enzyme concentration ($k_{cat} = v_{max}/[[\text{DHP}] \text{ or } [\text{Mb}]]$). For each set of reaction conditions, 10 replicates were performed and then averaged to determine the velocity at the varying [H$_2$O$_2$] (typically 20 ~ 480 µM) and fixed [TCP] (150 µM for Mbs and 500 µM for DHPs). All the kinetics experiments were performed at 4 °C. The
pH for reactions catalyzed by DHPs is 7.0 (100 mM potassium phosphate buffer). The turnover of Mbs were measured at pH 5.4 (50 mM sodium acetate buffer) since the reaction rates were extremely slow at pH 7.0 for detection of quantifiable yields of quinone products.

**Spectroscopic Techniques.**

UV-Vis absorption spectra were recorded with a Cary 400 spectrophotometer interfaced to a Dell PC. Magnetic circular dichroism (MCD) spectra were measured with a magnetic field strength of 1.41 T by using a JASCO J815 spectropolarimeter equipped with a JASCO MCD-1B electromagnet and interfaced with a Gateway PC through a JASCO IF-815-2 interface unit. Data acquisition and manipulation were carried out as previously described (152).

**Crystallography.**

The data set was collected at SERCAT 22ID beamline at the Advanced Photon Source (APS) in the Argonne National Laboratory. The data were indexed, integrated and scaled with the HKL2000 software package(60). Data collection and processing statistics are listed in Table 6.3. The structure of L100F•O₂ was determined using molecular replacement with the Phaser program (61) from the CCP4 suite of program (62) using the wt-DHP structure (PDB entry 1EW6) as the initial model. Structure rebuilding was carried out using Coot (77) and Turbo (63) Structure refinement and map calculation were performed using Refmac5 from the CCP4 (65).
RESULTS AND DISCUSSION

Effects of Fe-Distal His55 Nε Distance

DHPA has an almost 10- (or 6-) fold lower O₂ affinity (i.e., higher \( K_{O_2} \) value) than that of SW Mb \((124, 148)\) (or horse heart Mb \((124, 153)\)). This is likely the result of a compromise involved in obtaining the peroxidase activity during the evolution of DHP while maintaining a functional requirement as Hb. In addition, other physiological factors likely also play a role given that human hemoglobin (a tetrameric and allosteric heme protein \((124)\)) has approximately 10-fold lower O₂ affinity than DHP at about neutral pH. Note that Mb itself has a dehaloperoxidase activity that is much lower (~ 8% that of DHP at pH 5.4, with TCP as substrate \((15)\)) than that of DHP.

One structural factor that contributes to the lower O₂ affinity of DHP is almost certainly the longer distal His \(N^{\varepsilon 2}\) – heme iron distance in DHP compared to that in Mb (Figure 6.1 and Table of Content). In our previous study, the dehaloperoxidase activities of G65T, G65I and F43H/H64L Mb mutants, all of which have elongated distances (by 0.3 – 1.1 Å) between distal His and heme iron, were examined. The resulting \( k_{cat} \) values for these mutants increased along with the distal His \(N^{\varepsilon 2}\) – heme iron distance (Table 6.1) \((7)\). This is consistent with the significantly \((\times \sim 6)\) enhanced peroxidase activity of the F43H/H64L Mb mutants over that of wt Mb and may be true within a certain distance limit (< 6.6 Å, estimated based on L29H/H64L Mb, PDB:10FJ) as found by Watanabe and co-workers \((154)\). Conversely, the \( K_{O_2} \) values measured in this study show the opposite trend. As shown in Table 6.1, their O₂ affinities decrease as the distal His \(N^{\varepsilon 2}\) – heme iron distance increases, This is reasonable considering the H-bond formed between the distal His and dioxygen in the wt-Mb might be weakened in the mutants with the
distal His located further from the heme center (128-130). Thus, based on the present results with Mbs, we propose that “the longer the heme iron-distal His Nε2 distance, the weaker the O2 affinity” co-relation exists in general for O2-binding heme proteins when other structural and electronic factors are kept similar. This finding (Table 6.1) in turn strongly suggests that the longer heme iron-distal His Nε2 distance in DHP than in wt-Mb must have a considerable O2 affinity lowering effect even by a factor of an order of magnitude (Table 6.1). However, to maintain its primary function as an O2 carrier, such an unfavorable effect may need to be compensated for by DHP by altering other structural/electronic factors such as those described below. Any such adaptations that also involve further enhancement of the peroxidase activity would certainly be even more beneficial for DHP.

**Steric Effects in the Distal Pocket**

The L100F DHP variant, with the volume of the distal cavity reduced by a larger side chain amino acid residue, was designed in our previous study as a probe of the TXP (X = Br, Cl) substrate binding site of DHP (7). The crystal structure of the L100F•O2 complex was determined in this study; its parameters and refinement statistics are listed in Table 6.3. The structure of this mutant in the ferric state has been reported by Plummer et al (155). The structure of the L100F•O2 complex shows that Phe100, which is located at the back of the cavity, does not form contacts with the bound dioxygen molecule nor with the distal histidine [Figure 6.4(A)]. The increased affinity is likely due to entropic factors resulting from the cavity volume reduction meaning that fewer disordered water molecules need to be displaced upon O2 binding. Similar conclusions, based on a modeled position of dioxygen, were reached for the I107F mutant of human and sperm
whale Mbs (131, 132). Only the subtle difference in the turnover numbers for this mutant compared to that for the wild type enzyme suggested that the productive binding site of substrate halophenol is not in the distal pocket (49). It is also interesting to test the O₂ affinity of this mutant since the analogous I107F Mb mutant has been studied as a part of unsuccessful effort to engineer high O₂ affinity Mb using Ascaris suum globin (131). As found in this study, the effect is similar, albeit stronger, I107F Mb has 1.4-fold higher O₂ affinity than wt-Mb while L100F DHP exhibits a 3-fold higher O₂ affinity than wt-DHP (Table 6.2). Apparently, this type of mutation would not be effective in enhancing higher O₂ affinity or peroxidase activity for DHP.

Effects of the Electronic “push” from the Proximal His89

Dioxygen affinity has been shown to positively correlate with electron density on the heme iron (148, 153, 156) and negatively correlated with reduction potential (157). An electronic “push” from the proximal side toward the heme center could promote the formation of an Fe³⁺-O₂⁻ -like species and thus hinder the dissociation of O₂ from the Fe²⁺-O₂ complex (158). The M86E DHP mutant designed in our previous study has a negative charge inserted near the neutral proximal His in the wt-DHP. Subsequently, Franzen and coworkers have shown that its redox potential is considerably lowered [from 202 mV to 112 mV (vs. SHE)] compared to the wild type, confirming the increased electron density on the heme iron of this mutant (137). As expected, the O₂ affinity for this DHP mutant shows a significant increase by about five-fold while at the same time displacing an approximately three-fold increase in dehalogenase activity (Table 6.2). However, DHP did not use this mutational strategy for its functional adaptation, presumably because the gains by this alteration may still not be beneficial enough.
However, in making such an interpretation it should be considered that this mutation was done on DHP, which has already an adapted O₂ affinity.

