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Nuclear Magnetic Resonance Spectroscopic and Computational Investigations of Chloroperoxidase Catalyzed Regio- and Enantio-Selective Transformations

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FLORIDA INTERNATIONAL UNIVERSITY

Miami, Florida

NUCLEAR MAGNETIC RESONANCE SPECTROSCOPIC AND COMPUTATIONAL
INVESTIGATIONS OF CHLOROPEROXIDASE CATALYZED REGIO- AND
ENANTIO-SELECTIVE TRANSFORMATIONS

A dissertation submitted in partial fulfillment of

the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

CHEMISTRY

by

Rui Zhang

2013

To: Dean Kenneth G. Furton
College of Arts and Sciences

This dissertation, written by Rui Zhang, and entitled Nuclear Magnetic Resonance Spectroscopic and Computational Investigations of Chloroperoxidase Catalyzed Regio- and Enantio-selective Transformations, having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this dissertation and recommend that it be approved.

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Florida International University, 2013

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DEDICATION

I dedicate this dissertation to my wife, Yu Zhang; my father, Chengxiao Zhang; and my mother, Yaqin Wang. Without their patience, understanding, support, and most of all love, the completion of this work would not have been possible.

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I want to thank my dear family and friends for their support. I would not have survived this long journey without them.

ABSTRACT OF THE DISSERTATION

NUCLEAR MAGNETIC RESONANCE SPECTROSCOPIC AND COMPUTATIONAL
INVESTIGATIONS OF CHLOROPEROXIDASE CATALYZED REGIO- AND
ENANTIO-SELECTIVE TRANSFORMATIONS

by

Rui Zhang

Florida International University, 2013

Miami, Florida

Professor Xiaotang Wang, Major Professor

Chloroperoxidase (CPO) is the most versatile heme-containing enzyme that catalyzes a broad spectrum of reactions. The remarkable feature of this enzyme is the high regio- and enantio-selectivity exhibited in CPO-catalyzed oxidation reactions. The aim of this dissertation is to elucidate the structural basis for regio- and enantio-selective transformations and investigate the application of CPO in biodegradation of synthetic dyes.

To unravel the mechanism of CPO-catalyzed regioselective oxidation of indole, the dissertation explored the structure of CPO-indole complex using paramagnetic relaxation and molecular modeling. The distances between protons of indole and the heme iron revealed that the pyrrole ring of indole is oriented toward the heme with its 2-H pointing directly at the heme iron. This provides the first experimental and theoretical explanation for the "unexpected" regioselectivity of CPO-catalyzed indole oxidation. Furthermore, the residues including Leu 70, Phe 103, Ile 179, Val 182, Glu 183, and Phe 186 were found essential to the substrate binding to CPO. These results will serve as a lighthouse in guiding the design of CPO mutants with tailor-made activities for biotechnological applications.

To understand the origin of the enantioselectivity of CPO-catalyzed oxidation reactions, the interactions of CPO with substrates such as 2-(methylthio)thiophene were investigated by nuclear magnetic resonance spectroscopy (NMR) and computational techniques. In particular, the enantioselectivity is partly explained by the binding orientation of substrates.

In third facet of this dissertation, a green and efficient system for degradation of synthetic dyes was developed. Several commercial dyes such as orange G were tested in the CPO-H₂O₂-Cl⁻ system, where degradation of these dyes was found very efficient. The presence of halide ions and acidic pH were found necessary to the decomposition of dyes. Significantly, the results revealed that this degradation of azo dyes involves a ferric hypochlorite intermediate of CPO (Fe-OCl), compound X.

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ABBREVIATIONS AND ACRONYMS

Ala	Alanine
Asn	Asparagine
COSY	Correlation spectroscopy
CPDO	1,3-cyclopentanedione
CPO	Chloroperoxidase
CYP1A1	Cytochrome P450, family 1, member A1
Cys	Cysteine
DSS	4,4-dimethyl-4-silapentane-1-sulfonic acid
ee	enantiomeric excess
ELISA	Enzyme-linked immunosorbent assay
ENDOR	Electron nuclear double resonance
EPR	Electron paramagnetic resonance
Glu	Glutamic acid
GROMACS	Groningen Machine for Chemical Simulations
GROMOS	GROningen MOlecular Simulation
His	Histidine
Ile	Isoleucine
K_D	The dissociation constant
LC-MS	Liquid chromatography-mass spectrometry
Leu	Leucine
MCD	Magnetic circular dichroism

MD	Molecular dynamic
MM	Molecular Mechanics
MP2	Second-order Möller-Plesset perturbation theory
NMR	Nuclear magnetic resonance
NOE	Nuclear overhauser effect
P450 BM3	Cytochrome P450 BM3
P450	Cytochrome P450
Pca	Pyroglutamic acid
PDB	Protein data bank
Phe	Phenylaniline
PME	Particle mesh Ewald method
QM	Quantum Mechanics
RI	Resolution-of-the-identity
RMSD	Root-mean-square deviation
Rz	Reinheitzahl
SDS	Sodium dodecyl sulfate
SPC	Simple point charge
T_1	Longitudinal relaxation time
UV-Vis	Ultraviolet-Visible
Val	Valine

CHAPTER 1

GENERAL INTRODUCTION

Peroxidase is an important group of enzymes that catalyzes oxidation of substrates using a peroxide. These enzymes have been discovered and used for centuries. Among the peroxidases, chloroperoxidase (CPO) has attracted more attention for deciphering the mechanism of its unique reactions and for using this enzyme to serve our life. Although tremendous efforts have been made in the past few decades, the mechanism of CPO-catalyzed reactions remains mysterious and its application is facing the challenges in industry. Therefore, my dissertation is dedicated to exploring the regio- and enantio-selective transformations catalyzed by CPO and expanding the application of this enzyme.

To describe the general knowledge about the chloroperoxidase and its related peroxidase counterparts, in this chapter, the super-family of heme peroxidase that CPO belongs to are generally described, followed by a description of the known crystal structures of CPO and its complexes. To illustrate the versatility of CPO, CPO-catalyzed reactions are reviewed according to their reaction types. The mechanisms and catalytic cycle of CPO-catalyzed reactions are also described to the best of my knowledge. After that, the spectroscopic, bioengineering, and computational methods used to explore these mechanisms of CPO-catalyzed reactions are extensively reviewed, which rationales the methods that are employed in the following chapters. In addition, the current challenges for the applications of CPO are discussed. At the end of this chapter, the specific aims and major findings of this dissertation are simply stated.

1.1 Peroxidase and Chloroperoxidase (CPO)

1.1.1 Peroxidase and its superfamily

Peroxidases (EC number 1.11.1 [1]) are named for an enzyme that catalyzes the oxidation of various substances by peroxides [2]. Although hydrogen peroxide is not as universal as oxygen, the peroxidases are ubiquitous and abundant enzymes in all forms of life.

The ability of peroxidase that preferably uses peroxide as an oxidation reagent is possibly inherited from ancient species. In the early days of the earth, the level of oxygen was raised in the atmosphere, which could happen 2.32 Gyr ago [3]. From that time, both prokaryotes and eukaryotes have evolved to elaborate mechanisms for the utilization of the reduced oxygen species such as hydrogen peroxide. Furthermore, organisms naturally produce hydrogen peroxide as a by-product of oxidative metabolism. Consequently, nearly all livings (specifically, all obligate and facultative aerobes) possess enzymes known as catalase peroxidases, which harmlessly and catalytically decompose low concentrations of hydrogen peroxide to water and oxygen. Currently, 15 different EC numbers have been ascribed to peroxidase: from EC 1.11.1.1 to EC 1.11.1.16 (EC 1.11.1.4 was removed).

1.1.2 Heme peroxidase and heme cofactor

Heme peroxidase, a peroxidase that contains heme as a cofactor, is the most abundant and heavily investigated among all the peroxidases. Out of 10630 peroxidase sequences were recorded in PeroxiBase, and more than 78% of peroxidases are coded for heme peroxidases [4]. According to the phylogenetic trees, heme peroxidases are distributed into 9 superfamily [4] (Figure 1.1) such as catalase, cytochrome-c peroxidase, myeloperoxidase, dye-decolorizing peroxidase, peroxidase, and haloperoxidase.

Figure 1.1: Overview of all heme peroxidase superfamilies.

A wide variety of hemes have been found in nature (Figure 1.2). Among these hemes, Heme b (iron protoporphyrin IX) is the prosthetic group (i.e., tightly-bound cofactors) for the majority of heme peroxidases. Heme b also provides the starting point for synthesis of the other types of heme. Usually, the heme group is enclosed in heme peroxidases by ligation with a protein residue. For instance, heme in chloroperoxidase is ligated by cysteine, while in horseradish peroxidase it is ligated by histidine.

Figure 1.2: Structural variants of the heme cofactor in nature.

In the heme-containing peroxidases, cytochrome c peroxidase, horseradish peroxidase, lactoperoxidase, and myeloperoxidase are of interest due to their wide applications or unique reactions, and thus are intensively studied. Cytochrome c peroxidase was the

first heme peroxidase that has its crystal structure solved [5, 6], which provided the first insight into the active site of heme peroxidase [7]. On the other hand, the application of cytochrome c peroxidase is limited, since the function of cytochrome c requires two molecules of ferro-cytochrome c [8]. Horseradish peroxidase is widely used in techniques such as enzyme-linked immunosorbent assay (ELISA) [9] due to the ease of producing colored products. This enzyme is relatively less expensive than other peroxidase such as lactoperoxidase. However, the enantioselectivity of horseradish peroxidase-catalyzed sulfoxidations and epoxidations is quite low, except using horseradish-peroxidase mutant [10, 11]. In the contrast, myeloperoxidase is able to catalyze the enantioselective epoxidation of styrene and a number of substituted derivatives with a reasonable enantiomeric excess (up to 80%) [12]. However, under physiological conditions (pH 5-8) and in the presence of chloride ion [13], this enzyme catalyzes the formation of hypochlorite that can further oxidize the desired product. Recently, a new class of peroxidase, dye-decolorizing peroxidases, were identified and characterized by a variety of spectroscopic methods such as X-ray crystallography [14, 15, 16]. These enzymes can efficiently catalyze the cleavage of anthraquinone-type dyes [14], which inspired me to expand the application of chloroperoxidase in dye degradation.

1.1.3 Haloperoxidase and its classification

Chloroperoxidase is an enzyme that catalyzes the chlorination of organic compounds and belongs to the class of haloperoxidase. Haloperoxidases are peroxidases that catalyze the oxidation of halides (chloride, bromide, or iodide) by hydrogen peroxide. There are many enzymes in the haloperoxidase class that also catalyze chlorination reactions. In haloperoxidase class, the enzymes are named according to the most electronegative halide that can be oxidized [17]. That is, chloroperoxidases can catalyze oxidation of chloride, bromide, and iodide; bromoperoxidases can catalyze oxidation of bromide and iodide ions; and iodoperoxidases are specific to catalyze oxidation of iodide.

Haloperoxidases are categorized into three classes based on the types of cofactor; they are HalPrx, HalVPrx, and HalNPrx. HalPrx, which is often described as chloroperoxidase (CPO), contains heme b as a prosthetic group. HalVPrx contains vanadium instead of heme as a cofactor, while HalNPrx are non-heme enzymes. HalPrx (or chloroperoxidase) is mostly found in fungus, ascomycetes and basidiomycetes with three exceptions from phytophthora. The phylogenetic tree of chloroperoxidase (Figure 1.3) shows the wide diversity of these enzymes. In particular, the most intensively investigated haloperoxidase is chloroperoxidase from the ascomycete *Caldariomyces fumago*.

Figure 1.3: Phylogeny of heme-containing haloperoxidases. The evolutionary history was inferred using the Neighbor-Joining method [18]. The bootstrap consensus tree inferred from 1000 replicates [19] is taken to represent the evolutionary history of the protein (sequence) analyzed. The evolutionary distances were computed using the JTT matrix-based method [20]. Evolutionary analyses were conducted in MEGA5 [21, 22].

1.1.4 Chloroperoxidase from *Caldariomyces fumago*

Chloroperoxidase (EC number 1.11.1.10) from *Caldariomyces fumago* was first isolated by Morris and Hager in 1966 [23]. Since then a steady progress has been made toward the realization and utilization of CPO's catalytic properties. The number of articles published on topics related to CPO has also increased dramatically from 31 in the 60's to 2380 in the last decades (Figure reffig:1.4). Moreover, the appearance of numerous patents related to CPO, especially after 1990, indicates a great potential for the application of CPO (Figure reffig:1.4). Notably, the DNA sequence of CPO was determined by Fang et al. in early 1986 [24]. The crystal structure of CPO was first reported by Sundaramoorthy et al. in 1995 [25], while the crystal structure of CPO complexed with the substrate was solved by Kühnel et al. in 2006 [26]. These continuous efforts have made my in-depth study of CPO possible.

Figure 1.4: The publications and patents of CPO from 1966 to 2012

1.2 Crystal Structure of CPO

Chloroperoxidase (CPO) protein contains 299 amino acid residues and is heavily glycosylated [25, 26, 27]. This enzyme also has a cyclization of the N-terminal glutamic acid

(i.e., pyroglutamic acid) [25, 26, 27]. The structure of CPO folds into a unique tertiary structure comprised by eight α -helical segments, three short β helices, and two antiparallel β pairs (Figure 1.5).

Figure 1.5: The cartoon representation of the crystal structure of CPO [25]. Two channels (narrow, green; wide, blue) that connect the heme active site to the surface are shown.

The crystal structure of CPO complexes is only available for the tightly bound ligand including carbon monoxide, nitric oxide, cyanide, and thiocyanide; with an exception of the weakly bound ligand, 1,3-cyclopentanedione (CPDO). The small conformation changes between crystal structures of these CPO-ligand complexes reveal a rigid active-site architecture of CPO. Additionally, six bromide/iodide binding sites were identified in the crystal structure of CPO [26]. One of the binding sites on the surface of CPO has occupancies of 60% for bromide and 30% for iodide; therefore, this site likely serves as a docking site for halide anions. The crystal structure of CPO-iodide complex also shows an iodide binding site located near the active site with distances of approximately 4Å to the side chains of residues Leu 70, Phe 103, Ile 179, and Ala 267, respectively. There are two channels that connect the heme active site to the surface (Figure 1.5). Based on the crystal structure of

the iodide- and bromide-CPO complexes, Kühnel et al. postulated that access of halides to the heme occurs through the narrow channel that connects the heme to the surface.

In the active site of CPO, the prosthetic group, heme, is sandwiched between two helices, proximal and distal helices (Figure 1.5). The proximal helix contains an axial ligand, cysteine residue (Cys) 29, attached to the heme. In the contrast, the distal helix provides catalytic groups required for the CPO's activity. In the distal site, glutamic acid residue (Glu) 183 is the only polar group close enough to the heme to serve a catalytic role. The crystal structure of CPO-CPDO complex suggests that the organic substrate is sandwiched between phenylanline residue (Phe) 103 and Phe 186, in which CPDO has a distance of 3.5 Å to both the aromatic side chains of Phe 103 and 186.

1.3 Chemical Reactions Catalyzed by CPO

CPO is able to catalyze a wide variety of reactions including halogenation [28, 29, 30], oxidation [31, 32, 33, 34, 35, 36], *N*-demethylation [37, 38], dehalogenation [39, 40], and dismutation reactions [31] (see Figure 1.6). Significantly, the epoxidation and sulfoxidation reactions catalyzed by CPO are highly enantioselective [41, 42, 43, 44, 45, 46, 47], which suggests a potential application in pharmaceutical industry.

Figure 1.6: CPO-catalyzed reactions in the presence of hydrogen peroxide (H_2O_2).

1.3.1 Chlorination, bromination, and iodination

The biological function of CPO from *Caldariomycin fumago* was to synthesize caldariomycin (2,2-dichloro-1,3-cyclopentanediol) [48], which is a metabolic product of this organism [49]. The halogenation activity of CPO was first discovered in 1959 by Hager et al, in which CPO catalyzed the conversion of inorganic chloride and β -keto adipic acid to δ -chlorolevulinic acid [50]. In 1966, Hager et al. confirmed that the enzyme CPO catalyzes a two-step chlorination of 1,3-cyclopentanedione to caldariomycin [28].

Chloroperoxidase (CPO) can catalyze the formation of a carbon-halogen (C-X) bond of a suitable acceptor molecule in the presence of chloride, bromide, and iodide ion. The suitable halogen acceptors include β -keto acids [50], cyclic β -diketones [48], 2,3-unsaturated carboxylic acids [30] (such as 4-hydroxycinnamic acid), and flavonoids [51] (i.e., naringenin and hesperetin). However, CPO could not catalyze halogenation using fluoride ion [28]; fluoride ion was even shown to be an inhibitor of the halogenation reaction [28]. Moreover, Yaipakdee et al. reported that CPO can not catalyze the halogenation of substrates such as flavone [51], since the aromatic rings, which possess a strongly conjugated system, are quite stable.