**Effect of the Rotational Position of the Proximal His89**

H93K/T95H Mb was prepared in our previous study for the purpose of mimicking the positioning of the proximal His in DHP (vide supra) (7). Although its crystal structure is not presently available, a model based on the DHP structure indicates that the proximal His in this mutant is rotated about 60° from the eclipsed conformation (Figure 6.3). This is a conformation that favors ligand binding with high affinity (see Introduction section). Indeed, this mutant exhibits ~20-fold higher O₂ affinity (examined at pH 9.0 to avoid enhanced autoxidation of its oxy-complex at pH 7.0) than that of wt-Mb (Table 6.4), which in turn supports the structural model for the mutant, along with ~50-fold increase in peroxidase activity as reported previously (7) (see also Table 6.4). These remarkable increases in both O₂ affinity and dehaloperoxidase activity by the H93K/T95H double mutation in Mb explain reasonably well why DHP has adapted its proximal His plane to a position that is rotated about 60° from the eclipsed conformation. The Kₐ₂ data obtained in this study give more importance to this rotation, which not only markedly increases peroxidase function but also helps maintain moderate O₂ binding affinity by sufficiently compensating for the weakened O₂ affinity as a consequence of the longer distal His Nε²–heme iron distance.

**Effects of Distal His-55 Flexibility: Oxygen Affinity of T56G DHP and Effects of the Substrate TCP and Inhibitor 4-BP.**

As described above, the flexibility and location of the distal His55 in DHP are key determinants for both O₂ binding and enzymatic activity. In the L100F•O₂ complex, the imidazole of the distal histidine is disordered between two positions: one inside the
cavity where it forms a hydrogen bond with the distal atom of the dioxygen molecule, a situation previously observed in many oxy-globins \((159)\); the other position is outside the cavity where it forms a hydrogen bond with one of the heme propionates. While the His55 disorder is indisputable, it also appears that the dioxygen occupancy is close to full as suggested by strong electron density [Figure 6.4(B)] and temperature factors comparable to those of the heme atoms. This indicates that even without hydrogen bonding from the distal histidine to the \(O_2\) molecule the heme affinity for dioxygen is sufficient for saturation at the atmospheric pressure.

The Thr56 adjacent to the distal His55 is likely to also affect the dynamics of the distal His, and thus we would expect to see significant effects on the functions of DHP by changing this residue. By replacing Thr56 in DHP with much smaller-sized Gly that is found in Mb next to the distal His-64 (Figure 6.2), the distal His55 is expected to gain more mobility. Unfortunately, we could not crystallize this mutant and thus its crystal structure is not available. However, the analysis of the molecular dynamics simulations very recently reported indicates that in T56G DHP, the distal His is further away from the heme iron and much more flexible than in the wild type \((105)\). This might explain the \(~3.4\)-fold higher \(k_{\text{cat}}\) for this mutant than wt-DHP (Table 6.5). This is also consistent with the proposed trend of “the longer the Fe-\(N^e\) distance, the greater the peroxidase activity” (within a certain limit of the distance\((154)\)). With respect to the \(O_2\) affinity for this mutant, the \(K_{O_2}\) value is \(~2.4\)-fold larger (i.e., the \(O_2\) affinity is lower) than that for the wild type (Table 6.5), which is also consistent with the “the longer the Fe-\(N^e\) distance, the lower the \(O_2\) affinity” trend for globins.
Since it has been found that the binding of substrates and inhibitors affects the equilibrium between the two conformations of the distal His55 (“closed” and “open”) for wt-DHP (2, 15) adding substrate (TCP) or inhibitor (4-BP) to T56G DHP should also affect its $O_2$ affinity. As shown in Table 6.5, the $O_2$ affinity of T56G DHP becomes almost two-fold higher and about 1.5-fold lower on addition of 2 mM TCP and 2 mM 4-BP, respectively. In contrast, addition of either 2 mM TCP or 4-BP barely altered the $K_{O2}$ of wt-DHP. This indicates that the effects of TCP (or 4-BP) on the distal His position equilibrium are much stronger for T56G DHP than for the wild type protein and are enhanced by the higher TCP affinity for the mutant. Note that none of the $K_L$ values in Table 6.5 for the affinity of isocyanide ligand used in the $O_2$ affinity measurements are significantly affected by added TCP or 4-BP.

Spectrophotometric titrations for TCP binding to ferric T56G DHP (not shown) and wt DHP (see ref. 11 for a similar titration) and spectral (V-Vis and MCD) characterizations of the resulting complexes suggest that the two proteins have distinct binding modes (positions and locations) for TCP (Figure 6.5). The resulting absorption and MCD spectra of TCP-bound ferric T56G DHP (Figure 6.5, red solid line) closely resemble those of the acetate complex of ferric wt-DHP (at pH 5.4, black dotted line in Figure 6.5) and Mb (at pH 7, blue dashed line in Figure 6.5), both reported previously (7). Furthermore, the binding affinity for the TCP complex of T56G DHP (pH 7.0, 4 °C) is found to be extremely high ($K_d \approx 3 \mu M$, this study).

Previously, two modes of TCP binding were observed in crystallographic studies: one “internal”, deep within the distal cavity, the other “external” with a TCP molecule blocking access to the distal cavity and hindering ligand binding to the heme
iron (49). Both TCP binding modes are different from the 4-XP binding mode (2) which is consistent with the spectroscopic data reported here. The TCP binding mode observed in crystals depended on DHP mutant studied and even in some cases was different in the subunits of the DHP dimer. On the other hand the very high TCP concentration used, 200 mM (50/50 water/ethanol), makes it unlikely that another TCP binding site in the studied proteins (DHP, Y34N, Y34N/S95G and L100F) exists (49). At lower concentrations, such as 2 mM, TCP binding in crystals was below the observation threshold. TCP functions as a substrate with a $K_M$ of about 0.5 mM because its affinity for Compound I is higher than for DHP (49). Similar conclusion was reached for Mb based on inhibition studies of its dehaloperoxidase activity by phenol (160). Ferric T65G DHP has TCP affinity much higher than any other DHP variant studied so far. It is highly unlikely that the TCP mode of binding is external because this mode locks the dioxygen binding site while T65G oxygen affinity in the presence of TCP is higher than in its absence. Much more plausible is that T65G DHP•TCP complex resembles L100F DHP•TCP and wt DHP•TBP complexes in which the internal binding mode is observed (18, 49).