Yamada et al. elucidated that the specificity of CPO toward various 2,3-unsaturated carboxylic acids was related to the nucleophilicity/electron density of the π -bond carbon-carbon [30], which can be reduced by the adjacent electron withdrawing carboxyl group. Thus, CPO was inactive toward substrates such as 4-chlorocinnamic acid [30]. In contrast, with the electron-donating groups overcome the effect of the carboxyl group, 4-hydroxycinnamic acid can serve as a substrate of CPO.

As CPO-catalyzed halogenation is highly efficient, a spectroscopic assay was developed to rapidly assess CPO's activity by determining chlorination of monochlorodimedon to dichlorodimedon at pH 2.7 [28]. The decrease of the absorbance at 278 nm, which is associated with the formation of the dihalo derivative, is observed during the assay. Ad-

ditionally, CPO-catalyzed chlorination of monochlorodimedon can utilize NaClO_2 as both the oxidant and the halogen donor [52].

Halogenation catalyzed by CPO also has several limitations for use. Firstly, CPO shares the ability with other peroxidases to catalyze the oxidation of iodide to iodine [28]. In the absence of organic substrates, CPO catalyzes peroxidation of chloride or bromide ion to the molecular chlorine or bromine [53]. Secondly, CPO-catalyzed halogenation is not stereoselective, as Ramakrishnan et al. reported that 2-methyl-4-propyl-cyclopentane-1,3-dione was quantitatively chlorinated to 2-chloro-2-methyl-4-propylcyclopentane-1,3-dione, a racemic diastereomer [54].

1.3.2 Oxidation, sulfoxidation, and epoxidation

In the absence of halide ion, CPO exhibits the oxidation activity with a pH optimum around pH 5.0, however the pH optimum can vary depending on the particular substrate [31]. The substrates of CPO oxidation include a wide variety of organic compounds such as indole [33, 55], monoterpenes [56], 4-chloroaniline (to 4-chloronitrosobenzene) [32], and the classical peroxidase substrates such as guaiacol, pyrogallol, and leucomalachite green [31]. In particular, the primary alcohols can be selectively oxidized to aldehyde in the presence of CPO and hydrogen peroxide (H_2O_2) [34].

The speed at which the oxidant is added affects the yield of CPO-catalyzed oxidation, as the sensitivity of CPO for hydrogen peroxide has to be taken into account [34]. For instance, oxidation of 5-hydroxymethylfurfural was shown to approach completion only when the H_2O_2 was added slowly.

The presence of chloride, bromide, or iodide ion seems to increase the oxidation potential of CPO. In the absence of halide ion, terpene hydrocarbons didn't serve as substrates, while terpenes were converted into one or more products in the presence of chloride, bromide, or iodide ion [56]. In another case, oximes were effectively converted to halonitro compounds and ketones in the presence of KBr and KCl [57]. Furthermore, the presence of

chloride is also required so as to produce the reaction intermediates; for instance, CPO reacts with the thiols (2-nitro-5-thiobenzoic acid) or disulfides (5,5'-dithiobis(2-nitrobenzoic acid)) to give the corresponding sulfenyl chloride (3-carboxy-4-nitrobenzenesulfenic acid chloride) [36], which is decomposed subsequently to the corresponding sulfonic acid (3-carboxy-4-nitro-benzenesulfonic acid).

1.3.3 Enantioselectivity of the CPO-catalyzed reaction

A remarkable feature of CPO is its ability to catalyze the enantioselective epoxidation of disubstituted alkenes [35, 43], enantioselective oxidation of *cis*-cyclopropylmethanols [58], and asymmetry oxidation of sulfide [41, 45]. There are several factors known to control the enantioselectivity such as steric effect, chain length, and electronic property of substituents.

The electronic property of substituents, that is activating or poorly group deactivating on the aromatic ring, can dramatically influence the outcome of CPO-catalyzed reactions [41]. For instance, the chemical yield and enantiomeric excess (ee) of para substitution, methyl p-tolyl sulfide, were reported higher than ortho substitution, methyl o-tolyl sulfide [41]. Steric effects on substrates are also critical for CPO-catalyzed oxidation, for instant, replacement of a methyl group in methyl p-tolyl sulfide by an ethyl or isopropyl group decreased the chemical yield and the degree of asymmetric induction. In another case, trans-disubstituted olefins gave low yields of epoxide [42]. The chain lengthens has pronounced effects on the symmetry, but the increase in enantioselectivity has an upper limit. Hager et al. demonstrated that the increase of the chain length cause the increase of the ee up to 95% (5-bromo-2-methyl-1-pentene), but cause the decrease of the ee of 6-bromo-2-methyl-1-hexene (87%) and 7-bromo-2-methyl-1-heptene (50%) [44]. In addition, the oxidant used in CPO-catalyzed oxidation reactions was also found to effect the ee [59]. In asymmetric sulfoxidation of methyl p-tolyl sulfide [41], steric hindered oxidants, except *tert*-butyl hydroperoxide, such as cumyl hydroperoxide and trityl hydroperoxide together

with water-insoluble oxidants such as iodosylbenzene and *m*-chloroperbenzoic acid all afford racemic or almost racemic sulfoxide.

It is worth mentioning that aliphatic terminal alkenes surprisingly produces an inactive derivative, in which the heme of CPO is assumed to be *N*-alkylated [60]; thus, such CPO-catalyzed epoxidation gives poor yields of chiral epoxide. Although epoxidation of allyl propionate with CPO leads to the rapid formation of an inactive derivative, the formation of such a species was not detected during epoxidation of methyl allyl propionate [43].

1.3.4 Other reactions

Contrary to its primary biological function chlorination, CPO can dehalogenate substrates [39] such as trihalophenols, *p*-halophenols, and 4-fluorophenol, which may have important environmental implications. Additionally, CPO is able to transform tetra- and penta-chlorinated phenols and anilines, which are widely distributed pollutants [61].

The enzyme CPO catalyzes *N*-demethylation of *N,N*-dimethylaniline to *N*-methylaniline and formaldehyde in equimolar amounts with no other detectable products [37]. CPO can also catalyze *O*-demethylation, but the reaction is much slower than that of horseradish peroxidase [62].

In the absence of organic substrates, CPO reacts catalytically with hydrogen peroxide to form oxygen [31] similar to that of catalase. However, in the presence of a large excess of hydrogen peroxide, CPO undergoes inactivation, which highlights the importance of engineering more robust enzymes [63].

1.4 Mechanism and Characterization of CPO

Scientists have made 50 years of continuous efforts towards final realization of the mechanism of CPO by employing a variety of techniques including mutation, x-ray spectroscopy, nuclear magnetic resonance spectroscopy (NMR), and molecular dynamics. However, despite extensive knowledge obtained about the catalytic and structural information of CPO, the mechanism of CPO still remains elusive.

1.4.1 Catalytic cycle

The catalytic cycle of CPO [64] involves only the ferric and higher oxidation states of iron (Figure 1.7). As in the first step of the oxidation, dismutation, and halogenation reactions, the high-spin five-coordinate ferric state of CPO reacts with hydrogen peroxide (H_2O_2) to form the compound I [65], an oxo-ferryl(VI) cation radical ($\text{Fe}=\text{O}+\bullet$) intermediate. For oxidation reactions catalyzed by CPO, it was generally assumed that CPO carries out reactions in one-electron (peroxidase-like pathway) and/or two-electron (peroxygenase-like) pathway [66]. In the formal pathway, CPO compound I serves as a one-electron oxidant, converting the organic substrate (AH) into a radical product ($\text{A}\cdot$) with concurrent reduction of compound I to compound II, a oxoiron(IV) intermediate [67]. Compound II subsequently oxidizes the second substrate (AH) to regenerate the heme iron. In the latter pathway, the oxygen atom transferred is derived from H_2O_2 , and a direct oxygen transfer (two-electron) was proposed [68]. This direct transfer is supported by the experimental evidence that epoxidation of styrene took place by direct interaction of the substrate with iron-bound oxo atom [69]. For the dismutation of H_2O_2 , compound I oxidizes a second molecule of H_2O_2 to dioxygen (O_2) and returns to the ferric resting state. For the chlorination reaction, it was proposed that compound I catches the chloride ion to form a hypochlorite adduct ($\text{Fe}-\text{OCl}$) [70], compound X, that further chlorinates the organic compound. However, the exact details of the chlorination are still under debate.

1.4.2 Mutations of CPO amino acid residues

Mutation, a change in the nucleotide sequence of the genome of an organism, has been used to provide insights into the mechanism of CPO. The mutant of CPO, C29H, indicated that the proximal ligand (Cys 29) could be replaced with a histidine residue and retain most of the chlorination, peroxidation, epoxidation, and catalase activities [71]. This remaining of activity suggests that thiolate ligand cannot be only the factor that affects CPO-catalyzed reactions. The mutant E183H, a residue replaced Glu 138 by a histidine residue, was ob-

Figure 1.7: The catalytic cycle of CPO and the postulated structures of the intermediates.

tained in *Aspergillus niger* expression system to overcome expression failure in *Caldariomyces fumago* [72]. The mutant E183H diminishes activity of chlorination, peroxidation, and catalase activities. However, the epoxidation activity of the mutant was surprisingly shown to be enhanced ~2.5 fold compared to the native CPO [72]. Furthermore, the chemical modification of His 105, a residue adjacent to Glu 183, was found to destroy the CPO's halogenation activity [73]. In sum, how residues are involved in the mechanism is still not quite understood.

1.4.3 X-ray spectroscopy

In 2006, the crystal structure of the CPO complexed with hydrogen peroxide was determined by Kühnel et al. through controlled photo reduction of CPO-oxygen complex during x-ray data collection [74]. Significantly, the formation of the hydroperoxo intermediate (Fe-OOH), compound 0, induces no major conformational changes in the protein. Additionally, the X-ray absorption has been used to determine the Fe=O distances of compound I [75] and compound II [67] that are 1.63 Å and 1.82 Å, respectively.

1.4.4 Nuclear magnetic resonance (NMR) spectroscopy

NMR spectroscopy not only identifies the product of CPO-catalyzed reaction as a conventional method [41, 69, 76], but also represents a powerful method for structural characterization, which are complementary to x-ray crystallography in most cases [77]. For instance, on the basis of a large chemical shift differences near the heme propionate observed in overhauser effect (NOE) measurements, the primary structure difference for the two isoenzymes was suggested to be resided in the ring propionate substituents of the heme group [78]. This difference of isoenzyme in heme group was later confirmed by the crystal structure of CPO [25]. Furthermore, the NMR relaxation time measurement [79], which measures the spin-lattice relaxation time (T_1) of substrate and calculates the distance using Solomon-Bloembergen relationship, can provide the structural basis of the interaction of CPO with its substrates. The CPO complexed with substrates including chloride [80], phenyl sulfides [81], and phenol [82], and CPDO [79] were studied by the relaxation measurements. In particular, the distance between the heme iron and 4,5-position of proton of CPDO was reported to be $7.1 \pm 0.3 \text{ \AA}$, and the dissociation constant of the CPO-CPDO complex to be 33 mM. It was shown that the presence of chloride has no effect on binding of 1,3-cyclopentanedione to CPO. In addition, a two-dimensional NMR spectroscopy of CPO was reported and the proton of the active site of CPO was systematically assigned [77], which provides a suitable reference for other NMR spectroscopic study.

1.4.5 UV-Visible and MCD spectroscopy

To date, CPO and its intermediates in the mechanism have been characterized by diverse spectroscopic techniques involving electronic absorption, Mössbauer, resonance Raman, and EPR (see Table 1.1).

The MCD (magnetic circular dichroism) spectra of iron porphyrins are sensitive to the nature of axial ligands to the heme iron, and thus are suited for structural investigations of axial ligation [96]. The thiolate ligation of heme iron was assigned through comparisons

Table 1.1: Parameters and assignment for CPO and its intermediates (Compound 0, Compound I, and Compound II) obtained from spectroscopic methods.

Species	Assignment	Spectroscopy				Ref.
UV-Visible Spectroscopy						
		Soret (nm)		Visible (nm)		
CPO	π -radical	398 (396)		515, 650		[83]
Compound I		367		610, 666		[84, 85]
Compound II		436		542, 572		[85]
Mössbauer Spectroscopy						
		δ (mm/s)		ΔEQ (mm/s)		
CPO	Fe^{3+}	0.2		1.16		[86]
Compound I	Fe^{4+}	0.15		1.02		[87]
Compound II	Fe^{4+} -OH	0.10		2.06		[88]
Resonance Raman Spectroscopy						
		$\nu(Fe-S)$ (cm^{-1})	$\nu(Fe-O)$ (cm^{-1})	Fe-S $\text{\AA}$	Fe-O $\text{\AA}$	
CPO	Fe^{3+}	347		2.30		[89]
Compound I	$Fe^{4+}=O$		790 (792)	2.48	1.65	[90, 91]
Compound I	$Fe^{4+}=OH$		565	2.40	1.82	[92]
EPR Spectroscopy						
		g values		J/D		
CPO	Fe^{3+}	g=2.62,2.26,1.83				[66, 93]
Compound I	π -radical	g=1.72, $g_{ }$ =2.00		1.02 (0.97)		[93, 94, 95]

MCD spectra of P450 to that of CPO [97, 98]. Additionally, the ferric CPO undergoes an irreversible UV-Vis spectral change to an alkaline form above pH 7 [99]. MCD studies further indicate that the alkaline form has a nitrogen donor that is likely derived from histidine imidazole and coordinated to the heme iron as a distal ligand [100].

1.4.6 Mössbauer, EPR, and ENDOR spectroscopy

Rutter et al reported the first direct evidence of CPO compound I as a porphyrin radical cation in 1982 from EPR (electron paramagnetic resonance) studies [93]. In that work, broad EPR spectrum were observed [93], which results from the antiferromagnetic exchange interaction [93, 94, 95] between the ferryl iron ($S=1$) and the porphyrin π -radical cation ($S=1/2$). The compound I contains a porphyrin π -cation radical rather than an iron-bound cysteinyl radical was confirmed by electron nuclear double resonance (ENDOR)

studies of Kim et al., which shows that the radical is predominantly on the porphyrin with the spin density of sulfur (ρ_S) smaller than $\rho_{Smax} \approx 0.23$ [101]. Kim et al. further suggests that P450 compound I can similar properties. Interestingly, CPO compound I and P450 (CYP119) compound I have similar UV-Vis and Mössbauer spectra, whereas their EPR signatures are different [87].

The structural characteristics of compound II were revealed recently. In 2006, the Mössbauer spectrum of CPO compound II suggests two distinct ferryl species in an $\sim 70:30$ ratio, where density functional theory (DFT) calculations and cryogenic reduction and annealing experiments assigns the major species as an $\text{Fe}^{\text{IV}}\text{-OH}$ intermediate [88]. It is known that EPR spectra is not available for CPO compound II, since the $\text{Fe}^{\text{IV}}\text{-OH}$ ($S=1$) is EPR silent. However, radiolytic reduction of compound II at 77 K results in the formation of an EPR-active species [92]. Thus, the presence of iron (IV) hydroxide was confirmed by ENDOR spectra of radiologically reduced compound II [92].

In 1995, Dexter et al. found that the red-brown native CPO in the reaction with hydrogen peroxide in the presence of allylbenzene converted to a green-enzyme species that is inactive in chlorination and epoxidation [60]. Although the structure of this species has not been unraveled, it was proposed as a six-coordinate *N*-alkylhemin metallacycle on the basis of EPR spectra that have a strong octahedral crystal field splitting [102]. In addition, the spread of *g*-values of the green CPO was found considerably smaller than that of native CPO [102]. Mössbauer spectra of this green CPO has also been provided [102], where spectra parameters were interpreted in terms of the electronic structure by the crystal field model of Griffith [103].

1.4.7 Raman and low-frequency coherence spectroscopy

The Raman spectra of native CPO were first reported in the late 70's [104, 105], where the spectra of CPO are temperature sensitive and thus suggests a transition from high to low spin occurred at a low temperature (77 K). Later in 1986, the Fe-S stretching frequencies

of CPO was assigned at 347 cm^{-1} [89], which provides an evidence that CPO has a sulfur ligand that is identical to that of cytochrome P450.