The L100F•TCP structure allows more in depth mechanistic analysis because the TCP occupancy is high while in the reported DHP•TBP structure it is low, only 10%. In the L100F DHP•TCP complex there is a water molecule bound to the heme Fe and hydrogen bonded to the TCP hydroxyl. Although there is some overlap of the TCP hydroxyl positions in L100F DHP•TCP and the position of the distal atom of the dioxygen molecule in the reported here L100F DHP•$O_2$ structure (Figure 6.4), only a minor adjustment of the TCP position or tilting the $O_2$ molecule is needed to eliminate the hindrance. Also, the likely hydrogen bond between the hydroxyl and dioxygen can
explain the higher O₂ affinity of T65G DHP•TCP than T65G DHP as observed in this study, the assumption that the T65G•TCP complex is similar to the L100F DHP•TCP complex would suggest a water molecule as the distal ligand. The similarity of the MCD spectra of the TCP-bound T56G DHP with those of wt DHP•acetate complex in the ferric state (Figure 6.6) suggests an anionic oxygen ligand, perhaps hydroxide ion stabilized by the interaction with the TCP hydroxyl. No carboxylate-containing amino acid groups are available in the DHP heme vicinity as a candidate to become a heme ligand (distal side Asp54 is located > 10 Å away from the wt-DHP heme iron).

Implications of the Effects of TCP on the O₂ Affinity of DHP for the Functional Switch Mechanism

Our group previously proposed a functional switching mechanism for the conversion of DHP from an O₂ carrier to a peroxidase that is mediated by TCP radical generated by H₂O₂ and a trace amount of ferric DHP in the oxy-DHP sample (28). Recently, D’Antonio and Ghiladi proposed an alternative mechanism (15). In this mechanism, substrate (2,4,6-TXP) binding is proposed to cause a tautomerization of the distal H55 that destabilizes the bound dioxygen and thus shifts the equilibrium towards deoxyferrous DHP, which then reacts with H₂O₂ to form the catalytically active ferryl species Cpd II (15). Such reactions of ferrous heme proteins with H₂O₂ to yield ferryl species are known (161-163). However, the results demonstrated in this study indicate that the binding of TCP to DHP barely affects its dioxygen affinity, suggesting an alternative role of TCP in the functional switching such as a reductant.
CONCLUSION

Since the major Hb in the terebellid polychaete *Amphitrite ornata* was discovered to also have a physiological peroxidase activity, studies of the mechanism of this globin-catalyzed oxidative reaction have been the focus of much attention (15). As determined in this study, DHP has much lower O$_2$ affinity than SW Mb ($K_{O2(DHP)} = 3.23$ µM vs. $K_{O2(Mb)} = 0.27$ µM at 4°C), which is reasonable because Mb functions in O$_2$ storage while DHP transports O$_2$ as a coelomic Hb (73). The main findings in this study and their significance can be summarized as follows.

First, structural factors on both the distal and proximal sides of the heme prosthetic group that have been shown to affect the peroxidase activity of DHP (7) were also probed for their effects on O$_2$ affinity. We found a trend of “the longer the distal His Fe-$N^\varepsilon$ distance, the greater the peroxidase activity and weaker the dioxygen binding ability” for Mb. These trends help explain the intermediate globin and peroxidase function of DHP compared to HRP and Mb. On the proximal side, our results suggest that the rotational position and the lack of electronic push from the proximal His89 heme ligand in DHP is quite essential to maintain a proper O$_2$ affinity for its Hb function. These results demonstrate how DHP has gained significantly enhanced enzymic (peroxidase) capability in its evolitional process for protection from toxic living environment without compromising its primary function as an O$_2$ carrier.

Second, to understand a role(s) of the substrate TCP in the substrate triggered-functional switching mechanism for DHP, the influence on O$_2$ affinity upon the addition of the substrate-TCP has been evaluated. Destabilization of the O$_2$ complex of ferrous wt-DHP (i.e., weakening of the O$_2$ affinity) was not seen in the presence of even high
concentration (up to 2 mM) of TCP. These results challenge the newly proposed peroxidase cycle for DHP in which the oxyferrous derivative is the starting state and the release of O$_2$ is the trigger to activate peroxidase activity (50).
Table 6.1. O$_2$ affinity of SW Mbs (wt and mutants) and wt-DHP in 100 mM potassium phosphate buffer (pH 7) at 4 °C and their turnover numbers for the oxidative dechlorination with 150 µM TCP and varying H$_2$O$_2$ concentration in 50 mM sodium acetate buffer (pH 5.4) (Mbs) or 50 mM sodium citrate buffer (pH 5.4) (DHP) at 4 °C.

<table>
<thead>
<tr>
<th>Protein</th>
<th>$K_{O2}$ (µM) 4 °C, pH 7.0$^a$</th>
<th>Fe-His55 distance (Å)$^b$</th>
<th>$N^e$</th>
<th>$k_{cat}$ (min$^{-1}$) 4 °C, pH 5.4$^a,c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt-Mb</td>
<td>0.27 ± 0.01 (1.0)</td>
<td>4.3</td>
<td></td>
<td>19.0 ± 1.7 (1.0)</td>
</tr>
<tr>
<td>G65T Mb</td>
<td>0.39 ± 0.05 (1.4)</td>
<td>4.6</td>
<td></td>
<td>92.0 ± 7.0 (4.8)</td>
</tr>
<tr>
<td>G65I Mb</td>
<td>1.34 ± 0.21 (5.0)</td>
<td>5.1</td>
<td></td>
<td>138 ± 10 (7.3)</td>
</tr>
<tr>
<td>F43H/H64L Mb</td>
<td>6.12 ± 0.60 (23)</td>
<td>5.4</td>
<td></td>
<td>161 ± 9 (8.5)</td>
</tr>
<tr>
<td>wt-DHP</td>
<td>3.23 ± 0.37 (12)</td>
<td>5.4</td>
<td></td>
<td>243 ± 3 (13)</td>
</tr>
</tbody>
</table>

$^a$ The values in parentheses are relative $K_{O2}$ or $k_{cat}$ vs. those of wt-Mb. $^b$ PDB accession codes for metaqua structures of proteins listed in this table: wt-Mb (1A6K), G65T Mb (3O89), G65I Mb (3SDN), F43H/H64L Mb (1OFK) and DHP (1EW6). SW wt-Mb (1A6K), G65T Mb (3O89), G65I Mb (3SDN), F43H/H64L Mb (1OFK) and DHP (1EW6). $^c$ From ref (7).
Table 6.2. O₂ affinity of DHPs and their turnover numbers for the oxidative dechlorination with 500 µM TCP and varying H₂O₂ concentration in 100 mM potassium phosphate buffer (pH 7) at 4 °C.