Compound I of CPO is relatively short-lived intermediates that has a half-life of $\approx 1\text{ s}$ at room temperature [85], thus compound I was prepared by low temperature trapping [66] for EPR [93, 94] and Mössbauer [94] spectroscopy. In contrast, Raman spectroscopy was characterized at ambient temperature [90, 91]. It is now established that the Fe=O Raman frequency of CPO intermediates can serve as a sensitive indicator of the environments around the bound oxygen. The Fe=O frequency of CPO Compound I was identified at 790 cm^{-1} [75, 91]. Furthermore, Green et al. reported direct evidences for the existence of an $\text{Fe}^{\text{IV}}\text{OH}$ species in compound II using Raman spectroscopy [92]. In addition, resonance Raman spectra of the CPO complexes has been studied and systematically assigned [90]. It is worth mention that low-frequency modes are difficult to detect by conventional frequency domain Raman techniques. However, Gruia et al. recently reported low-frequency coherence spectra using ultrafast laser spectroscopy techniques, where the spectrum obtained were similar to its Raman spectr [106].

1.4.8 Computational characterization

The computational method is an important complement to the experimental approaches for gaining structural information that enables the exploration of biological systems at an atomic level. Although the use of computational method for the study of CPO is limited at status quo, this computational method is promising because of the rapid-growing computer technology. Recently, Morozov et al proposed that the simple steric considerations from the hydrophobic core (residues Phe 103, Ile 179, Val 182, and Phe 186) around the oxyferryl heme center govern the stereoselectivity of CPO [107, 108], based on the molecular model of *cis*- β -methylstyrene, a substrate can be oxidized enantioselectively by CPO and H_2O_2 .

1.5 Challenges in applications

chloroperoxidase (CPO) from *Caldariomyces fumago* is the most prominent enzyme due to its ability to catalyze a variety of reactions, its high total turnover number (mol of product produced by mol of biocatalyst) values [66], and its stability for weeks at room temperature under pH control [109]. However, both fundamental and practical issues, such as enzyme stability and availability, have delayed the development of industrial applications. Therefore, CPO application remains a challenge, and extensive efforts have been focused on the aspects of lowering the cost of enzyme, maintaining the CPO activity, and enhancing the stability of CPO.

1.5.1 Isolation and purification of CPO

In early days, many studies were taken to reduce to the high cost of isolation and purification of CPO. It was found that relatively large amounts of CPO could be obtained from the medium by employing fructose, instead of glucose, as a carbon source for the growth [110]. In 1989, Steven R. Blanke developed a continuous flow bioreactors, which increased the production to 1-1.5 g/liter CPO [111]. However, the commercial price of CPO is still not inexpensive as \$200 per ml.

1.5.2 Immobilization of CPO

In addition to the high cost of CPO, the synthetic use of CPO suffers from its deactivation when using high concentrations of the oxidant, hydrogen peroxide [63]. Considerable attention was given to the immobilization of CPO to improve the catalytic stability as well as recovering the active enzyme after the completion of the catalytic process. In 1990, CPO was covalently bound to aminopropyl-glass [112], while the specific activity of the immobilized enzyme was highest, 36% of theoretical, at low enzyme-to-carrier ratios. To date, CPO had been adsorbed on materials such as monoaminoethyl-N-aminoethyl agarose gels [113], polyurethane foam [114], mesoporous molecular sieve [115, 116], and microporous solids such as talc [117]. Alternatively, CPO could be microencapsulated in a microp-

orous silica gel cage built from tertramethylorthosilicate, which was first reported for the enantioselective oxidation of sulfides to sulfoxides [118].

1.5.3 Directed evolution of CPO

To improve the catalytic stability and the activity of CPO, a strategy of directed evolution, which mimics natural evolution in the laboratory (in *vivo*), was used to express the recombinant CPO in its natural host, *Caldariomyces fumago* [119, 120]. The isolated CPO mutants were found to have enhanced epoxidation and chlorination activities, and all of the mutants showing improved chlorination activity carried a mutation in Cys 29 residue.

1.5.4 Reactions in organic solvent and ionic liquid

Another issue that limits the applications of CPO in industry is that the organic substrates usually have a low aqueous solubility. To overcome this restriction, one way is to perform the reaction in aqueous co-solvent mixtures [121]. However, common solvents such as methanol [34] and dimethylsulfoxide [122] are both reported to be the substrates of CPO. Van Deurzen et al. reported that *tert*-butanol/water (50:50, v/v) could be a good solvent system [121], which could increase the solubility of the organic compound and maintain most of the CPO activity. A ternary system formulated with organic solvents such as α -pinene or n-hexane, short chain alcohols and water, were reported as a reaction media which could maintain the activity of CPO as well [123].

The ionic liquid, a solvent entirely composed of ions (mixtures of cation and anion) whose melting point is below 100°C, has been investigated as the alternative solvents for CPO catalyzed reactions [124, 125, 126]. It was found that CPO is active in the presence of ionic liquid as co-solvent [127]. However, the study showed that the CPO activity decreases with the increased concentration of ionic liquid for a variety of ionic liquids (except ethylammonium nitrate) [128].

Chloroperoxidase (CPO) has been the subject of intensive investigations due to its ability to catalyze many reactions with high stereo- and regioselectivity. Knowledge about the structure of CPO-substrate complex is critical to the understanding of the structure-function relationship of heme proteins in general and the structural basis of the diverse catalytic activity, strict substrate specificity, and high regio- and enantio-selectivity displayed by CPO in particular. Furthermore, knowledge about the factors controlling the degradation of azo dyes is important to design the novel biodegradation system. Thus, the motivation of this dissertation work includes: 1) providing the structural basis of high regio- and enantio-selectivity displayed by CPO; 2) serving as a lighthouse for designing more efficient CPO mutants with tailor-made activities for biotechnological applications; 3) and providing a starting point for understanding the mechanism of dye degradation catalyzed by CPO.

The main work in this dissertation relies on NMR spectroscopy and molecular modeling methods to investigate the interaction of CPO with substrates. The method of NMR spectroscopy has advantages for revealing the structural information, while the computer method benefits the study in the rationale of the findings. In the application of CPO, a green and efficient system for the degradation of dyes was developed. The decolorization reactions were mainly characterized by UV-Vis spectroscopy due to its simplicity. The decolorization products were identified by proton NMR spectroscopy.

In Chapter II, the structure of CPO-indole complex was studied using NMR relaxation measurements and computational techniques. The specific aim of this chapter is to reveal the orientation of indole binding in the active site of CPO, to identify the residues involved in the formation of the CPO-indole complex, and to elucidate the mechanism of the CPO-catalyzed regioselective oxidation of indole. The dissociation constant (K_D) of the CPO-indole complex was calculated to be approximately 21 mM. The distances (r) calculated between protons of indole and the heme iron and molecular docking revealed that the pyrrole ring of indole is oriented toward the heme with its 2-H pointing directly at

the heme iron. Both K_D and r values are independent of pH in the range of 3.0-6.5. The stability and structure of the CPO-indole complex are also independent of the concentration of chloride/iodide ion. Molecular docking suggests the formation of a hydrogen bond between the N-H of indole and the carboxyl O of Glu 183 in the binding of indole to CPO. Simulated annealing of the CPO-indole complex using r values from NMR experiments as distance constraints reveals that the van der Waals interactions were much stronger than the Coulomb interactions in indole binding to CPO, indicating that the association of indole with CPO is primarily governed by hydrophobic rather than electrostatic interactions.

In Chapter III, the interaction of CPO with sulfides/styrene-derivatives substrates was studied using the NMR relaxation and molecular docking. The specific aim of this chapter is to define the orientation of sulfides binding to CPO and to explore the mechanism of enantioselective sulfoxidation and epoxidation catalyzed by CPO. The styrene derivatives were selected as model substrates for epoxidation, while the 2-(methylthio)thiophene was chosen as a model substrate for sulfoxidation. To increase the solubility of CPO substrates, *cis*- β -methylstyrene and 2-(methylthio)thiophene, a series of organic solvents has been tested. Particularly, the substrates show the best solubility in tert-butanol among all the solvents tested, although the solubility of styrene is still not adequate in tert-butanol for NMR experiments. Alternatively, the hydrophilic derivatives of styrene were tested for solubility using UV-Vis absorption spectroscopy, and were further studied by NMR spectroscopy. However, the substrates, 3-aminostyrene and 4-aminostyrene, cannot be studied by NMR relaxation experiments due to a poor fit of $E_0[1/T_{1obs} - 1/T_{1f}]^{-1}$ versus substrates concentrations (S_0). The substrate styrene-4-carboxylic acid to CPO was found not binding in the active site of CPO. In the study of enantioselective sulfoxidation, the dissociation constant of the CPO-2-(methylthio)thiophene complex was calculated to be ~ 22 mM. Molecular docking of sulfides showed that the electronic and steric substituent on the benzene ring can dramatically affect on the binding orientation of substrates.

In Chapter IV, the biodegradation of a variety of synthetic dyes in the CPO-H₂O₂-Cl⁻ system was studied. The specific aim of this chapter is to examine various effects on the degradation of synthetic dyes catalyzed by CPO, to identify the degradation products in the CPO-H₂O₂-Cl⁻ system, and to provide the mechanism proposed for halide-dependent biodegradation. The decolorization in the system is fast and efficiently not only for azo dyes but also for triarylmethane and anthraquinone dyes. The optimum pH of decolorization is found around pH 2.7. Significantly, only in the presence of chloride/bromide can CPO efficiently catalyze decolorization of azo dyes. This dependence of degradation/decolorization on halide ion has never been address before. Using NMR spectroscopy, the degradation products of orange G in the CPO-H₂O₂-Cl⁻ system were identified, revealing a cleavage of naphthalenyl carbon-nitrogen bond of orange G. A mechanism of CPO-catalyzed degradation of orange G was proposed that involves the formation of compound X.

CHAPTER 2

PARAMAGNETIC RELAXATION AND MOLECULAR MECHANICS STUDIES OF CHLOROPEROXIDASE-INDOLE COMPLEX

2.1 Introduction

Chloroperoxidase (CPO), a heme-thiolate protein secreted by the marine fungus *Caldariomyces fumago* [23], is a versatile enzyme [25] capable of catalyzing a broad spectrum of chemical reactions including halogenation [28], dehalogenation [39], N-demethylation [37], dismutation [23], epoxidation [42], and oxidation [34, 41, 129, 130, 131]. Therefore, CPO has been the subject of numerous biotechnological investigations due to its potential applications in synthetic chemistry as well as pharmaceutical industry [44, 46, 132, 133]. Most interestingly, CPO-catalyzed oxidation and epoxidation reactions proceed with high regio- and enantio-selectivity [41, 42, 134]. Tremendous efforts have been made during the past few decades to understand the mechanism of CPO's regio- and enantio-selectivity [66, 64, 135]. Most of such studies are focused on the structures of CPO-substrate complexes. The structural feature of the CPO-substrate complexes will shed light into the structure-function relationship of heme proteins in general and the structural basis of the diverse catalytic activity, strict substrate specificity, and high regio- and enantio-selectivity displayed by CPO in particular. The definitive structure of the CPO-substrate complexes will also provide pivotal information regarding the topology of the heme active center of CPO, the residues involved in the catalytic cycle, the location/orientation of substrate binding, and the mechanisms of CPO-catalyzed reactions. Such information will serve as a lighthouse for designing more efficient CPO mutants that target specific substrates and produce desired products that are difficult to obtain under mild conditions [136]. Unfortunately, the structural information on CPO-substrate complexes remains scarce despite the great efforts made with the application of the most powerful techniques available including X-ray crystallography and NMR spectroscopy.

X-ray crystal crystallography is one of the most powerful techniques for elucidating the three dimensional structure of enzymes and their complexes with substrates. The crystal structure of CPO has demonstrated the presence of a substrate-binding site with an opening above the heme that enables organic substrates to approach the oxoferryl oxygen of CPO compound I [25]. It is generally expected that substrate binding would result in noticeable structural changes to the protein. Surprisingly, the structures of CPO-ligand complexes are indistinguishable from that of the ligand-free protein [27]. On the other hand, the crystal structure of CPO complexed with its natural substrate, 1,3-cyclopentanedione (CPDO), (PDB code: 2CIX) revealed another surprising picture in which the active methylene group of CPDO is not oriented towards the heme iron [26]. This is in good agreement with the results reported from an NMR relaxation study of CPO-CPDO complex in solution [79]. To date, the crystal 2CIX is the only crystal structure of CPO complexed with a substrate. The limited crystal structural data on CPO-substrate complexes are mainly attributed to the difficulty of diffusing organic substrates into CPO crystals [26].

Nuclear magnetic resonance (NMR) spectroscopy is an alternative technique to X-ray crystallography for probing the structural information of proteins and their complexes with substrates in solution. Particularly, in heme proteins, the enhanced nuclear relaxation induced by the paramagnetism of the heme [137, 138] was often used to establish the structure and stability of enzyme-substrate complex [137, 139, 140]. NMR relaxation studies have been successfully used in determining the structural features of CPO complexed with various substrates including phenols [82], sulfides [81], and the natural chlorination substrate, CPDO [79].

Molecular mechanics studies have become an important complement to NMR and X-ray experiments in exploring heme proteins and their interactions with substrates at the atomic level [141, 142]. The NMR-determined distances between the substrate and the heme can be used as restraints in simulation studies utilizing a simulated annealing protocol

[143, 144]. This protocol has been successfully applied to studies of substrate binding to several other hemoproteins [145, 146]. This success has prompted us to investigate the mechanisms of the CPO-catalyzed regioselective oxidation of indole using both NMR and computational techniques.

The CPO-catalyzed oxidation of indole to oxindole was first reported in 1979 [33]. Van Deurzen et al. further reported that the oxidation of substituted indoles yields the corresponding oxindoles and the reactivity of the substituted indoles depends on the nature and the position of its substituent [55]. However, to the best of my knowledge, the structural basis for the unusual regioselectivity of indole oxidation catalyzed by CPO remains undefined. Based on the structure of indole, the oxidation would be expected to occur at the 3-position of indole, which possesses the highest electron density and is thus most susceptible to attack by oxidizing agents and by other electrophilic reagents [33]. It is obvious to suspect the indolic N-H group as a structural feature controlling the position of oxidation, even though indole is an extremely weak base. However, the presence of a hydrogen-bond between the indole N-H group and an unknown group at the CPO active site has proven to be insufficient to explain the regiospecificity of indole oxidation [33]. Therefore, a detailed structural characterization of the CPO-indole complex would offer great insight into the mechanisms of the CPO-catalyzed oxidations and solve the long lasting puzzle of the unusual product from indole oxidation.

The aims of the present work are to reveal the orientation of indole binding at the active site of CPO, to identify the residues involved in the formation of the CPO-indole complex, and to elucidate the mechanism of the CPO-catalyzed regioselective oxidation of indole. The interaction of indole with CPO was probed using longitudinal NMR relaxation and two computational methods, molecular docking and simulated annealing. The dissociation constant of the CPO-indole complex and the distances between the protons of indole and the heme iron of CPO are obtained. The effect of pH and halide ion on the binding of indole

with CPO is presented. Simulated annealing and molecular docking of indole in the active center of CPO are provided. My results showed that a complex with a K_D of approximately 21 mM is formed between CPO and indole with indole's 2-H pointing towards the heme iron. This structural feature satisfactorily explains the unusual product observed from the CPO-catalyzed oxidation of indole. It is concluded that formation of the CPO-indole complex is responsible for the escorted delivery of oxygen from CPO compound I directly to the 2-position of indole.

2.2 Materials and Methods

2.2.1 Materials

Caldariomyces fumago (ATCC number: 16373) was purchased from ATCC (Manassas, VA). Unless otherwise specified, all chemicals were of analytical grade and were purchased from Sigma-Aldrich (St. Louis, MO).

2.2.2 Chloroperoxidase expression and purification

The enzyme CPO was isolated and purified according to published protocols [147, 148] with slight modifications. Only CPO preparations with Reinheitszahl values (R_z , A_{398} nm/ A_{278} nm ratios) of 1.4 or higher were used in all the experiments. The stock solution of CPO was prepared by repetitive (>6 times) isotope exchange of aqueous protein solution with D_2O in a Centriprep-30 centrifugal filter device. The concentration of CPO was determined by measuring the absorption at 398 nm using a molar extinction coefficient of $9.12 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$ [23].

2.2.3 Preparation of stock solutions and NMR samples

A stock solution of indole (14.0 mM) was prepared by dissolving 32.8 mg indole in 20.0 mL of warm D_2O in a vortex mixer. Stock solutions of 1.0 M chloride and iodide ion were prepared from their potassium salts in D_2O . Solutions of 1.0 M and 0.1 M DCl were prepared by dissolving DCl (35% w/w) in D_2O to adjust the pH of all test solutions. The samples used for NMR experiments contained 2-12 mM indole and 0.1 (or 0.01) mM CPO

in 100 mM phosphate buffer with 99.9% D₂O. The final volume of all NMR samples was 500 μ L.