<table>
<thead>
<tr>
<th>Protein</th>
<th>$K_{O_2}$ (µM) 4 °C, pH 7.0 a</th>
<th>$k_{cat}$ (min⁻¹) 4 °C, pH 7.0 a,b</th>
</tr>
</thead>
<tbody>
<tr>
<td>DHP</td>
<td>3.23 ± 0.37 (1.0)</td>
<td>25 ± 1.0 (1.0)</td>
</tr>
<tr>
<td>L100F DHP</td>
<td>1.03 ± 0.07 (1/3.1)</td>
<td>20 ± 1.7 (0.8)</td>
</tr>
<tr>
<td>M86E DHP</td>
<td>0.64 ± 0.08 (1/5.1)</td>
<td>78 ± 5.7 (3.1)</td>
</tr>
</tbody>
</table>

a The values in parentheses are relative $K_{O_2}$ or $k_{cat}$ vs. those of wt-DHP. b From ref (58).
<table>
<thead>
<tr>
<th>DHP complex</th>
<th>L100F•O₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDB code</td>
<td>4kjt</td>
</tr>
<tr>
<td>X-ray source</td>
<td>APS ID 22</td>
</tr>
<tr>
<td>Wavelength (Å)</td>
<td>1.000</td>
</tr>
<tr>
<td>Number of Frames</td>
<td>180</td>
</tr>
<tr>
<td>Oscillation Range (degree)</td>
<td>1.0</td>
</tr>
<tr>
<td>Temperature (K)</td>
<td>100</td>
</tr>
<tr>
<td>Space group</td>
<td><em>P</em>₂,₁,₂,₂₁</td>
</tr>
<tr>
<td>Unit cell dimensions</td>
<td></td>
</tr>
<tr>
<td>a (Å)</td>
<td>59.128</td>
</tr>
<tr>
<td>b (Å)</td>
<td>67.782</td>
</tr>
<tr>
<td>c (Å)</td>
<td>68.132</td>
</tr>
<tr>
<td>Volume (Å³)</td>
<td>273061</td>
</tr>
<tr>
<td>Mosaicity (deg.)</td>
<td>0.45</td>
</tr>
<tr>
<td>Resolution range (Å)</td>
<td>47.8-1.44</td>
</tr>
<tr>
<td>(highest shell)</td>
<td>(1.46-1.44)</td>
</tr>
<tr>
<td>Redundancy (highest shell)</td>
<td>6.9 (4.7)</td>
</tr>
<tr>
<td>Average I/σ(I)</td>
<td>15.0</td>
</tr>
<tr>
<td>Total number of reflections</td>
<td>342270</td>
</tr>
<tr>
<td>Number of unique reflections</td>
<td>47333</td>
</tr>
<tr>
<td>Completeness (%) (hi. shell)</td>
<td>98.3 (68.9)</td>
</tr>
<tr>
<td>Total linear R-merge (hi. shell)</td>
<td>6.4 (33.2)</td>
</tr>
<tr>
<td>R-value (%) (highest shell)</td>
<td>13.3 (19.0)</td>
</tr>
<tr>
<td><strong>R_{free}</strong> -value (%) (highest shell)</td>
<td>18.0 (26.7)</td>
</tr>
<tr>
<td>----------------------------------------</td>
<td>------------</td>
</tr>
<tr>
<td><strong>Ramachandran statistics</strong></td>
<td></td>
</tr>
<tr>
<td>% of residues</td>
<td></td>
</tr>
<tr>
<td>In most favored regions</td>
<td>94.0</td>
</tr>
<tr>
<td>In additional allowed</td>
<td>6.0</td>
</tr>
<tr>
<td>In generously allowed</td>
<td>0</td>
</tr>
<tr>
<td>Average B factor for protein</td>
<td>19.0</td>
</tr>
<tr>
<td>Average B factor for solvent</td>
<td>36.3</td>
</tr>
</tbody>
</table>
Table 6.4. O$_2$ affinity of SW wt-Mb and its H93K/T95H mutant in 100 mM potassium phosphate (pH 9) at 4 °C and their turnover numbers for the oxidative dechlorination with 150 µM TCP and varying H$_2$O$_2$ concentration in 50 mM sodium acetate buffer (pH 5.4) at 4 °C.

<table>
<thead>
<tr>
<th>Protein</th>
<th>$K_{O_2}$ (µM)</th>
<th>$k_{cat}$ (min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4 °C, pH 9.0</td>
<td>4 °C, pH 5.4 a,b</td>
</tr>
<tr>
<td>wt-Mb</td>
<td>0.33 ± 0.02 (1.0)</td>
<td>19.0 ± 1.7 (1.0)</td>
</tr>
<tr>
<td>H93K/T95H Mb</td>
<td>0.015 ± 0.003 (1/22)</td>
<td>104 ± 13 (5.5)</td>
</tr>
</tbody>
</table>

*a The values in parentheses are relative $K_{O_2}$ or $k_{cat}$ vs. those of wt-Mb. b From ref (7) .
Table 6.5. $O_2$ affinities ($K_{O_2}$) of wt-DHP and T56G DHP and turnover numbers for the oxidative dechlorination with 500 µM TCP and varying $H_2O_2$ concentration (100 mM potassium phosphate buffer, pH 7.0, 4 °C). See text for the definitions of $K_L$ (isocyanide equilibrium dissociation constant) and $K_P$ ($O_2$/isocyanide partition constant) for deoxyferrous DHP.

<table>
<thead>
<tr>
<th>Protein</th>
<th>$k_{cat}$ (min$^{-1}$)</th>
<th>$K_{O_2}$ (µM)</th>
<th>$K_L$ (µM) and $K_P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt-DHP</td>
<td>25 ± 1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>w/o TCP</td>
<td></td>
<td>3.23 ± 0.37</td>
<td>$K_L$ 3.47 ± 0.48</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$K_P$ 0.93 ± 0.06</td>
</tr>
<tr>
<td>+ TCP (2 mM)</td>
<td></td>
<td>3.53 ± 0.24</td>
<td>$K_L$ 3.60 ± 0.19</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$K_P$ 0.98 ± 0.02</td>
</tr>
<tr>
<td>+ 4-BP (2 mM)</td>
<td></td>
<td>2.95 ± 0.04</td>
<td>$K_L$ 3.17 ± 0.32</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$K_P$ 0.93 ± 0.15</td>
</tr>
<tr>
<td>T56G DHP</td>
<td>86.4 ± 6.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>w/o TCP</td>
<td></td>
<td>7.83 ± 0.83</td>
<td>$K_L$ 0.45 ± 0.03</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$K_P$ 17.40 ± 0.92</td>
</tr>
<tr>
<td>+ TCP (2 mM)</td>
<td></td>
<td>4.23 ± 0.52</td>
<td>$K_L$ 0.43 ± 0.04</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$K_P$ 9.83 ± 0.82</td>
</tr>
<tr>
<td>+ 4-BP (2 mM)</td>
<td></td>
<td>11.73 ± 0.89</td>
<td>$K_L$ 0.47 ± 0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$K_P$ 25.00 ± 2.83</td>
</tr>
</tbody>
</table>
Table 6.6. O$_2$ affinity of wild-type Sperm Whale Mb and DHP in 100 mM potassium phosphate buffer (pH 7.0) at 4 °C and ~ 22 °C.

<table>
<thead>
<tr>
<th>Protein</th>
<th>$K_{O2}$ (µM) $\quad 4 , ^{\circ}\mathrm{C}, , \mathrm{pH} , 7.0$</th>
<th>$K_{O2}$ (µM) $\quad \sim 22 , ^{\circ}\mathrm{C}, , \mathrm{pH} , 7.0$</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT Mb</td>
<td>0.27 ± 0.01</td>
<td>0.83 ± 0.06</td>
</tr>
<tr>
<td>DHP</td>
<td>3.23 ± 0.37</td>
<td>7.70 ± 0.45</td>
</tr>
</tbody>
</table>
Table 6.7. Effect of TCP on O\textsubscript{2} affinity of T56G DHP in 100 mM potassium phosphate buffer (pH 7.0) at 4 °C. See the main text for the definitions of K\textsubscript{L} (isocyanide equilibrium dissociation constant) and K\textsubscript{P} (O\textsubscript{2}/isocyanide partition constant).

<table>
<thead>
<tr>
<th>[TCP], mM</th>
<th>K\textsubscript{O2} (µM) \textit{4 °C, pH 7.0}</th>
<th>K\textsubscript{L} (µM) and K\textsubscript{P} \textit{4 °C, pH 7.0}</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>7.83 ± 0.83</td>
<td>K\textsubscript{L} 0.45 ± 0.03</td>
</tr>
<tr>
<td></td>
<td></td>
<td>K\textsubscript{P} 17.40 ± 0.92</td>
</tr>
<tr>
<td>0.4</td>
<td>6.17 ± 0.72</td>
<td>K\textsubscript{L} 0.46 ± 0.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td>K\textsubscript{P} 13.42 ± 0.77</td>
</tr>
<tr>
<td>0.8</td>
<td>5.60 ± 0.33</td>
<td>K\textsubscript{L} 0.47 ± 0.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td>K\textsubscript{P} 11.92 ± 0.98</td>
</tr>
<tr>
<td>2.0</td>
<td>4.23 ± 0.52</td>
<td>K\textsubscript{L} 0.43 ± 0.04</td>
</tr>
<tr>
<td></td>
<td></td>
<td>K\textsubscript{P} 9.83 ± 0.82</td>
</tr>
</tbody>
</table>
Figure 6.1. A comparison of distal side of sperm whale myoglobin (PDB: 1a6k), DHP A (PDB: 1ew6) and horseradish peroxidase (PDB: 1gwu) in the ferric state. Note that, in DHP A, two conformations, open (toward the front) and closed (toward the back) forms for the distal His-55, are shown.
Figure 6.2. Ferric DHP A (blue) and sperm whale Mb (red) X-ray structures alignments for entire peptide folds (A) and heme and its environmental amino acids that are subject of this study (B) created by superposing the common heme ring atoms. PDB accession codes are 1EW6 (DHP) and 1A6K (Mb). Note that, in DHP A, two positions (open and closed) are shown for the distal His-55. This figure was prepared using Pymol.
Figure 6.3. Proximal histidine rotational positions in (A) SW Mb, (B) Soybean leghemoglobin a (Lba) and (C) DHP. The dashed line is parallel to the plane of His ring, showing eclipsed (A) and staggered (B) conformations.
Figure 6.4. Stereoview of the structure (A) and electron densities (B) of the L100F DHP A•O2 complex and its heme distal side environment. Phe100 is located deep in the distal pocket and does not form contacts with dioxygen or His55. Two positions of His55 are stabilized through hydrogen bonding with O2 and a heme propionate. The final 2FoFc map is contoured at a 1σ level. Somewhat broaden density of the distal O2 atom of the dioxygen molecule may be related to the disorder of His55. This figure was prepared using Pymol and Turbo.
Figure 6.5. MCD (top) and UV-Vis (bottom) absorption spectral of TCP-bound ferric T56G DHP (red solid line, 0.8 mM TCP) in 0.1M potassium phosphate buffer pH 7.0, acetate-bound ferric DHP (black dotted line, 82 µM acetate) in 50 mM sodium citrate buffer pH 5.4 [re-plotted from (9)], acetate-bound ferric Mb (blue dashed line, 6 M sodium acetate) in 0.1 M potassium phosphate buffer pH 7.0 [re-plotted from (9)] and phenol-bound ferric Lba (green dot-dashed line) [re-plotted from (57)]. All spectra were recorded at 4 °C.
CHAPTER 7

STRUCTURAL AND FUNCTIONAL STUDIES OF K42N AND K42Y MYOGLOBINS

This work was supported by NSF Grant 0820456. Data were collected at the Southeast Regional Collaborative Access Team (SER-CAT) and Structural Biology Center (SBC) 22-ID beamlines at the Advanced Photon Source, Argonne National Laboratory. Use of the Advanced Photon Source was supported by the U.S. Department of Energy, Office of Basic Energy Sciences, under Contract No. W-31-109-Eng-38.