2.2.4 Structural characterization of indole by NMR

Proton NMR measurements were conducted on a Bruker Avance 600 MHz NMR spectrometer at 298.0 K. Proton chemical shifts were referenced to the internal reference 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS). The NMR data were processed using Topspin, version 2.1. The line width was obtained from proton spectra by fitting the proton peak to a Lorentzian line shape.

2.2.5 T_1 Relaxation experiments

The longitudinal relaxation time (T_1) was determined using the standard inversion-recovery method with 180° - τ - 90° pulse sequence [149]. Eleven spectra were recorded for each sample, with the inter-pulse delay τ ranging from 0.5 to 25.0 s. For each spectrum, 32 scans were acquired. The data were processed using Topspin, version 2.1. The T_1 values were calculated by fitting Equation 2.1, where τ is the interval between 180° and 90° pulses, M_z is the Z-component of nuclear magnetization (represented by the intensity of the peak) when the interval is τ , M_0 is the Z-component of the nuclear magnetization when the interval is infinite, and ρ is a parameter that equals to 2.0 at an exactly 180° pulse.

$$M_z = M_0(1 - \rho e^{-\frac{\tau}{T_1}}) \quad (2.1)$$

2.2.6 Dissociation constants of CPO-indole complex

The longitudinal relaxation rate (T_{1obs}^{-1}) is the weighted average of the relaxation rates of the free substrate (T_{1f}^{-1}) and the bound substrate (T_b^{-1}) [150]. Thus, the relaxation-time values (T_{1obs}^{-1} , T_{1f}^{-1} , and T_b^{-1}) are related as shown in Equation 2.2 when only one molecule of substrate binds to a molecule of enzyme, where T_{1obs} is the relaxation time of the indole obtained from the relaxation experiment, T_{1f} is the relaxation time of indole obtained from the relaxation experiment in the absence of CPO, T_{1b} is the relaxation time of the CPO-bound indole, K_D is the dissociation constant of CPO-indole complex, E_0 is the initial

CPO concentration, and S_0 is the initial indole concentration.

$$E_0[\frac{1}{T_{1obs}} - \frac{1}{T_{1f}}]^{-1} = K_D[\frac{1}{T_{1b}} - \frac{1}{T_{1f}}]^{-1} + S_0[\frac{1}{T_{1b}} - \frac{1}{T_{1f}}]^{-1} \quad (2.2)$$

2.2.7 Location of indole binding site

The location of indole in the active site of CPO can be determined from the distances (r) between the indole protons and the heme iron of CPO calculated according to the Solomon-Bloembergen equation (Equation 2.3) [137, 139, 151], where μ_0 is the permeability of free space, γ is the gyromagnetic ratio of the proton, g is the electronic g-factor, μ_B is Bohr magneton, S is the spin state of the heme iron of CPO, ω_I and ω_S are the nuclear and electronic Larmor frequency, respectively, r is the distance from proton nuclei to the heme iron, and τ_c is the correlation time that describes the dipolar interaction between the ligand and the paramagnetic iron in solution. A value of $\tau_c = 8.8 \times 10^{-11}$ s was used for CPO [81]. When the observed proton is in the extreme narrowing conditions ($\omega_I^2 \tau_c^2 \ll 1$, $\omega_S^2 \tau_c^2 \gg 1$), as is usually the case for high-spin hemoproteins [81, 152], Equation 2.3 is simplified to Equation 2.4, which was used in calculating the distances in this work.

$$\frac{1}{T_{1m}} = \frac{2}{15} \left(\frac{\mu}{4\pi} \right)^2 \frac{\gamma^2 g^2 \mu_B^2 S(S+1)}{r^6} \left[\frac{\tau_c}{1 + (\omega_I - \omega_S)^2 \tau_c^2} + \frac{3\tau_c}{1 + \omega_I^2 \tau_c^2} + \frac{6\tau_c}{1 + (\omega_I + \omega_S)^2 \tau_c^2} \right] \quad (2.3)$$

$$r(cm) = [(8.66 \times 10^{-31}) T_{1m} \tau_c]^{\frac{1}{6}} \quad (2.4)$$

Additionally, T_{1m} is related to T_{1b} through Equation 2.5, where T_{1d} is the relaxation time in diamagnetic states, T_{1m} is the relaxation time in paramagnetic states, and τ_M is the lifetime of the enzyme-substrate complex. Since both T_{1d}^{-1} and τ_M are negligible compared to T_{1b} in my case [79, 81, 82], T_{1b} is approximately the same as T_{1m} .

$$\frac{1}{T_{1b}} - \frac{1}{T_{1d}} = \frac{1}{T_{1m} + \tau_M} \quad (2.5)$$

2.2.8 Molecular docking

Molecular docking for the preferred orientation of the indole and 1-methylindole within the active site of CPO was performed with the software package AutoDock [153], version

4.2.3. The results from molecular docking studies provided the starting position of indole for distance-restrained simulated annealing. The structure of CPO for docking indole and 1-methylindole were taken from the X-ray structure (PDB codes: 2CPO). Additionally, 14 of the glycosylation sites were removed from the CPO structure, and the pyroglutamic acid (Pca) residue were replaced by a proline (Pro) residue, since parameters for sugars and the Pca residue are unavailable in the GRONingen MOlecular Simulation (GROMOS) force field [154] that was used in my simulated annealing. The removal of sugars from CPO should have negligible effect on substrate binding, because CPO without glycosylation is still highly active [155]. Furthermore, the manganese ion was removed from the CPO structure, as manganese-free CPO [156] was used in my NMR experiment. The crystallographic water in the PDB file was removed to reduce any interference from water.

Both indole and 1-methylindole structure were built by MarvinSketch, version 5.9, in the JChem software package (ChemAxon, Ltd.) and further optimized with ORCA [157], version 2.9, using second-order Möller-Plesset perturbation theory (MP2) [158] and the def2-SVP basis set [159]. AutoDockTools [160], version 1.5.4, was used to add Gasteiger charges to CPO (+1.00 was added manually on Fe), indole, and 1-methylindole, respectively. During simulation, the CPO structure was kept rigid. Indole or 1-methylindole was simulated in a box centered at the heme iron, which was confined using a grid size of 30 Å × 30 Å × 30 Å box with 0.375 Å spacing. A docking of 60 separate simulation runs was performed with 25 million energy evaluations per run.

2.2.9 Distance-restrained simulated annealing

Distance-restrained simulated annealing and energy minimization for the CPO-indole complex were performed with the Groningen MAchine for Chemical Simulation (GROMACS) package, version 4.6. The CPO-indole complex was simulated with the GROMOS96 53a6 force field [154]. The force field was augmented with the parameters developed in this work (see Table 2.5). The topology parameters of indole were built using the

PRODRG2 server [161]. The partial atomic charges of indole were assigned by the analogy with the equivalent functional group, tryptophan, in the GROMOS96 53a6 force field [162]. The protonation states of the titrable amino acid residues were assigned as follows: +1 for His, Lys; -1 for Glu, Asp; and neutral for Tyr.

The CPO-indole complex was solved with simple point charge (SPC) waters [163]. The protein has a net charge of -16, and so 16 of Na^+ ions were added to create a neutral system for simulation. Periodic boundary conditions were imposed using a truncated octahedron box created from a $82 \text{ \AA} \times 82 \text{ \AA} \times 82 \text{ \AA}$ cube. After deletion of overlapping waters, 12644 waters remained. A shift method with a $14 \text{ \AA}$ cutoff was used for the calculation of Lennard-Jones interactions. Electrostatic interactions were calculated with the particle mesh Ewald method (PME) [164], using a cutoff of $12 \text{ \AA}$ for real-space interactions, a Gaussian width parameter of $3.84195 \text{ \AA}$, an FFT grid determined with the fourier spacing method using a maximum spacing of $1.2 \text{ \AA}$, and fourth-order interpolation for reciprocal-space interactions. A relative dielectric constant ($\epsilon_r = 1.0$) was used. All bond lengths were constrained using LINCS algorithm [165]. Positional restraints were placed on the α -carbon backbone during simulated annealing. Indole was restrained with a force constant of $600 \text{ kJ mol}^{-1} \text{ nm}^{-2}$ using the NMR-derived distances between the protons of indole and the heme iron (see Table 2.6). Brief energy minimization of the CPO-indole complex was performed using the conjugate gradient method to eliminate unrealistic van der Waals contacts. A time constant of 50 ps was applied to the distance restraint. The system was rapidly heated to 800 K, cooled to 300 K over 150 ps, and allowed to equilibrate for 100 ps at 300 K, using a 2 fs timestep. The Coulomb and Lennard-Jones potential energies during the last 50 ps were calculated using GROMACS. The root-mean-square deviation (RMSD) was calculated with respect to the starting structure. Snapshots of the indole molecule bound to CPO taken during the last 50 ps of MD simulation and were used to determine the indole binding modes.

2.3 Results and Discussion

2.3.1 Proton NMR spectrum of indole in the presence of chloroperoxidase(CPO)

Figure 2.1 compares the ^1H NMR spectra of 5.7 mM indole in the absence (Figure 2.1A) and presence of varying amount (0.01, 0.02, 0.05, 0.08, and 0.1 mM, Figure 2.1B-F) of CPO at pH 6.0. The six main peaks (Figure 2.1A) can be easily assigned, based on the splitting pattern and shift positions, to the protons of the indole in the absence of CPO (Figure 2.2): 7.32 ppm (2-H), 6.51 ppm (3-H), 7.63 ppm (4-H), 7.08 ppm (5-H), 7.17 ppm (6-H), and 7.47 ppm (7-H). The 1-H was not observed due to its fast exchange with D_2O . After addition of CPO, the doublet from 2- and 3-H (Figure 2.1A) was changed to two broad singlets due to the paramagnetism of the heme in CPO (Figure 2.1B), suggesting the close promximity of these protons to the heme iron. Further increasing the concentration of CPO led to more significant broadening of these peaks (Figure 2.1B-F) and noticeable shift in their positions. For instance, the chemical shift of 4-H was changed from 7.63 to 7.61 ppm. The change in chemical shifts results from the dipole-dipole interaction between indole and the heme iron [138], while line broadening of the NMR signals is due to paramagnetic enhancements of the transverse nuclear relaxation rates (T_2^{-1}) of indole caused by the heme iron [138]. The results shown in Figure 2.1 indicate that a reasonably stable CPO-indole complex is formed with indole bound near the heme center of the protein. More importantly, the NMR signal from all indole protons can be clearly observed, making it possible to obtain the orientation of indole in the active center of CPO because multiple distances between substrate protons and the heme iron can be experimentally determined.

As the concentration of CPO increased from 0.01 to 0.05 mM, the linewidth of the 2-proton increased from 2.47 to 54.37 Hz (Figure 2.1B-D). The peak of 2-H was broadened so severely that it became hardly observable when the concentration of CPO reaches 0.08 mM or higher (Figure 2.1E-F). The linewidth of 3-H also increased dramatically from 4.27 to 59.64 Hz (Figure 2.1B-F) as the concentration of CPO was increased from 0.01 to 0.1 mM,

Figure 2.1: The effect of the concentration of CPO on the proton NMR spectra of indole. The spectra (A-F) was obtained in 10 mM deuterated phosphate buffer (pH 6.0) containing 5.7 mM indole and a fixed concentration of CPO.

although the broadening effect was not as profound for that observed for 2-H. In contrast, the other peaks were less broadened than those of the 2- and 3-H. For instance, the linewidth of 4-H increased gradually from 4.00 to 15.71 Hz in the range of the CPO concentration tested (Figure 2.1B-F). This suggests that protons in the pyrrole ring are closer to the heme iron than protons in the benzene ring of indole with 2-H being the closest. The systematic broadening of indole proton resonances observed here is consistent with the previous study of CPO-CPDO complex [79].

2.3.2 Dissociation constant of CPO-indole complex

To evaluate the binding affinity between CPO and indole, the longitudinal relaxation time (T_1) of indole protons was measured in the presence of 0.1 mM CPO and different concentrations of indole (2.8-11.0 mM) at pH 6.0. Figure 2.3 shows the nice exponential fit of signal intensities (M_z) as a function of the delay time (τ) as defined in Equation 2.1, using

Figure 2.2: CPO-catalyzed oxidation of indole to 2-oxindole using H_2O_2 . The indole structure is labeled with proton chemical shifts, and proton number assignments are also shown.

Table 2.1: The dissociation constant (K_D) of CPO-indole complex and the distances (r) between the protons of indole and CPO heme iron.^a

position	δ (ppm)	T_{1b} ^b	r (Å)	K_D (mM)
2-H	7.31	6.8×10^{-5}	4.2	21
3-H	6.50	3.7×10^{-4}	5.5	26
4-H	7.61	7.2×10^{-3}	9.1	21
5-H	7.06	1.6×10^{-2}	10.4	22
6-H	7.15	1.8×10^{-2}	10.5	20
7-H	7.44	9.9×10^{-3}	9.5	19

^a The relaxation experiments were performed in 10 mM deuterated phosphate buffer (pH 6.0) containing 0.01 mM CPO to measure the δ and T_1 of 2- and 3-H or 0.1 mM CPO for the other protons by changing the concentration of indole from 2.8 to 11.0 mM. ^b The average was from two independent experiments.

the resonance at 7.61 ppm (4-H) as an example. Similar fits are obtained for other protons of indole. Therefore, T_{1obs} of 4-H (Figure 2.3A-E) can be obtained from Equation 2.1 (1.51 s, 1.63 s, 1.79 s, 1.85 s, and 1.92 s, respectively). The increase in T_{1obs} is attributed to the increase of the fraction of free indole as its concentration is increased.

Figure 2.3F shows a plot of $E_0[1/T_{1obs} - 1/T_{1f}]^{-1}$ versus S_0 for 4-H. The fitted straight line demonstrates the reliability of the data ($R^2=0.9957$). The K_D value can be calculated as the intercept of the fitted line divided by its slope using Equation 2.2. The K_D calculated from Figure 2F was approximately 21 mM as listed in Table 2.1.

Figure 2.4 shows plots of $E_0[1/T_{1obs} - 1/T_{1f}]^{-1}$ versus S_0 for other protons. It should be noted that linear fits could only be obtained for 2- and 3-H in the presence of 0.01 mM CPO. Protons in the benzene ring of indole did not give reliable results in 0.01 mM CPO solution and thus do not fit into Equation 2.2 possibly due to the weak paramagnetic effect at low enzyme concentrations or long distances from these protons to the heme iron. Therefore, a

Figure 2.3: NMR relaxation experiments for 4-H of indole in the presence of CPO. (A-E) The plots of peak intensities at 7.61 ppm as a function of inversion recovery time (τ). The results (dots) was obtained in 100 mM deuterated phosphate buffer (pH 6.0) containing 0.1 mM CPO and (A) 2.8 mM indole, (B) 5.5 mM indole, (C) 8.3 mM indole, (D) 9.7 mM indole, and (E) 11.0 mM indole. (F) The plot of $E_0[1/T_{1obs} - 1/T_{1f}]^{-1}$ as a function of the concentration of indole (S_0).

higher concentration of CPO (0.1 mM) was used to measure the relaxation rates of protons on the benzene ring of indole. Table 2.1 lists the dissociation constant (K_D) of CPO-indole complex at pH 6.0. The K_D values obtained from the relaxation property of different protons in indole are quite consistent, further proving the validity of the relaxation method in probing the structural properties of hemoprotein-substrate complexes. The average of K_D calculated from all protons of indole is ~ 21 mM, indicating a weak binding of indole to CPO. This value is comparable to the reported K_D of 33 mM for the binding of CPDO to CPO [79]. The stability of CPO-indole complex also compares nicely with that of indole-P450 BM3 complex [166].

Figure 2.4: The plots of $E_0[1/T_{1obs} - 1/T_{1f}]^{-1}$ as a function of the concentration of indole (S_0) for (A) 2-H, (B) 3-H, (C) 5-H, (D) 6-H, and (E) 7-H of indole.