All the data reported here is performed by Chunxue Wang
ABSTRACT

Sperm whale Myoglobin (Mb), as an oxygen carrier, possesses weak peroxidase activity which enables it to carry out H$_2$O$_2$-dependent dehalogenation reaction. Hemoglobin/dehaloperoxidase from *Amphitrite ornata* (DHP) is a dual function protein, represented by two isoproteins DHP A and DHP B, which peroxidase activity is at least 10 times stronger and plays physiological role. The “DHP A-like” K42Y Mb mutant and “DHP B-like” K42N Mb mutant were engineered to mimic the heme environment in DHP A and DHP B, respectively. Peroxidase reaction rates increased ~3.5 fold and ~5.5 fold in K42Y and K42N versus wild type Mb, respectively. The crystal structures of metaquo- K42Y and K42N mutants reveal these substitutions at position 42 slightly elongate not only the distances between the distal His 55 and the heme iron, but also the hydrogen bonding distances between His 55 and the Fe-coordinated water. The enhanced peroxidase activity in K42Y and K42N is thus probably attributed to the weaker binding of the water molecule which competes with hydrogen peroxide for the binding site at the heme. Furthermore, positive cooperativity in K42N Mb was observed when the kinetic studies were conducted as a function of substrate trichlorophenol (TCP). The unique cooperativity in K42N was rationalized by protein dimerization induced by the excess H$_2$O$_2$. 
Sperm whale myoglobin (Mb), a monomeric heme protein of 153 amino acids (15.5 kDa molecular weight), has been intensively and thoughtfully studied during the past several decades. It functions as oxygen storage protein thorough binding dioxygen reversibly in the ferrous state (124). More recently, Mb was found to carry out the oxidative dehalogenation of halopheonls in the presence of hydrogen peroxide when its heme was in the ferric state (7, 29). Hemoglobin/dehaloperoxidase (DHP) discovered in *Amphitrite ornata* is a homodimeric globin that consists of 137 amino acids in each subunit (1, 2, 4). DHP has two physiological functions: the primary as an oxygen carrier to store and transport oxygen and secondary as an enzyme - dehaloperoxidase (15, 28). The peroxidase activity in DHP is over one order of magnitude higher than Mb, but its oxygen affinity is about 10 fold lower than that of Mb (7, 27, 29). The objective of this study was to identify the structural factors that modulate the two functions (2).

As observed previously, the replacement of Tyr34 (DHP A) with Asn34 (DHP B) enhances both peroxidase activity and oxygen affinity in DHP B. The amino acid alignment of DHP A and DHP B with Mb shows that Lys42 in Mb corresponds to Tyr34 in DHP A and Asn34 in DHP B, respectively (Figure 7.1). In order to support our hypothesis that this residue is important for the function, the Mb variants K42Y and K42N were generated and studied to mimic DHP A and DHP B at position 34. In the present studies, we not only determined the crystal structures of K42Y and K42N Mb mutants but also investigated the functional properties (peroxidase and oxygen carrier functions) of these two mutants.
MATERIALS AND METHOD

Site-directed mutagenesis and protein purification

The Mb mutants were generated using the Quick Change Method. The sequences of mutagenic primers are listed in Table 7.1. The pUC19 plasmid containing wild type Sperm Whale Mb was a gift from Watanable (the institute of Molecular Science, Nagoya University). DNA with desired mutations were verified by LiCor DNA sequencing (Engencore at the University of South Carolina) and then transformed into Escherichia coli BL21 (DE3) cells. Mb mutants were over expressed in BL Escherichia coli (DE3) cells and purified with the established protocol (56, 91).

Homogenous Mb proteins in the ferric state were prepared by adding potassium ferricyanide (57). The excess ferricyanide was removed using a Bio-Gel P-6 DG desalting column. In order to obtain oxyferrous Mb, the ferric proteins were treated with excess sodium dithionite followed by running a P-6 DG desalting column (56).

Crystallization

The Mb crystals were grown using a hanging drop method at 277 K. The proteins were buffer exchanged into 20 mM Tris pH 9.0 and concentrated to 20 mg/ml. For crystallization, 2 µL of protein was mixed with 2 µL of 20 mM Tris, 1 mM EDTA (pH 8.5 or 9.0) and 2.6 - 2.8 M ammonium sulfate, as reported previously (7, 164). The crystals were transferred into a cryoprotectant, which was the mother liquor with additional 20% ethylene glycol, and subsequently flash-frozen in liquid nitrogen at 100 K.
**Data collection and structure determination**

All X-ray diffraction data were collected at the SERCAT 22ID beamline at the Advanced Photon Source (APS) in the Argonne National Laboratory and then processed using the HKL2000 suit (60). The structures were determined by molecular replacement with CCP4 software (62). The Mb G65T structure (PDB entry 3OCK) was utilized as the starting models for solving the structures of other Mb mutants. The model rebuilding was carried out using Coot (77) and Turbo-Frodo software (63). The refinements and map calculations were performed using Refmac5 (64) from the CCP4 suit. The parameters and statistics for data collection and processing are summarized in Table 7.2. The superposition were conducted using Lsqkab (78) from the CCP4 suit. The superposition were conducted using Lsqkab (78) in the CCP4 suit. Figures 7.1 and 7.10 were prepared using PyMOL and Figures 7.3-7.6 were prepared using Turbo-Frodo.

**Dehaloperoxidase Activity Assay**

The dehaloperoxidase activity assay was performed on a Cary 400 spectrophotometer at 4 °C using a UV-vis absorption spectroscopy assay as described previously (7). The 272 nm absorbance peak of the 2,4-dichloroquinone product was monitored versus time (68). The initial rate for each reaction was calculated from the linear initial portion of the trace automatically using the Cary Win UV software set to kinetic mode. All the activity assays of Mb were measured at optimum pH 5.4, in 50 mM sodium citrate buffer. To measure the turnover number of Mb mutant, the concentration of Mb and TCP in each reaction was set to 5,0 µM and 150 µM, respectively. The $K_m$ of Mb for TCP was measured under the same conditions as that of DHP. The initial rates as a function of $\text{H}_2\text{O}_2$ or TCP concentration were fit to the Michaelis-Menten equation using the Prism 5 software.
RESULT AND DISCUSSION

In general, the structures of K42Y and K42N Mb are very similar to wt metaquo-Mb, yielding 0.7 Å r.m.s and 0.48 Å r.m.s deviations in Cα positions, respectively (Figure 7.2). The active sites of K42Y and K42N superposed with wt-Mb are shown in (Figure 7.3). In Mb, the Fe atom is located at a distance of 2.14 Å from the Nε of the proximal histidine, His93, and 2.13 Å from the coordinated water. In both K42Y and K42N, the Fe atom is located 2.07 Å from Nε of His93 and 2.08 Å from the coordinated water (Figure 7.4 and 7.5). The distal His-Fe distances in K42N and K42Y were slightly increased by 0.05 Å and 0.09 Å relative to Mb (4.30 Å), while distances between the heme Fe and the Cα are elongated to 8.63 Å and 8.65 Å from 8.56 Å in Mb, respectively (Table 7.3). The hydrogen bonding distance of 2.83 Å between Nε of His64 and the water coordinated to Fe in K42Y and K42N is 0.15 Å larger than that distance in Mb (2.67 Å). The length of this hydrogen bond, apparently affected by the main chain position, is negatively correlated to the strength of the bond. Thus the weaker bond between the distal histidine and the Fe-coordinated water facilitates the replacement of water by hydrogen peroxide and leads to the increased peroxidase activity. This is consistent with the trend observed for wild type metaquo-DHP, in which the distance from Nε of His64 to the coordinated water is fairly long (3.1 Å) whereas the peroxidase activity is relatively high. In the mutation-site, the NH3 of Lys 42 forms a 2.72 Å hydrogen bond with the carbonyl of Lys 98 in Mb (Figure 7.6). This hydrogen bond not present upon the replacement of this lysine with a tyrosine or asparagine. The side chains of Tyr42 and Asn42 do not form hydrogen bonds directly to surrounding amino acids. Rather, it appears that water molecules mediate Tyr42 and Asn42 hydrogen-bonding
interactions with other amino acids. In the structure of K42Y, the hydroxyl group of Tyr42 forms a 2.68 Å bond with a water molecule which forms another 2.77 Å hydrogen bond with the side chain of Tyr 103 (Figure 7.4). In the structure of K42N, a new hydrogen bond was induced involving NH$_2$ of Asn42 and a water molecule, which in turn forms a 2.88 Å hydrogen bond with the carbonyl of His97 (Figure 7.5). Notably, the hydrophobic residues Ile99 and Tyr103 around the mutation site were also slightly affected which probably in turn modulates the heme environment. In particular, in K42N the side chain of asparagine turns to face the pyrrole plane and is closer to the heme active site.

Kinetic data for the dehalogenation of TCP are shown in Table 7.4. A comparison of $k_{cat}$ values shows ~3.5 fold higher peroxidase activity for K42Y than for Mb. The activity of K42N is enhanced even more ~5.5 fold relative to Mb; this parallels the activity of the corresponding DHP enzymes. A comparison of heme environment in their crystal structures shows possible causes for the increased peroxidase activity of these two mutants. In addition to the destabilization of the bound water molecule discussed above the longer distal His-Fe distances in K42Y and K42N may provide more proper positions of the distal histidine for binding and heterolysis of peroxide.

Interestingly, the initial rates for K42N did fit better to the allosteric sigmoidal equation than to the standard Michaelis–Menten equation when we measured its activity as a function of TCP concentration (Figure 3.7 and 3.8). The sigmoidal curve indicates positive cooperativity in K42N Mb. In order to figure out whether there is Mb monomer aggregation driven by TCP, size-exclusion chromatography analysis was conducted in the presence and absence of 100 mM TCP (Figure 3.9). The elution volumes of K42Y
and K42N with and without TCP are the same within experimental errors indicating that their molecular weights are almost the same and no monomer aggregation occurs in the physiological condition. The co-crystallization of K42N with TCP was also tried but the structure of K42N co-crystallized with TCP doesn’t show any differences when compared to the structure of native K42N. Thus, the phenomenon of positive cooperativity of K42N is not due to the presence of substrate TCP. Previous studies showed that sperm whale myoglobin dimerized by a dityrosine cross-link in the presence of H$_2$O$_2$ (165-167). The computer docking experiment and mass spectrometry analysis showed the formation of the dityrosine covalent bond was involved in the Tyr151 of one Mb and Tyr103 of the other (166). Based on the crystal structure of Mb, Tyr103 is located in the surface of Mb (Figure 3.10) and the 42-mutation site is very close to Tyr103, both of which are accessible to solvent. Compared to lysine in Mb and tyrosine in K42Y, it appears that the relative small side chain of asparagine in K42N reduces the steric hindrance of cross-linking between Tyr103 and Tyr151. Thus, the probability of dimer formation in K42N increases statistically. The positive cooperativity of K42N Mb in the dehalogenation reaction is likely caused by dimerization in the presence of excess H$_2$O$_2$, but there is no available experimental evidence to prove that. Overall, both structural and kinetic analyses suggest that two more active peroxidases, which mimic DHP A and DHP B respectively, were engineered successfully. Also, this study probes the importance role of residue 42 on peroxidase activity in Mb.

**CONCLUSION**

We prepared two Mb mutants K42Y and K42N designed to mimic the Asn34 and Tyr34 in DHP A and DHP B, respectively. The mutants are better peroxidases than
Mb as predicted. The structures of these two Mb mutants reveal changes relative to wild-type Mb that are likely associated with the increased peroxidase activity. First, hydrogen-bonding networks in the vicinity of heme are slightly altered due to the mutations. Second, the distal histidine in K42Y and K42N Mb mutants is located further from the heme iron. Third, the hydrogen-bonding distances between the coordinated water molecule and the distal histidine His55 are longer. These changes observed here are likely associated with the enhanced catalytic activity in the mutants. Thus the residue at position 42 is important for the peroxidase function of Mb. In addition, the possibility of dimmer formation in K42N was explained by the replacement of lysine with asparagine that has a smaller side chain.
Table 7.1. DHP site-directed mutagenesis primers

<table>
<thead>
<tr>
<th></th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>K42N Mb</td>
<td>CCG GAA ACT CTG GAA AAT TTC GAT CGT TTC AAA</td>
</tr>
<tr>
<td>K42Y Mb</td>
<td>CCG GAA ACT CTG GAA TAT TTC GAT CGT TTC AAA</td>
</tr>
</tbody>
</table>
Table 7.2. Crystallographic data and refinement statistics for Mb mutants.