2.3.3 Effect of pH on indole binding to CPO

To check the effect of pH on indole binding to CPO, the K_D and the distances from 2- and 3-H to the heme iron were determined in the pH range of 3.0-6.5 (Table 2.2). The relaxation time of 3-H of indole cannot be determined at $\text{pH} \leq 4.0$ due to acid-catalyzed tautomerization of the N-protonated species [167] that leads to the exchange of 3-H with deuterated solvent. Therefore, the K_D values listed in Table 2.2 were derived from T_1 of 2-H only. Surprisingly, both the dissociation constant, K_D , and the distance, r , between the 2-H of indole and CPO heme iron were independent of pH in the pH range tested (Table 2.2), indicating that no ionizable group is involved in indole binding to CPO. This is at odds with the case of CPDO where an ionizable group with a pK_a between 4.5 and 6.5 is involved in the binding of CPDO to CPO [79]. The ionizable group has been suggested

Table 2.2: The dissociation constant (K_D) of CPO-indole complex and the distances (r) of 2- and 3-protons between the protons of indole and CPO heme iron^a at different pH

pH	T_{1b} (s)		r (Å)		K_D /mM ^c
	2-H	3-H	2-H	3-H	
3.0	7.0×10^{-5}	n.d. ^b	4.2	n.d.	25
3.5	5.6×10^{-5}	n.d.	4.0	n.d.	25
4.0	6.2×10^{-5}	n.d.	4.1	n.d.	23
4.5	5.6×10^{-5}	3.2×10^{-4}	4.0	5.4	25
5.0	6.0×10^{-5}	4.1×10^{-4}	4.1	5.6	23
5.5	6.1×10^{-5}	3.8×10^{-4}	4.1	5.5	23
6.0	6.8×10^{-5}	3.7×10^{-4}	4.2	5.5	21
6.5	6.0×10^{-5}	4.1×10^{-4}	4.1	5.6	23

^aThe relaxation experiment were performed in 10 mM deuterated phosphate buffer (pH 3.0-6.5) containing 0.01 mM CPO to measure T_1 of 2- and 3-protons by changing the concentration of indole from 2.0 to 12.0 mM. ^bn.d. means not determined owing to deuterated exchanged of the 3-proton. ^cThe dissociation constants were derived from relaxation experiments of 2-proton.

to be either a carboxylate residue (Glu 183) or a histidine imidazole group (His 105) [79]. Since CPDO is polar, it may require hydrogen bonding with Glu 183 or His 105 to stabilize its binding with CPO. In contrast to CPDO, indole is neutral and hydrophobic; therefore, its binding with CPO would naturally be dominated by hydrophobic interactions with the enzyme. The X-ray structure of CPO has revealed the presence of a putative hydrophobic channel for the approach of organic substrates to the heme active site [25]. At the bottom of this hydrophobic channel, two phenylalanines, Phe 103 and Phe 186, serve as the gate keeper in controlling substrate selectivity of CPO. Therefore, these two residues are likely to be the principal contact sites for the hydrophobic interactions between CPO and indole. Additionally, my results imply that the effect of pH on CPO's catalytic activity is primarily due to changes in the rate of formation of CPO compound I rather than the formation of CPO-substrate complexes, especially for hydrophobic organic substrates.

2.3.4 Association of indole with CPO in the presence of halide ion

It is well known that halides (including chloride, bromide, and iodide) are co-substrates of CPO-catalyzed halogenations [28]. Although CPO from *Caldariomyces fumago* is incapable of catalyzing the halogenation of indole, halide binding sites have indeed been

identified by a recent X-ray diffraction study of CPO [26]. To probe the effect of chloride and iodide on the binding of indole to CPO, the K_D and the distances from 2- and 3-H to the heme iron in the presence of chloride and iodide were measured. Both K_D and the distances displayed negligible dependence on the presence of chloride or iodide (Tables 2.2 and 2.3), indicating that chloride and iodide do not affect the binding affinity and orientation of indole to CPO. This is consistent with the previous report of CPO-CPDO complex in the presence of chloride ion [79] and is supported by the absence of chloride binding sites in chloride-soaked crystals of CPO [25]. However, the essentially identical K_D of CPO-indole complex in the presence of varying concentrations of iodide is somewhat surprising as CPO does have three iodide binding sites at the distal heme pocket [26]. The effect of iodide on substrate binding was confirmed by the previous study on CPO-CPDO complex, which displayed a dramatic increase in K_D (from 33 to 123 mM) as the concentration of iodide is increased [79]. This was later rationalized by possible steric clashes between CPDO and CPO bound iodide, since the distance between the carbonyl oxygen of CPDO and CPO bound iodide is only 1.9 Å [26]. It is thus proposed that the effect of iodide ion on the binding of substrates to CPO depends on the specific structure and nature of the substrates. Substrates such as CPDO that undergo halogenation reactions are affected by the presence of iodide, while substrates undergoing other transformations are not, implying that iodide binding results in little or no structural rearrangement for the substrate binding site of CPO. For CPO-catalyzed chlorination reactions, chloride ion is captured by compound I [168, 169] without the need for a bound chloride ion at the active site of CPO. This is plausible because the physiological environment where CPO functions has sufficient chloride ion to satisfy its need for chlorination reactions.

2.3.5 Molecular docking models of indole bound to CPO

To address the preferred orientations of indole within the active site of CPO, computer-aided docking of the molecules including indole and its *N*-methyl derivative into the active

Table 2.3: The dissociation constant (K_D) of CPO-indole complex and the distances (r) between the protons of indole and CPO heme iron^a in the presence of chloride and iodide ion.

C_{ions} (mM)	T_{1b} (s)		r (Å)		K_D /mM ^b
	2-H	3-H	2-H	3-H	
Cl ⁻		at pH 6.0			
10	6.5×10^{-5}	4.2×10^{-4}	4.1	5.6	24
20	5.5×10^{-5}	3.4×10^{-4}	4.0	5.4	25
30	5.6×10^{-5}	3.4×10^{-4}	4.0	5.4	24
40	6.2×10^{-5}	3.4×10^{-4}	4.1	5.4	22
Cl ⁻		at pH 4.0			
20	7.1×10^{-5}		4.2		20
I ⁻		at pH 6.0			
10	6.6×10^{-5}	3.8×10^{-4}	4.1	5.5	25
20	6.4×10^{-5}	3.4×10^{-4}	4.1	5.4	20
30	5.4×10^{-5}	4.0×10^{-4}	4.0	5.6	26
40	6.8×10^{-5}	3.7×10^{-4}	4.1	5.5	21
I ⁻		at pH 4.0			
20	8.6×10^{-5}		4.3		20

^aThe relaxation experiment were performed in 10 mM deuterated phosphate buffer (pH 4.0 and pH 6.0) containing 0.01 mM CPO and a certain concentration (C_{ion}) of chloride or iodide ion to measure T_1 of 2- and 3-protons by changing the concentration of indole from 2.0 to 12.0 mM. ^aThe dissociation constants were evaluated from relaxation measurements of 2-proton.

site of CPO was performed. During 60 Autodock runs, only one cluster of poses for indole (or its derivative) was observed. The key issue described here is the position and orientation of the substrate with respect to the heme and Glu 183 in the active site of CPO, since Glu 183 is postulated to be the acid-base catalyst in compound I formation [72, 170] and thus tune the catalytic activity of CPO [108]. Figure 2.5A shows CPO-indole complex deduced from molecular docking. The pyrrole ring of indole faces the heme with the 2-H being the closest to the heme iron. The orientation of indole observed here is consistent with that derived from the distances between the protons of indole and the heme iron in my relaxation experiments (Table 2.1). Additionally, the N-H of indole is 1.7 Å from the γ -carboxyl group of Glu 183, indicating that, potentially, hydrogen bond can be formed between the N-H of indole and Glu 183. This hydrogen bond, even if it does form, is not sufficient to be

the major factor in keeping indole in its binding site in CPO [33]. However, it is reported that the hydrogen bond between Glu 183 and alcoholic OH group directs benzyl alcohol or 2-phenylethanol to a specific orientation [171]. To further elucidate the function of a hydrogen bond formed between the N-H of indole and Glu 183, 1-methylindole was also docked into the active site (Figure 2.5B). It is observed that the methyl group of 1-methylindole points toward the heme, not Glu 183. Thus, the orientation of 1-methylindole is different from that of indole within the active site of CPO (Figure 2.5A). This is attributed to both the hindrance from a bulkier methyl group and the disruption of the putative hydrogen bond between indole and Glu 183. This also confirms that the N-H of indole does play a role in the binding of indole to CPO, since 1-methylindole is indeed hardly oxidized by CPO [33]. Therefore, the observed negligible effect of pH on the K_D of the CPO-indole complex from my NMR relaxation studies can be attributed to the extremely low pKa of Glu 183 within the active site of CPO.

Figure 2.5: Ribbon and stick representations of (A) indole and (B) 1-methylindole docking in the active site of CPO. There was only one orientation of indole and 1-methylindole found in docking. The indole and 1-methylindole are colored green. Heme is shown as red sticks, and Glu 183 is shown as yellow sticks.

The binding energy, which is the free energy released by the formation of the complex between the substrate and the enzyme, is an important parameter for evaluating the bioaffinity of the substrate for the enzyme [172]. The docking study resulted in binding energies of -23.2 kJ/mol for CPO-indole complex and -21.9 kJ/mol for CPO-1-methylindole complex.

Compared to the binding of substrates to P450, such as indole (-53.5 kJ/mol) [173] and ticlopidine (-32.6 kJ/mol) [174], the binding of indole to CPO is apparently weaker. This agrees with the conclusion from my NMR relaxation experiments (Table 1). The magnitude of the binding energy of 1-methylindole to CPO is slightly lower than that of indole, probably because formation of a hydrogen bond with Glu 183 is not possible.

2.3.6 Distance-restrained model of CPO-indole complex

To further investigate the interaction between indole and CPO, simulated annealing of CPO-indole complex was performed using the distances calculated from my NMR relaxation studies at pH 5.0. Mean interaction energies of CPO with indole were calculated for the last 50 ps of the simulated-annealing. The root-mean-square deviation (RMSD) values of CPO-indole complex reach constant values (~ 1.4 Å) in the last 50 ps, indicating that CPO-indole complex stabilizes. Figure 2.6 depicts the structural model of indole in the active site of CPO deduced from simulated annealing. Ten equally-spaced snapshots from the last 50 ps of simulation are overlaid. The orientation of the indole is seen to be quite stable. The distances between the protons of indole and the heme iron observed in Figure 2.6 are ~ 2 Å smaller than that of NMR results. This is consistent with previous results that the distance between the protons of CPDO and the heme iron of the crystal structure of CPO-CPDO complex [26] (4.9 Å) is much smaller than that of relaxation measurement [79] (7.1 Å). Furthermore, the difference of obtained distances between the distance-restrained model and NMR results is also seen in the study of CYP1A1-phenacetin complex [175]. This may be attributed to the fact that NMR results reflect an ensemble of multiple orientations of the substrate in the active site [174]. Nevertheless, there should be only one major orientation of indole as indicated by the significant broadness of NMR peaks of 2- and 3-protons of indole compared to other protons (Figure 2.1). It is observed that residues within 5 Å radius of indole are Val 67, Ile 68, Leu 70, Ala 71, Asn 74, Phe 103, Ile 179, Val 182, Glu 183, Phe 186, and Ala 267. The side-chain RMSDs of these residues, except Glu

183, averaged over the last 50 ps are comparable to the corresponding root-mean-square fluctuation (RMSF). The RMSD of Glu 183 turned out to be much larger than the corresponding RMSF, suggesting that the substantial motion of the polar Glu 183 side chain is a prerequisite for indole binding at the active site. This is consistent with the previous study [108] showing that the mobility of Glu 183 is critical to the catalytic reactivity of CPO. Furthermore, the residues in the active site strongly interacting with indole are of critical importance, which can serve as a lighthouse for designing better CPO mutants for the oxidation of not only indole but also similar types of organic substrates. Therefore, interaction energies between the active-site residues and the indole molecule are examined (Table 2.4).

Figure 2.6: The distance-restrained model of indole in the active site of CPO. Residues within a 5 Å radius of indole are Val 67, Ile 68, Leu 70, Ala 71, Asn 74, Phe 103, Ile 179, Val 182, Glu 183, Phe 186, and Ala 267. The side chains of Leu 70, Phe 103, Ile 179, Val 182, Glu 183, and Phe 186 are shown for the sake of clarity. To differentiate the protein residues from the substrate, the indole molecule are colored green, the heme is colored red, and the carbons of the residues are colored blue.

Table 2.4: The calculated energy (kJ/mol) in the last 50 ps of the simulation.

Residues	Average Energy ^{a,b}		RMSD	
	Coul	LJ	Coul	LJ
Val 67	-2.1±0.3	-2.7±0.3	1.1	1.2
Ile 68	-0.4±0.03	-2.4±0.3	0.2	1.1
Leu 70	1.1±0.1	-7.2±0.1	0.5	1.5
Ala 71	1.7±0.2	-4.0±0.3	0.9	3.1
Asn 74	-1.2±0.3	-2.8±0.1	1.3	0.8
Phe 103	-3.1±0.2	-10.4±0.1	1.0	1.8
Ile 179	-1.3±0.1	-8.0±0.1	1.6	2.7
Val 182	0.02±0.06	-8.8±0.1	0.5	2.5
Glu 183	-25.7±1.1	-5.6±0.2	7.8	3.0
Phe 186	-1.4±0.1	-6.6±0.2	1.1	2.2
Ala 267	-0.9±0.1	-1.6±0.2	0.5	0.8

^aLJ, Lennard-Jones potential energy; Coul, coulomb potential energy (rcoulomb=1.2 nm, rvdw=1.4 nm). ^bStatistics over 50001 steps (150.0 through 250.0 ps).

The calculated Coulomb and Lennard-Jones potential energies of indole bound to CPO (Table 2.4) can be interpreted as representing electrostatic and hydrophobic interactions, respectively. A strong Coulomb interaction (-25.7 kJ/mol) was found between indole and Glu 183, indicating the presence of a hydrogen bond between bound substrate and Glu 183. This agrees with my molecular docking studies of this system. His 105 in CPO has been postulated to form a hydrogen bond with and modulate the acidity of Glu 183 [78,190]. The interaction between His 105 and Glu 183 helps hold Glu 183 in its place to interact with iron-bound hydrogen peroxide that facilitates the formation of compound I. It has been demonstrated that chemical modification of His 105 is detrimental to the CPO-catalyzed oxidations [73]. In order to learn whether His 105 can affect indole binding to CPO, the interaction between His 105 and indole was checked. It was found that only a small repulsive Coulomb interaction (4.3 kJ/mol) exists between His 105 and indole, indicating that His 105 has little direct effect on indole binding to CPO, although His 105 can indirectly influence indole binding via its effect on the orientation and pKa of Glu 183. Additionally, a weak Coulomb interaction (-2.1 kJ/mol) was found between indole and Val 67. Surprisingly, a negligible Coulomb interaction (-1.2 kJ/mol) was found between indole

and Asn74, indicating that the interaction between Asn 74 and indole is not essential for substrate binding. This is quite different from the case of polar substrates such as ethylene glycol and dimethyl sulfoxide, which interact with Asn 74 extensively [26].

The van der Waals interaction (-103 kJ/mol) was much higher than the Coulomb interaction between entire CPO and indole (-30 kJ/mol) from my simulated annealing studies. This indicates that the formation of the CPO-indole complex is mainly governed by hydrophobic interaction, rather than hydrogen bond or electrostatic interactions. Specifically, indole interacts strongly with the several key hydrophobic residues including Leu 70, Phe 103, Ile 179, and Val 182, and Phe 186 at the active center of CPO. These residues contribute -6.6 to -9.4 kJ/mol per residue to the formation of CPO-indole complex (see Table 2.4). This agrees with reports that these hydrophobic residues interact significantly with hydrophobic substrates such as *cis*- β -methylstyrene [107, 108] and anthracene [176].

2.3.7 Mechanism of CPO-catalyzed oxidation of indole

On the basis of my NMR relaxation measurements and the distance-restrained models presented in Figure 2.6, a mechanism for the CPO-catalyzed regioselective oxidation of indole is proposed (Figure 2.7). As for all CPO catalyzed reactions, the oxidation of indole is initiated by the binding of a neutral hydrogen peroxide molecule to the heme iron of CPO (Fe^{III} , Figure 2.7, I). Binding of H_2O_2 to the heme iron is facilitated by the transfer of one of the peroxide hydrogens to the carboxyl oxygen of Glu 183 [25]. The hydrogen ion is then delivered to the distal oxygen of the peroxide as the peroxide bond is heterolytically cleaved to produce an oxoferryl π -cation radical intermediate ($\text{Fe}^{\text{IV}}=\text{O}\cdot+$, Figure 2.7, II) known as compound I. Formation of compound I has been observed and characterized through many different techniques including electron paramagnetic resonances (EPR) [94], Mössbauer [94], X-ray absorption [75], and electron nuclear double resonance spectroscopy [101]. Single electron reduction of compound I by indole converts compound I to compound II (Figure 2.7, III, [67]) and generates an indole radical intermediate (Figure 2.7, IV). The

presence of substrate intermediates as carbon-centered radicals has been established in the P450-catalyzed reaction [177]. Additional evidences in favor of such a radical intermediate is provided by EPR studies of lactoperoxidase in the sulfoxidation [178] and by ^1H NMR and EPR studies of a synthetic heme system in the epoxidation of styrene [179]. In addition, the hydrogen-atom abstraction of the substrate is also presumed to be the mechanism in CPO-catalyzed demethylation of *N,N*-dimethylanilines [180]. Green and Bukowski et al. suggested that the hydrogen-atom abstraction from reducing substrates is facilitated by the axial thiolate ligand [181, 182]. Reduction of compound II results in the transfer of the ferryl oxygen to the substrate and the regeneration of the initial ferric resting state of CPO. Consequently, 2-hydroxyindole is formed (Figure 2.7, V), which spontaneously tautomerizes to the stable oxindole (Figure 2.7, VI). Similar mechanisms have been reported in CPO-catalyzed oxidative dehalogenation [40]. Furthermore, my results clearly indicate that the consecutive electron transfers occur between the 2-position of indole and CPO intermediates, since 2-H of indole is pointing directly to the heme iron. Therefore, CPO-catalyzed oxidation of indole has a regioselectivity at 2-position.