<table>
<thead>
<tr>
<th>DHP mutant</th>
<th>Aquomet K42Y</th>
<th>Aquomet K42N</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDB code</td>
<td></td>
<td></td>
</tr>
<tr>
<td>X-ray source</td>
<td>APS SER-CAT</td>
<td>APS SER-CAT</td>
</tr>
<tr>
<td></td>
<td>22-ID</td>
<td>22-ID</td>
</tr>
<tr>
<td>Wavelength(Å)</td>
<td>1.0000</td>
<td>1.0000</td>
</tr>
<tr>
<td>Number of Frames</td>
<td>125</td>
<td>110</td>
</tr>
<tr>
<td>Oscillation Range (degree)</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Temperature(K)</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Space group</td>
<td>$P2_12_12_1$</td>
<td>$P6$</td>
</tr>
<tr>
<td>Unit cell dimensions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>a (Å)</td>
<td>35.002</td>
<td>90.300</td>
</tr>
<tr>
<td>b (Å)</td>
<td>47.515</td>
<td>90.300</td>
</tr>
<tr>
<td>c (Å)</td>
<td>85.243</td>
<td>45.300</td>
</tr>
<tr>
<td>Volume (Å³)</td>
<td>141771</td>
<td>320025</td>
</tr>
<tr>
<td>Mosaicity (deg.)</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>Resolution range (Å) (hi. shell)</td>
<td>42.6-1.24 (1.26-1.24)</td>
<td>78.2-1.24 (1.26-1.24)</td>
</tr>
<tr>
<td>Redundancy (highest shell)</td>
<td>4.0 (1.9)</td>
<td>5.8 (2.1)</td>
</tr>
<tr>
<td>Average $I/\sigma(I)$</td>
<td>40.7 (476.5)</td>
<td>36.1 (270.4)</td>
</tr>
<tr>
<td>Total number of reflections</td>
<td>154441</td>
<td>343048</td>
</tr>
<tr>
<td>Number of unique reflections</td>
<td>38881</td>
<td>58965</td>
</tr>
<tr>
<td>Completeness (%) (hi. shell)</td>
<td>93.6 (54.1)</td>
<td>98.6 (84.7)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>------------------------------</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td><strong>Total linear R-merge</strong></td>
<td>4.8 (25.7)</td>
<td>5.8 (20.9)</td>
</tr>
<tr>
<td><strong>R-value (%) (highest shell)</strong></td>
<td>15.8</td>
<td>12.1</td>
</tr>
<tr>
<td><strong>R_{free} -value (%) (highest shell)</strong></td>
<td>20.8</td>
<td>14.6</td>
</tr>
<tr>
<td><strong>Ramachandran statistics</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Residues in most favored regions (%)</td>
<td>92</td>
<td>93.5</td>
</tr>
<tr>
<td>Residues in additional allowed regions (%)</td>
<td>8.0</td>
<td>6.5</td>
</tr>
<tr>
<td>Residues in generously allowed regions (%)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Residues in disallowed regions (%)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Average B factor for protein</td>
<td>16.0</td>
<td>9.62</td>
</tr>
<tr>
<td>Average B factor for solvent</td>
<td>33.3</td>
<td>32.3</td>
</tr>
</tbody>
</table>
Table 7.3. Comparison of selected distances at the heme for metaquo Mb, metaquo K42Y and metaquo K42N.

<table>
<thead>
<tr>
<th></th>
<th>Wt-Mb (1A6K)</th>
<th>K42N</th>
<th>K42Y</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe-His89N(^{r2}) (Å)</td>
<td>2.14</td>
<td>2.07</td>
<td>2.07</td>
</tr>
<tr>
<td>Fe-His55N(^{r2}) (Å)</td>
<td>4.30</td>
<td>4.35</td>
<td>4.39</td>
</tr>
<tr>
<td>Fe-Water (Å)</td>
<td>2.13</td>
<td>2.08</td>
<td>2.08</td>
</tr>
<tr>
<td>Water-His55N(^{r2}) (Å)</td>
<td>2.67</td>
<td>2.83</td>
<td>2.83</td>
</tr>
<tr>
<td>C(_\alpha)-Fe (Å)</td>
<td>8.56</td>
<td>8.63</td>
<td>8.65</td>
</tr>
</tbody>
</table>
Table 7.4. Turnover number ($k_{\text{cat}}$) for dehalogenation reaction in 50 mM sodium citrate buffer (pH 5.4) of Mb variants

<table>
<thead>
<tr>
<th>Variant</th>
<th>$k_{\text{cat}}^{\text{H}_2\text{O}_2}$ (min$^{-1}$)</th>
<th>$K_m^{\text{H}_2\text{O}_2}$ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt-Mb</td>
<td>19 ± 2.0</td>
<td>-</td>
</tr>
<tr>
<td>K42Y Mb</td>
<td>67.1 ± 3.13</td>
<td>2.695</td>
</tr>
<tr>
<td>K42N Mb</td>
<td>103.9 ± 5.39</td>
<td>2.727</td>
</tr>
<tr>
<td>DHP A</td>
<td>243 ± 3</td>
<td>-</td>
</tr>
</tbody>
</table>

The $k_{\text{cat}}$ values of wt-Mb and DHP A are from ref (7).
Figure 7.1. The amino acid sequences alignment of DHP A, DHP B and Mb. The amino acid differences at positions 34 and 91 between DHP A and DHP B are highlighted in red and another three differences at positions 9, 32 and 81 are highlighted in yellow. The Lys42 in Mb was shown in blue, which corresponds to the amino acid in position 34 in DHP.
Figure 7.2. Stereoview ribbon diagram of superposition of wild type Mb (green; PDB code: 1A6K), K42Y Mb (pink) and K42N Mb (turquoise). The heme and the mutation site at position 42 are shown in stick.
Figure 7.3. Superposition of wild type Mb (green; PDB code: 1A6K), K42Y Mb (pink) and K42N Mb (turquoise). The hydrophobic amino acids affected by the mutation were shown.
Figure 7.4. The active site of K42Y Mb and the environment of Try 42 in K42Y Mb.
Figure 7.5. The active site of K42N Mb and the environment of Asn 42 in K42N Mb.
Figure 7.6. The active site of wt-Mb and the environment of Lys42 in wt-Mb.
Figure 7.7. The dehaloperoxidase kinetic of K42Y Mb as a function of TCP. The initial rate was autocaculated by fitting the linear portion of first 10s of the reaction.

<table>
<thead>
<tr>
<th>Michaelie-Menten</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Best-fit values</strong></td>
<td></td>
</tr>
<tr>
<td>VMAX</td>
<td>0.1528</td>
</tr>
<tr>
<td>KM (μM)</td>
<td>49.91</td>
</tr>
<tr>
<td><strong>Std. Error</strong></td>
<td></td>
</tr>
<tr>
<td>VMAX</td>
<td>0.006</td>
</tr>
<tr>
<td>KM</td>
<td>5.5</td>
</tr>
<tr>
<td>Kcat (mol prod)(mol ENZ)^(-1) min^-1</td>
<td>30.56</td>
</tr>
</tbody>
</table>
Figure 7.8. The dehaloperoxidase kinetics of K42N Mb as a function of TCP. The initial rate was autocaculated by fitting the linear portion of first 10s of the reaction.
Figure 7.9. Size exclusion chromatography analysis of K42Y and K42N Mbs in the presence or absence of 100 mM TCP. The analysis was conducted using Superdex 75 and proteins were eluted with 20 mM Tris pH 8.0.
Figure 7.10. Spatial position relationship of Tyr 103 and the amino acid at position 42. Both of them are located at the edge of the protein.


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Title: Complex of Myoglobin with Phenol Bound in Proximal Cavity

Author: Xiao Huang, Chunxue Wang, Lesa R. Celeste, Leslie L. Lovelace, Shengfang Sun, John H. Dawson, and Lukasz Lebioda

Publication: Acta Crystallogr Sect F Struct Biol Cryst Commun

Publisher: IUCr Journals

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