Figure 2.7: Model of Mechanism of CPO-Catalyzed Oxidation of Indole.

2.3.8 The parameterization of GROMOS force field

The Gromos96 force field in GROMACS was defined as a set of equations (called potential functions) used to generate the potential energies and the forces, as shown in Equation 2.6 and 2.7 [154]. V^{bond} and V^{nonb} are bonded and non-bonded potential energy; K_b , K_θ , K_ξ and K_ϕ are the force constant of bond stretching, bending, improper, and dihedral torsion, respectively; b_0 , θ_0 , and ξ_0 are the equilibrium bond distance, bond angle, improper dihedral, respectively; in trigonometric dihedral-angle torsion terms, n is the multiplicity and ϕ_0 is the phase shift; and $C^{(12)}$ and $C^{(6)}$ are constants taken from a matrix of Lennard-Jones parameters to determine the van der Waals interactions, q_i and q_j are partial charges of atoms i and j , f is a constant equal to $138.935485 \text{ kJ}\cdot\text{mol}\cdot\text{nm}\cdot\text{e}^{-2}$. The improper dihedral terms (K_ξ and ξ_0) are included mainly to keep planar groups (e.g. benzene ring) planar.

$$V^{bond}(r) = \sum_{n=1}^{N_b} \frac{1}{4} K_{b_n} [b_n^2 - b_{0_n}^2]^2 + \sum_{n=1}^{N_\theta} \frac{1}{2} K_{\theta_n} [\cos \theta_n - \cos \theta_{0_n}]^2 + \sum_{n=1}^{N_\xi} \frac{1}{2} K_{\xi_n} [\xi_n - \xi_{0_n}]^2 + \sum_{n=1}^{N_\phi} K_\phi [1 + \cos(n\phi_n - \phi_{0_n})] \quad (2.6)$$

$$V^{nonb}(r) = \sum_{pairs(i,j)} \left\{ \left[\frac{C_{ij}^{(12)}}{r_{ij}^{12}} - \frac{C_{ij}^{(6)}}{r_{ij}^6} \right] + f \frac{q_i q_j}{\epsilon_r r_{ij}} \right\} \quad (2.7)$$

The force constants for bond stretching and angle bending can be obtained from the hessian matrix using Seminario's method [183]. In this method, the corresponding force δF on atom A due to a displacement δx of atom B can be expressed with the k_{AB} hessian. In a 3×3 hessian matrix, the diagonalization of $[k_{AB}]$ gives the eigenvalues λ_i^{AB} and the eigenvectors μ_i^{AB} , as shown in Equation 2.8. Therefore, the bond stretching force constant of the bond A-B can be obtained according to Equation 2.9, where μ is the normalized vector pointing towards from A to B. Similarly, the angle bending force can be derived according to Equation 2.10, where $\hat{\mu}^{PA}$ ($\hat{\mu}^{PA} = \hat{\mu}_N \times \hat{\mu}^{AB}$, and $\hat{\mu}^{PC}$ ($\hat{\mu}^{PC} = \hat{\mu}_N \times \hat{\mu}^{CB}$) are vectors perpendicular to the bonds A-B and C-B on the plane ABC, and $\hat{\mu}_N$ is the

vector perpendicular to the plane ABC. In addition, the harmonic force constants have to be converted to the Gromos force field form, i.e. to the fourth power bond stretch potential and cosine based angle bend potential, according to Equations 2.11 and 2.12, where $k^{b,harm}$ and $k^{\theta,harm}$ are the harmonic form of the force constant, k_b and k_θ are the Gromos force constant, and b_{ij} and θ_{ijk} are the equilibrium bond of the distance and angle, respectively.

$$\begin{bmatrix} \delta F_{Ax} \\ \delta F_{Ay} \\ \delta F_{Az} \end{bmatrix} = - \begin{bmatrix} \frac{\partial^2 E}{\partial x_A \partial x_B} & \frac{\partial^2 E}{\partial x_A \partial y_B} & \frac{\partial^2 E}{\partial x_A \partial z_B} \\ \frac{\partial^2 E}{\partial y_A \partial x_B} & \frac{\partial^2 E}{\partial y_A \partial y_B} & \frac{\partial^2 E}{\partial y_A \partial z_B} \\ \frac{\partial^2 E}{\partial z_A \partial x_B} & \frac{\partial^2 E}{\partial z_A \partial y_B} & \frac{\partial^2 E}{\partial z_A \partial z_B} \end{bmatrix} \times \begin{bmatrix} \delta x_B \\ \delta y_B \\ \delta z_B \end{bmatrix} \quad (2.8)$$

$$k_{AB} = \sum_{i=1}^3 \gamma_i^{AB} |\hat{\mu}^{AB} \cdot \hat{v}_i^{AB}| \quad (2.9)$$

$$\frac{1}{k_\theta} = \frac{1}{R_{AB}^2 \sum_{i=1}^3 \lambda_i^{AB} |\hat{\mu}^{PA} \cdot \hat{v}_i^{AB}|} + \frac{1}{R_{CB}^2 \sum_{i=1}^3 \lambda_i^{CB} |\hat{\mu}^{PC} \cdot \hat{v}_i^{CB}|} \quad (2.10)$$

$$2k^b b_{ij}^2 = k^{b,harm} \quad (2.11)$$

$$k^\theta \sin^2(\theta_{ijk}^0) = k^{\theta,harm} \quad (2.12)$$

For bonded parameters, the heme structure was taken from a high-resolution X-ray crystal structure (PDB code: 2CIW, resolution at 1.15 Å) and ligated with methylthiolate (representing cysteine). The side chains of the heme including four methyl groups, two vinyl groups and two carboxyl groups were omitted to simplify the calculation, since the experimental geometries of porphyrin models can be reproduced to a high accuracy without simple side chains (e.g., alkyl and vinyl groups) [184]. Hydrogen atoms were added manually using the package MOLDEN [185], version 5.0. *Ab initio* DFT geometry optimizations at the spin-unrestricted TPSS [186, 187] level was performed by ORCA (version 2.9) [157] with both the def2-SVP [159] and def2-SVP/J [188] basis set on all atoms. The TPSS functional was used because of its high accuracy and fast calculation speed for reproducing the experimental geometry [184]. The def2-SVP/J basis set was used for the resolution-of-the-identity (RI) approach to speed up the calculations significantly [188].

Table 2.5: The calculated energy (kJ/mol) in the last 50 ps of the simulation.

Groups	Equilibrium values	Force constant
Bond	Bond Length (Å)	(kJ/mol/nm ²)
Fe-N	2.10	4.62×10^5
Fe-S	2.30	6.35×10^5
N-C(heme)	0.14	6.18×10^6
Angle	Bending Angle (°)	(kJ/mol)
N-Fe-N	87.1	293.38
Fe-N-C	126.1	656.75
Fe-S-CH ₃	105.4	216.21
S-Fe-N	103.0	265.06
Dihedral Angle	multiplicity (n)	(kJ/mol)
N-Fe-S-CH ₃	4 ($\phi_0=0$)	0.1525

The vibrational frequency was calculated by two-sided numerical differentiation of analytical gradients. The hessian matrix was analyzed by Seminario's method [183] to derive the force constants for bond stretching and angle bending. While the dihedral terms can be fit to vibrational frequencies by Seminario's method, the harmonic approximation is particularly poor for the torsional degrees of the freedom [189]. Instead, the terms are derived from a relaxed potential energy scan (PES) [190]. A series of constrained geometry optimizations was performed by freezing different N-Fe-S-C dihedral angles at each step, while the other coordinates were relaxed. The optimized structures were then energy minimized with GROMACS [191] (Groningen Machine for Chemical Simulations), version 4.5.5, using a conjugate gradient algorithm to a gradient of less than 5 kcal/mol/Å in which the dihedral terms were set to zero. The difference in energy between the two scans (QM and MM) was then fit to a trigonometric expansion to derive the dihedral constant. My *ab initio* calculation indicates a total barrier of 1.50 kJ/mol, and my molecular simulation shows a non-bond energy contribution of 0.28 kJ/mol to the dihedral barrier. The bond stretching, angle and dihedral-angle parameters are list in Table 2.5.

Non-bonded parameters were determined as follow. Van der Waals parameters for the heme were taken directly from the GROMOS53a6 force field. The atomic partial charges of the heme system were obtained by CHELPG (CHarges from Electrostatic Potentials using a Grid based method) [192] as implemented in ORCA. The heme structure from the X-ray crystal structure (PDB code: 2CPO) was optimized at the same level of theory with the def2-SVPD [193] basis set. For this purpose, the side chains of heme were attached to the structure. The charges of equivalent atoms were symmetrized with the constraint that the total charges on the heme-thiolate system are negative two.

2.4 Conclusion

The work presented here provides insights into the mechanism of the CPO-catalyzed regioselective oxidation of indole. Association of indole with CPO was confirmed from both linewidth and longitudinal relaxation time measurements of the proton NMR signals of indole. Significantly, the precise orientation of indole in the CPO heme cavity was deduced from my results. The dissociation constant of the CPO-indole complex was calculated to be 21 mM, and the distances between protons of indole (2-, 3-, 4-, 5-, 6-, and 7-H) and the heme iron of CPO were 4.3 Å, 5.6 Å, 9.2 Å, 10.5 Å, 10.7 Å, and 9.7 Å, respectively. In the pH range of 3.0-6.5, the dissociation constant of CPO-indole complex and the position of indole in the active site are independent of solution pH. It was also found that the presence of halide (chloride and iodide) ion negligibly affect the stability of CPO-indole complex and the binding geometry of indole in the active site of CPO at pH 6.0. Molecular docking of indole in the active site of CPO reveals that the pyrrole ring of indole points to the heme and the 2-H of indole is the closest to the heme iron, confirming the results from NMR studies of CPO-indole complex. This indicates a direct-insertion mechanism of oxygen into the C-H bond at the 2-position of indole. On the other hand, molecular docking of 1-methylindole reveals that the methyl group points toward the heme iron, not Glu 183 as in the case of indole. These docking studies suggest that the N-H of indole plays an important

role in the binding of indole to CPO. The observed pH independence of indole binding to CPO in my pH effect experiments may be attributed to the low pKa of Glu 183 that remains fully deprotonated within the pH range tested in my experiments. Furthermore, simulated annealing of CPO-indole complex indicates that the Coulombic interaction between indole and Glu 183 is much stronger than that between Val 67, Asn 74, and His 105. Finally, my study demonstrated the association of CPO with indole is mainly governed by hydrophobic interactions rather than electrostatic interactions. This work provides the first experimental and theoretical explanation for the observed "unexpected" regioselectivity of CPO-catalyzed oxidation of indole. My results will also serve as a lighthouse in guiding the engineering of CPO into an efficient biocatalyst for synthetic and pharmaceutical applications.

CHAPTER 3

NMR SPECTROSCOPIC AND MOLECULAR DOCKING INVESTIGATIONS OF CHLOROPEROXIDASE COMPLEXED WITH SUBSTRATES IN ENANTIOSELECTIVE TRANSFORMATIONS

3.1 Introduction

The enantioselective oxidation of sulfides produces chiral sulfoxides, which are efficient chiral auxiliaries due to their configurational stability and high asymmetric transformations exerted by the sulfinyl fragment [194]. The chiral sulfoxides are important building blocks in the pharmaceutical industry. For instance, esomeprazole [195], a medicine that contains a chiral sulfoxide, is one of the most widely prescribed medicines. Therefore, it is not surprising that the development of new methods leading to chiral sulfoxides with high enantioselectivity remains a subject of fundamental interest. In the last few years, biocatalyst has emerged as a valuable method for preparation of chiral sulfoxide. In particular, chloroperoxidase(CPO)-catalyzed oxidation of a prochiral sulfide is unquestionably a green and economical method for synthesizing enantiomerically pure sulfoxides [196], because CPO can use hydrogen peroxide as the oxidant without requiring a cofactor. In 1986, Kobayashi, et al. first reported the enantioselective oxidation of methyl phenyl sulfides by CPO and hydrogen peroxide [197]. In 1992, a series of substituted sulfides suitable for enantioselective sulfoxidation was reported by Colonna et al [198]. Moreover, Deurzen reported that the sulfoxidation can be performed efficiently in *tert*-butanol/water mixture (50:50, V/V) that increase the solubility of sulfides in water [109].

Chloroperoxidase (CPO) itself catalyzes a broad class of reactions including halogenation [28], dehalogenation [39], *N*-demethylation [37], epoxidation of alkenes [42], dismutation of peroxide [31], and oxidation of organic compounds such as sulfides [197], alcohols [34], and amines [130]. A remarkable feature of CPO is to catalyze oxygen insertions with a high enantioselectivity in sulfoxidation [41, 198] and epoxidation reactions [42, 43], which offers a great potential in the synthesis of chiral compounds [46, 109, 133].

Therefore, the mechanism of these enantioselective transformations has received much attention. Since considerable efforts have been engaged in comprehending the mechanism, the CPO intermediates are now identified and characterized that occurs during the catalytic cycle [67, 70, 74, 199, 200]. However, the origin of the enantioselectivity in CPO-catalyzed sulfoxidation and epoxidation reactions are still not well understood. Furthermore, the interactions of CPO with substrates are poorly defined. Hence, a clear description of substrates binding to CPO in these chiral transformations is urgently needed.

In 1992, Casella, et al. first investigated the binding of alkyl aryl sulfides to the CPO's active site [81] and determined the distances between the protons of the sulfides and the heme iron using paramagnetic relaxation. Unfortunately, the examination of the interaction of CPO active-site residues with substrates was limited in that report, since the crystal structure of CPO was unknown until 1995 [25]. In 1998, Sundaramoorthy, et al. first used computer simulations to address which factors give rise to the pronounced enantioselectivity in epoxidation reactions [27] and suggested that the high enantioselective product is related to favorable interactions between the substrates and Glu 183. Furthermore, according to molecular dynamic simulations of CPO-cis- β -methylstyrene complex, Morozov, et al. proposed that enantiomer is likely due to a subtle balance of steric interactions caused by residues Phe 103, Ile 179, Val 182, and Phe 186 around the oxyferyl heme center [107, 108]. All the works reported so far have added up to questioning the structure of CPO-substrates complex. Unfortunately, X-ray crystal structure of CPO complexes is only available for tight-binding ligands (such as carbon monoxide) [27] except 1,3-cyclopentanedione due to the difficulty of diffusing organic substrates into CPO crystals. Hence, the mechanism remains a mystery to be elucidated. This work uses a combination of the paramagnetic relaxation and molecular docking to explore the structure of CPO-substrate complex. Such a structural information will provide the paradigm for understanding the mechanism of the high enantioselectivity displayed by CPO.

The aim of this chapter is to unravel the mechanism of CPO-catalyzed enantioselective epoxidation and sulfoxidation reactions by exploring the structure of CPO-substrates complexes. Styrene derivatives were selected as model substrates for epoxidation, while 2-(methylthio)thiophene was chosen for sulfoxidation, as both substrates produce a high enantiomeric excess (ee) in CPO-catalyzed reactions. The binding of the chosen substrates to CPO was probed by NMR spectroscopy and molecular docking. To perform relaxation experiments, the solvents for hydrophobic substrates were examined. The dissociation constant of the CPO-2-(methylthio)thiophene complex and the distances between the protons of 2-(methylthio)thiophene and the heme iron of CPO were determined using paramagnetic relaxation. The molecular docking of sulfides including 2-(methylthio)thiophene, 2-(methylthio)pyridine, 4-(methylthio)toluene, 1-(methylthio)-4-nitrobenzene, 1-methoxy-2-(methylthio)benzene, and (propylthio)benzene are presented. In particular, the factors that potentially control the enantioselectivity of CPO were examined. The results will provide a deep insight into the mechanism of CPO-catalyzed chiral transformations.

3.2 Materials and Methods

3.2.1 Materials

Unless otherwise stated, all the chemical reagents were purchased from Sigma-Aldrich (St Louis, MO) and all of analytical grade. Millipore Milli-Q water (18.2 M Ω) was used throughout.

Chloroperoxidase(CPO) isolation: stock cultures of *Caldariomyces fumigo* were inoculated on potato dextrose agar plate and grown for a week at 28°C as a surface mat. The surface mat from three plates of inoculum was used to inoculate 300 mL of fructose peptone salts medium supplemented with 50 mg/L carbenicillin and homogenized for 30 sec with a sterile tissue homogenizer Omni TH115 (Kennesaw, GA). The homogenate were equally divided into six 125 mL erlenmeyer flasks plugged with autoclaved cotton balls. After 7 days of growing at 22°C on the rotary shaker, the mold mycelium was homogenized again

and inoculated into six 4 L baffled culture flasks, each containing 2 L of fructose peptone salts medium. The inoculum was grown at 19°C on the shaker with a rotation speed of 200 rpm. The production of CPO was monitored kinetically by the standard monochlorodimedone chlorination assay [28] three times a week. The *Caldariomyces fumigo* medium containing secreted CPO was harvested when the monochlorodimedone chlorination activity stop increasing, followed by isolation and purification of CPO from the growth medium according to the method established by Morris et al. [23] with minor modifications, using acetone instead of ethanol in the solvent fractionation step.

CPO purification: previous harvested CPO crude media were further purified by gel filtration column and ion exchanged column [111]. The purified CPO solution was added in Centriprep centrifugal filter Devices (Billerica, MA), concentrated by Beckman Coulter high performance centrifuge (Pasadena, CA) with 1500 g for 4 hours, and stored at 4°C. CPO was collected in solution prepared with 100 mM phosphate buffer (pH 5.8). The concentrations of native CPO were determined by Cary Bio-300 UV-Vis spectrometer at 250-700 nm using an extinction coefficient of $91\,200\text{ M}^{-1}\text{ cm}^{-1}$ at 398 nm [23]. The Reinheitszahl value (R_z , $A_{398\text{ nm}}/A_{280\text{ nm}}$) was used to check the purity of the final sample. Only CPO with R_z values of 1.4 or higher were used in NMR experiments. In order to avoid solvent effects and simplify the NMR spectra, CPO was deuterium exchanged with 0.1 M deuterated phosphate buffer (pH 5.8). The exchanges were processed by adding deuterated phosphate buffer into CPO solution, and centrifuging solutions to discard excess H_2O . The exchange process was repeated at least 6 times.

The CPO mutant, F103A, was kindly supplied by my colleague, Dr. Zheng Wang.

The solution with 25% (V/V) co-solvent: 25 % (V/V) co-solvent solutions for testing the solubility of *cis*- β -methylstyrene were prepared by mixing 75 μL of co-solvent with 0.39 μL of *cis*- β -methylstyrene in 2925 μL of water.

The buffer with 25% (V/V)*tert*-butanol: 25% (V/V) *tert*-butanol co-solvent was prepared by mixing *tert*-butanol with 1 M KH_2PO_4 and water in a volume ratio of 5:2:13.

3.2.2 Preparation of NMR samples

A stock solution of 2-(methylthio)thiophene (0.1 M) was prepared by dissolving 13.0 mg 2-(methylthio)thiophene in 1.0 mL of *tert*-butanol- d_{10} . NMR samples contained 5-15 mM 2-(methylthio)thiophene and 0.1 mM purified CPO in 100 mM deuterated phosphate buffer with 25% (V/V) *tert*-butanol- d_{10} . The final volume of the NMR tube was 500 μL .

3.2.3 NMR characterization of 2-(methylthio)thiophene

Proton NMR measurements were conducted on a Bruker Avance 600 MHz NMR spectrometer at 298.0 K. The proton chemical shifts were referenced to the internal reference 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) (1H, δ 0.00). The NMR data were processed using Topspin, version 2.1.

3.2.4 Molecular Docking

The molecular docking for the preferred orientation of the 2-(methylthio)thiophene, 2-(methylthio)pyridine, 4-(methylthio)toluene, (propylthio)benzene, 1-(methylthio)-4-nitrobenzene, and 1-methoxy-2-(methylthio)benzene within the active site of CPO was performed by the software package AutoDock [153], version 4.2.5.

The molecular docking was performed as described in Chapter 2. Briefly, the structure of CPO for docking was taken from the X-ray structure (PDB code: 2CPO). Substrates 2-(methylthio)thiophene, 2-(methylthio)pyridine, 4-(methylthio)toluene, (propylthio)benzene, 1-(methylthio)-4-nitrobenzene, and 1-methoxy-2-(methylthio)benzene were built by MarvinSketch (Version 5.11) in JChem software package (ChemAxon, Ltd.) and further optimized by ORCA [157] (version 2.9) using the spin-component scaled second-order Möller-Plesset perturbation theory and def2-SVP basis set [159]. AutoDockTools [160] (version 1.5.4) was used to add Gasteiger charges to CPO (+1.00 was added manually on Fe) and sulfides. During the simulation, the protein structure was kept rigid. The sulfide

was simulated in a box centered at the heme iron that was confined with a grid size of $30 \times 30 \times 30 \text{ \AA}^3$ box with $0.375 \text{ \AA}$ spacing. A docking of 100 separate simulation runs was performed with 2.5 million energy evaluations per run and 300 population size.

3.3 Results and Discussion

3.3.1 Searching the solvents for styrene derivatives

To obtain high signal-to-noise ratios and avoid large errors in fitting relaxation results, based on my experiences, NMR relaxation experiments usually require the concentration of the organic substrates to be higher than 1 mM. Therefore, the appropriate solvents for *cis*- β -methylstyrene was searched based on the requirement of the solubility of *cis*- β -methylstyrene and styrene > 1.0 mM

3.3.1.1 Solubility of *cis*- β -methylstyrene and styrene in water

To obtain a high quality result of NMR experiments for *cis*- β -methylstyrene, the solubility of *cis*- β -methylstyrene and styrene in water was first checked due to their hydrophobicity. When $0.39 \text{ }\mu\text{L}$ of *cis*- β -methylstyrene or $0.35 \text{ }\mu\text{l}$ of styrene was added into 3.0 mL of water, a cloudy emulsion was observed. This indicates that the solubility of *cis*- β -methylstyrene in water is lower than 1 mM, and water is a poor solvent for *cis*- β -methylstyrene and styrene. Therefore, to increase the solubility of *cis*- β -methylstyrene and styrene in water, I tested organic co-solvents in the following sections.

3.3.1.2 Solubility of *cis*- β -methylstyrene and styrene in co-solvent/water

The enzyme CPO can be denatured and lost its activity in the presence of a high concentration of hydrophobic solvents [121], whereas the solubility of the organic substrates in hydrophilic solvents is usually limited. The key issue here is to balance the activity of CPO maintained in solution and the solubility of the substrate *cis*- β -methylstyrene. When concerning the solvent for increasing solubility, the rule "like dissolves like" is frequently referred. The "like dissolves like" means that a polar substance will dissolve a polar substance, and nonpolar substance will dissolve other nonpolar substances, but nonpolar and

polar substances will not dissolve one another. Obviously, *cis*- β -methylstyrene and styrene contain a benzene ring, and thus are strong hydrophobic organic compounds. Therefore, I chose the co-solvents that contain both the hydrophobic moiety for *cis*- β -styrene and the hydrophilic group for CPO. These solvents include cyclopentanone, cyclohexanone, and acetophenone. To check whether these solvents are qualified as co-solvent, the emulsion of 1 mM of *cis*- β -methylstyrene and styrene in 25% (V/V) co-solvents was tested as described in Section 3.2.1. It was found that the solubility of *cis*- β -methylstyrene in 25% (V/V) co-solvents was not improved significantly. Additionally, some nonpolar solvents including benzene, toluene, nitrobenzene, and cyclohexane were also tested. However, the maximum solubility of *cis*- β -methylstyrene in water containing 25% nonpolar solvents (V/V) was found less than 1 mM. Therefore, all these solvents were not used for NMR experiments.

Other approach for enhancing the solubility of hydrophobic styrene and its derivatives in aqueous solution is to use the water-soluble surfactants. Thus, sodium dodecyl sulfate (SDS) and Triton X-100, a nonionic commercial surfactant that has a hydrophilic polyethylene oxide chain and an aromatic hydrocarbon lipophilic group, were tested for *cis*- β -methylstyrene-CPO system. The results showed that Triton X-100 and sodium dodecyl sulfate can increase the miscibility of *cis*- β -methylstyrene in water. However, Triton X-100 was found oxidized by H₂O₂ in the presence of CPO, indicating that Triton X-100 is a substrate of CPO. Since CPO was found to be denatured by a small fraction of SDS (=10%, W/V), no suitable amount of SDS can be used in my experiment.

To increase the solubility of *cis*- β -methylstyrene/styrene in water, a series of hydrophilic organic solvents that can homogeneously mix with water were also tested as co-solvent. These solvents include acetic acid, acetone, acetonitrile, pyridine, ethylene glycol, ethyl acetate, glycerol, 1-propanol, 1,4-dioxane, tetrahydrofuran, N,N-dimethylformamide, dimethyl sulfoxide, methanol, and ethanol, since their deuterated counterparts are commercially

available. However, I found that these solvents (except the pyridine), whose concentrations are at least 25% (V/V) or even 50% (V/V), were inappropriate for solvating *cis*- β -methylstyrene and styrene. In particular, when 1 mM of *cis*- β -methylstyrene was prepared in 30% (V/V) pyridine solvent without pH adjustment (pH >7.0), no cloudiness was observed. However, the solution pH needed to be adjusted to acidic range by adding 6 M hydrogen chloride (HCl), since pyridine is a base and CPO is denatured if pH above 7.0 [66]. When pH was adjusted lower than 7, a precipitate was formed in 1 mM *cis*- β -methylstyrene solution prepared by 30% (V/V) pyridine co-solvent. Moreover, pyridine was reported to form a complex with CPO that may interfere the binding of *cis*- β -methylstyrene to CPO [100]. Therefore, pyridine cannot be used as co-solvent for my NMR experiments. Fortunately, no eye-observed cloudiness was found using tert-butanol as co-solvent for 5 mM *cis*- β -methylstyrene in 25% (V/V) tert-butanol-water co-solvent. Therefore, tert-butanol was further tested as co-solvent by UV-Vis spectroscopy.

The paramagnetic relaxation experiments for studying the interaction of CPO with substrates generally takes several hours. Therefore, the stability of the substrates in the searched co-solvent, 25% (V/V) tert-butanol-water, should be examined. To evaluate the actual stability (or miscibility), the absorption spectra of the substrate *cis*- β -methylstyrene in pure tert-butanol and in 25% (V/V) tert-butanol-water co-solvent were recorded, respectively. The UV-Vis spectrum of 0.05 mM *cis*- β -methylstyrene in tert-butanol showed that the maximum absorption wavelength (λ_{max}) of *cis*- β -methylstyrene appeared at 241 nm. It should be noted that a low concentration of *cis*- β -methylstyrene was used, as an appropriate absorption was required. The spectra of 0.05 mM *cis*- β -methylstyrene in tert-butanol were found stable over time as expect. This indicates that a stable absorption spectrum of *cis*- β -methylstyrene at 0.05 mM in tert-butanol can be obtained.

Figure 3.1 shows UV-Vis spectra of *cis*- β -methylstyrene ($\sim$ 0.05 mM) in *tert*-butanol-water co-solvent over time. All the spectra samples were prepared separately, but in the

Figure 3.1: The change of UV-Vis spectra of *cis*- β -methylstyrene in 25% *tert*-butanol co-solvent over time. The tested solutions containing 0.05 mM *cis*- β -methylstyrene were diluted with 25% *tert*-butanol co-solvent from the same 5 mM *cis*- β -methylstyrene stock solution prepared with 25% *tert*-butanol co-solvent.

same way, by a dilution of 5 mM *cis*- β -methylstyrene solution that was prepared in 25% *tert*-butanol-water (V/V) solvents. If the concentration of *cis*- β -methylstyrene is stable to be 5 mM in solution, the absorbance observed should be the same for all the samples. Unexpectedly, the absorbance obtained is linearly decreased over the tested time (Figure 3.1), indicating that the concentration of *cis*- β -methylstyrene in the stock solutions is decreased during the time course. Thus, the stability of *cis*- β -methylstyrene in 25% (V/V) *tert*-butanol is inadequate for performing relaxation experiments. Similarly, the absorbance of styrene is also linearly decreased over time (Figure 3.2), indicating that the stability of styrene in 25% (V/V) *tert*-butanol is also inadequate. In sum, the co-solvent for *cis*- β -methylstyrene and styrene has not been found. Nevertheless, the *tert*-butanol provided the best solubility of *cis*- β -methylstyrene and styrene among other tested solvents. Therefore, *tert*-butanol can be a potential co-solvent for other substrates such as 2-(methylthio)thiophene.

Figure 3.2: The change of UV-Vis spectra of styrene in 25% *tert*-butanol co-solvent over time. The tested solutions containing 0.2 mM styrene were diluted from the same 2 mM styrene stock solution.

3.3.2 Searching for hydrophilic styrene derivatives

It is obvious that the solubility of hydrophobic styrene derivatives in water can be increased if the styrene derivatives are attached with a hydrophilic group such as -OH, -NH₂, -COOH, and -SO₃H. Therefore, the hydrophilic derivatives of styrene were tested for solubility using UV-Vis absorption spectroscopy and further studied in NMR experiments. Four hydrophilic styrene derivatives (Figure 3.3) including 3-aminostyrene, 4-aminostyrene, styrene-4-carboxylic acid, and 4-styrene sulfonic acid were examined. The substrate 4-vinylphenol was not tested, since -OH group is also oxidized in CPO-catalyzed reaction [129].

3.3.2.1 Solubility and NMR characterizations of 3-aminostyrene and 4-aminostyrene

To check the solubility of 4-aminostyrene and 3-aminostyrene in water, UV-Vis spectroscopy of 4-aminostyrene (0.1 mM) and 3-aminostyrene (0.05 mM) in water were recorded that shows a maximum absorbance at 271 nm for 4-aminostyrene and 330 nm for 3-aminostyrene. As expected, the absorbance of all the tested sample (0.1 mM 4-aminosty-

Figure 3.3: The change of UV-Vis spectra of styrene in 25% *tert*-butanol co-solvent over time. The tested solutions containing 0.2 mM styrene were diluted from the same 2 mM styrene stock solution.

rene and 0.05 mM 3-aminostyrene) that are diluted from 20 mM 3-aminostyrene and 30 mM 4-aminostyrene at different time are found to be the same. Concerning the substrate solubility, 4-aminostyrene and 3-aminostyrene should be qualified for NMR study. Additionally, the reaction of 4-aminostyrene in the presence of CPO and H_2O_2 was checked using UV-Vis absorption spectroscopy. The result showed that the absorbance of 4-aminostyrene at 260 nm is decreased significantly in a short time. This indicates that 4-aminostyrene can be oxidized by H_2O_2 using CPO catalysis. Therefore, 4-aminostyrene and 3-aminostyrene, were further studied by NMR experiments.

Figure 3.4 shows proton NMR spectra of 3-aminostyrene (10 mM) in the absence (A) and presence (B) of 0.1 mM CPO in 0.1 M deuterated phosphate buffer at pH 6.0. From Figure 3.4, it can be seen six main peaks that can be assigned to six types of the protons of 3-aminostyrene molecule (see Figure 3.3). The chemical shift of these protons observed in the presence of CPO is same to that in the absence of CPO.

To calculate the dissociation constant (K_D) of CPO-3-aminostyrene complex, the $E_0[1/T_{1obs} - 1/T_{1f}]^{-1}$ values as a function of the concentration of 3-aminostyrene (S_0) is plotted as shown in Figure 3.5. It can be seen that the $E_0[1/T_{1obs} - 1/T_{1f}]^{-1}$ values for all protons is unable to fit linearly to Equation 2.2. This could be attributed to my finding that 3-

Figure 3.4: Proton NMR spectra of 3-aminostyrene (10 mM) in the (A) absence and (B) presence of 0.1 mM CPO at pH 6.0

aminostyrene is slowly convert to white gel-like substances in deuterated phosphate buffer at pH 4-6. The NMR relaxation experiments were also performed for 4-aminostyrene. The result from the plot of $E_0[1/T_{1obs} - 1/T_{1f}]^{-1}$ values as a function of the concentration of 4-aminostyrene is same as that of 3-aminostyrene and CPO system. Thus, 3-aminostyrene and 4-aminostyrene are inappropriate as substrates for my study.

3.3.2.2 Solubility and NMR characterizations of styrene-4-carboxylic acid and 4-styrene sulfonic acid

Styrene-4-carboxylic acid that contains a strong hydrophilic group, $-\text{COOH}$, and 4-styrene sulfonic acid that contains another strong hydrophilic group, $-\text{SO}_3\text{H}$, both have satisfied solubility in water; even though the solubility of these two compounds is pH dependent due to their acid group. Nevertheless, in the pH range from 1 to 5.5, the solubility of styrene-4-carboxylic acid in water (12-83 mM, Scifinder database) is sufficient for the NMR T_1 measurement.

To verify whether styrene-4-carboxylic acid is the substrate of CPO, a kinetic assay of styrene-4-carboxylic acid was carried out in the presence of CPO. Since the maximum-

Figure 3.5: Plots of $E_0[1/T_{1obs} - 1/T_{1f}]^{-1}$ as a function of the concentration of 3-aminostyrene (S_0). Where E_0 is the concentration of CPO, T_{1obs} is T_1 measured by relaxation experiments, and T_{1f} is T_1 of 3-aminostyrene in the absence of CPO. Measurement condition: 5-proton at 7.13 ppm, 0.1 M deuterated phosphate buffer, 0.1 mM CPO, at pH 6.0

absorption wavelength (λ_{max}) of styrene-4-carboxylic acid is 262 nm [201], if styrene-4-carboxylic acid is oxidized to 4-(2-oxoiranyl)-benzoic acid by CPO-catalyzed oxidation, the absorption observed at 262 nm is expected to decrease dramatically in the presence of CPO and H_2O_2 . Thus, I observe the absorption of styrene-4-carboxylic acid at 262 nm in the presence of CPO and H_2O_2 . Figure 3.6 shows the time course of absorption of 0.1 mM styrene-4-carboxylic acid at 262 nm in 0.1 M phosphate buffer with addition of H_2O_2 (2 mM, final concentration) at pH 5.0 (A) in the presence of 4.7 μ M native CPO and (B) in the presence of 1 nM CPO mutant, F103A, respectively. From the Figure 3.6A, it can be seen that the absorption remains the constant after addition of H_2O_2 , indicating that no oxidation of styrene-4-carboxylic acid was taken place. Therefore, styrene-4-carboxylic acid is not a substrate of native CPO. In contrast, as shown in Figure 3.6B, the absorption is slowly decreased after addition of H_2O_2 , indicating that styrene-4-carboxylic acid is oxidized by H_2O_2 in the presence of the CPO mutant. It should be note that a low concentration of CPO mutant was used due to small amount of F103A CPO (mutant) available. My results clearly demonstrate that only the CPO mutant is able to catalyze the oxidation of styrene-

4-carboxylic acid. This may be attributed to that the carboxylic acid is bulky and prevent the substrate to enter the substrate binding site of native CPO, while the mutant changes the structure of CPO. It seems that styrene-4-carboxylic acid cannot bind to native CPO in the active site. Therefore, NMR studies were carried out to check styrene-4-carboxylic acid binding to native CPO.

Figure 3.6: The change of UV absorbance of 0.1 mM styrene-4-carboxylic acid at 262 nm over time course in the presence of native CPO (A) or F103A CPO mutant (B) with addition of 2 mM H_2O_2 at pH 5.0. The absorption were monitored in 0.1 M phosphate buffer at pH 5.0 (A) in the presence of 4.7 μM native CPO with addition of 2 mM (final concentration) H_2O_2 and (B) in the presence of 1 nM F103A CPO (mutant).

Figure 3.7 shows proton NMR spectra of styrene-4-carboxylic acid (30 mM) in the absence (A) and presence (B) of 0.1 mM CPO in 0.1 M deuterated phosphate buffer at pH 6.0. NMR proton assignment of styrene-4-carboxylic acid in the absence of CPO is shown in Figure 3.3. From Figure 3.7, it can be seen that the shapes of the NMR spectra of styrene-4-carboxylic acid obtained in presence of CPO are same to that in the absence of CPO, indicating that the binding of styrene-4-carboxylic acid to CPO is, if not possible, very weak.

To check pH-dependent of the longitudinal relaxation time (T_1), T_1 of styrene-4-carboxylic acid were firstly examined at different pH (Table 3.1). T_1 of *trans*-position proton (*trans*- β -H) is increased from 1.84 s at pH 4.7 to 1.85 s at pH 5.2 and to 1.88 s at pH 7.0, indicating that T_1 values of styrene-4-carboxylic acid ($\text{pK}_a=4.24$) protons are pH

Figure 3.7: Proton NMR spectra of styrene-4-carboxylic acid (30 mM) in 0.1 M deuterated phosphate buffer (A) in the absence and (B) in presence of 0.1 mM native CPO at pH 6.0.

Table 3.1: Relaxation time (T_1) of 5 mM styrene-4-carboxylic acid in the absence of CPO at different pH.

Position	T_1 (s) at pH 4.7	T_1 (s) at pH 5.2	T_1 (s) at pH 7.0
<i>trans</i> - β -H	1.84	1.85	1.88
<i>cis</i> - β -H	1.41	1.41	1.42
α -H	3.42	3.45	3.55
2-H	2.49	2.50	2.57
3-H	3.99	4.19	4.27

^aRelaxation experiments were carried out in 100 mM deuterated phosphate buffer containing 5 mM styrene-4-carboxylic acid in the absence of CPO at pH 4.7, pH 5.2, and pH 7.0.

dependent. T_1 of other protons are increased similarly with the increasing of solution pH. This is attributed to the deprotonation of the carboxyl group of styrene-4-carboxylic acid. Additionally, the difference of T_1 would be small when pH difference is small (Table 3.1, T_1 at pH 4.7 and pH 5.2). Nevertheless, pH of styrene-4-carboxylic acid solution should be carefully controlled.

To investigate the binding between styrene-4-carboxylic acid and CPO, the relaxation experiment was carried out in 0.1 M deuterated phosphate buffer containing 10-50 mM styrene-4-carboxylic acid and 0.1 mM CPO at pH 6.0. Table 3.2 lists the T_1 of styrene-4-

Table 3.2: The relaxation time (T_1) of styrene-4-carboxylic acid at different concentration (S_0) obtained in 0.1 M deuterated phosphate buffer in the presence of 0.1 mM CPO at pH 6.0.

S_0 (mM)	<i>trans</i> - β -H	<i>cis</i> - β -H	α -H	2-H	3-H
10	1.88	1.45	3.89	2.63	4.39
20	1.84	1.42	3.63	2.63	4.38
30	1.88	1.42	3.65	2.63	4.24
40	1.85	1.42	3.58	2.69	4.34
50	1.86	1.41	3.54	2.61	4.41

-carboxylic acid obtained in the presence of 0.1 mM CPO at pH 6.0. The results showed that T_1 was approximately the same at different styrene-4-carboxylic acid concentration and was unable to fit to Equation 2.2. This confirms that styrene-4-carboxylic acid is not a substrate of native CPO. Furthermore, these results suggest that the bulky group such as carboxyl acid in styrene-4-carboxylic acid is not preferable group to attach on substrates.

To investigate the binding of 4-styrene sulfonic acid to CPO, 4-styrene sulfonic acid was studied similarly to that described in styrene-4-carboxylic acid by NMR spectra and relaxation experiments. Figure 3.8 shows proton NMR spectra of 4-styrene sulfonic acid (100 mM) in the absence and presence of 0.05 mM CPO in 0.1 M deuterated phosphate buffer at pH 6.0. The NMR proton assignment of 4-styrene sulfonic acid in the absence of CPO is shown in Figure 3.3. As shown in Figure 3.8, the chemical shifts observed in the presence of CPO is same to that in the absence of CPO. This indicates that the binding of 4-styrene sulfonic acid to CPO is very weak. Additionally, it was found that T_1 obtained is unable to fit to Equation 2.2. This may be attributed to the fact that 4-styrene sulfonic acid also contains a bulky sulfonic group. This also agree with the poor Equation fits of styrene-4-carboxylic acid as described above.

In a brief summary of this section, I found that the bulky group such as sulfonic and carboxyl acid can exclude styrene from entering the active site of CPO (see Figure 3.6 and Table 3.2). Therefore, although the solubility of these two compounds is satisfied for NMR

Figure 3.8: Proton NMR spectra of 100 mM 4-styrene sulfonic acid in 0.1 M deuterated phosphate buffer(A) in the absence of and (B) in presence of 0.05 mM CPO at pH 6.0.

experiments, the binding of styrene-4-carboxylic acid and 4-styrene sulfonic acid to CPO cannot be studied by relaxation experiments.

3.3.3 Checking the co-solvent for 2-(methylthio)thiophene

3.3.3.1 CPO activity in *tert*-butanol as a co-solvent

To check solvent effects of *tert*-butanol on the CPO activity, UV-vis spectra of 0.01 mM CPO in 25% (V/V) *tert*-butanol co-solvent was measured over time course at pH 5.0. The result showed that the absorbance of CPO maintains constant within 8 hours, and the Soret peak at 398 nm is the same to that observed in the aqueous solution without *tert*-butanol. This indicates that the structure of CPO is stable in 25% (V/V) *tert*-butanol solution. This stability is consistent with the fact that *tert*-butanol is not a substrate for CPO owing to its bulky structure [121]. Furthermore, it was reported that in 50% (V/V) *tert*-butanol solution the sulfoxidation of alkyl aryl sulfides and related compounds catalyzed by CPO has the same enantioselectivity to the sulfoxidation in water [121]. Therefore, *tert*-butanol can be used without seriously disturbing the CPO activity.

3.3.3.2 Solubility of 2-(methylthio)thiophene in *tert*-butanol co-solvent

To check the solubility of 2-(methylthio)thiophene in the presence of the co-solvent *tert*-butanol, 15 mM 2-(methylthio)thiophene in 25% *tert*-butanol (V/V) was prepared and tested as described above. The UV absorbance of 0.15 mM 2-(methylthio)thiophene is essentially the same for all the tested samples that are diluted 100 times from the same stock solution of 15 mM 2-(methylthio) thiophene. This indicates that the concentration of 2-(methylthio)thiophene was constant in 2-(methylthio)thiophene stock solutions over time. Obviously, the solubility of 2-(methylthio)thiophene is at least up to 15 mM in 25% *tert*-butanol solution. Therefore, the 25% *tert*-butanol solution can be as a co-solvent for my NMR experiments.

3.3.4 NMR characterization of CPO-sulfide complex

3.3.4.1 Proton assignment of 2-(methylthio)thiophene

Proton NMR spectra of 5 mM 2-(methylthio)thiophene in the absence and presence of 0.1 mM CPO at pH 5.0 show six well-defined peaks (Figure 3.9). The chemical shifts of 2-(methylthio)thiophene in the absence of CPO are assigned according to the NMR spectra Database, <http://nmrdb.org> (7.388 (2, 1H, dd, J=8.142, J=1.340), 7.163 (3, 1H, dd, J=8.142, J=5.011), 7.341 (4, 1H, dd, J=5.011, J=1.340), 2.406 (7, 3H)). The three NMR peaks that are in the aromatic region of the spectrum (6-8 ppm) were assigned to the thiophene ring of 2-(methylthio)thiophene, while the singlet at 2.30 ppm was assigned to the protons on the methyl group. In the presence of 0.1 mM CPO (Figure 3.9B), the peaks assigned to 2-(methylthio)thiophene are broaden compared with that in the absence of CPO, indicating that 2-(methylthio)thiophene is bound to CPO.

3.3.4.2 Deriving the dissociation constant and distances from T_1 relaxation data

To evaluate the binding affinity between CPO and 2-(methylthio)thiophene, the longitudinal relaxation time (T_1) of 2-(methylthio)thiophene was measured in the absence and presence of 0.1 mM CPO at pH 5.0. Figure 3.10 shows the plots of $E_0(1/T_{1obs} - 1/T_{1f})^{-1}$

Figure 3.9: Proton NMR spectra of 2-(methylthio)thiophene. The data was obtained in 0.1 M deuterated phosphate buffer containing 5 mM 2-(methylthio)thiophene and 25%(V/V) *tert*-butanol in the (A) absence of and (B) presence of 0.1 mM CPO at pH 5.0.

versus the concentration of 2-(methylthio)thiophene, in which a straight line was obtained by the linear least-squares fitting of relaxation-experiment data. The correlation efficient of the fits was higher than 0.979, indicating a very accurate fit, while the fitted straight line is consistent with the 1:1 stoichiometry of 2-(methylthio)thiophene binding to CPO. Table 3.3 lists the relaxation time of 2-(methylthio)thiophene bound to CPO (T_{1b}) and the calculated dissociation constant (K_D). The average of the K_D values from all the protons was calculated to be 22 mM while was 25 mM from the protons of thiophene ring. This is nearly consistent with our previous report of indole bound to CPO at $K_D=21$ mM (Table 2.1) and that of CPDO at $K_D=33$ mM [79] obtained by relaxation experiments. Furthermore, the K_D value obtained in millimole- per-liter level suggests that the binding affinity between CPO and 2-(methylthio)thiophene is weak.

To deduce the orientation of 2-(methylthio)thiophene in the active site of CPO, T_{1b} of 2-(methylthio)thiophene was used to calculate the distance between the heme iron and the proton of 2-(methylthio)thiophene, as described in Section 3.2.4. Table 3.3 shows the dis-

Figure 3.10: Proton NMR spectra of 2-(methylthio)thiophene. The data was obtained in 0.1 M deuterated phosphate buffer containing 5 mM 2-(methylthio)thiophene and 25%(V/V) *tert*-butanol in the (A) absence of and (B) presence of 0.1 mM CPO at pH 5.0.

Table 3.3: The dissociation constant (K_D) of CPO-2-(methylthio)thiophene complex and the calculated distances (r) estimated between protons of 2-(methylthio)thiophene and the heme iron.

	3-H	4-H	5-H	methyl-H
T_{1b} (s)	5.58×10^{-3}	4.76×10^{-3}	5.02×10^{-3}	8.78×10^{-3}
K_D (mM)	23.1	28.8	24.2	11.9
r (Å)	8.7	8.4	8.5	9.4

tances between the protons of 2-(methylthio)thiophene and CPO calculated according to Equation 2.4. The distances reveal that the 3-proton and methyl protons are far from the heme iron compared with 4-, and 5- protons, indicating the active sulfur group is facing away from the heme iron. This is consistent with the distances found by Casella et al [81]. It should be note that the distances obtained here are inconsistent to derive one specific orientation fitting in the active site pocket, indicating the presence of multiple orientations. This also suggests a dynamic binding of 2-(methylthio)thiophene to CPO. The multiple orientations are also commonly found in substrate such as midazolam [146] and amidopyrine [145] binding to cytochrome P450. Since the NMR-derived distances usually give the

average of the multiple orientations of the substrates [174], the computer model is present to probe the preferable orientation and position of 2-(methylthio)thiophene in the active site of CPO.

3.3.5 Molecular docking model of CPO-sulfide complex

To understand the binding of sulfides to CPO and explore the origin of the enantioselectivity, a series of sulfides (Figure 3.11) including 2-(methylthio)thiophene, 2-(methylthio)pyridine, and 4-(methylthio)toluene, 1-(methylthio)-4-nitrobenzene, 1-methoxy-2-(methylthio)benzene, and (propylthio)benzene were docked in CPO's active site as described in Section 2.2.4. Substrates 2-(methylthio)thiophene, 2-(methylthio)pyridine, 4-(methylthio)toluene were reported to produce a high enantiomeric excess ($ee > 65\%$) and conversion yield ($> 60\%$) in CPO-catalyzed sulfoxidation. In the contrast, 1-(methylthio)-4-nitrobenzene, 1-methoxy-2-(methylthio)benzene, and (propylthio)benzene are poor substrates of CPO that have a lower ee ($< 30\%$) and conversion ($< 33\%$).

Figure 3.11: The structure of the docked sulfides. The 2-(methylthio)thiophene, 2-(methylthio)pyridine, and 4-(methylthio)toluene can be enantioselective oxidized using CPO as a catalyst. 1-(methylthio)-4-nitrobenzene and 1-methoxy-2-(methylthio)benzene has a low sulfoxidation yield in the CPO-catalyzed oxidation, while (propylthio)benzene has a very low enantioselectivity (27%) and poor product yield (3%).

3.3.5.1 The multiple binding orientations of sulfides in CPO-sulfide complex

The key issue here is to explore the position and orientation of the sulfides in the active site of CPO with the aid of the distances between the protons of sulfides and the heme iron from NMR experiments. Thus, substrates, 2-(methylthio)thiophene (see Table 3.3) and 2-(methylthio)pyridine [81], were docked in the active site of CPO (Figure 3.12). In docking of 2-(methylthio)thiophene, the conformations were ranked in two clusters with mean binding energies of -18.8 kJ/mol and -18.0 kJ/mol. As shown in Figure 3.12A, the lowest-energy docked 2-(methylthio)thiophene (-19.2 kJ/mol) binds in a productive orientation, i.e., the sulfur group locates near the heme center so that it can easily approach the oxyferryl ($\text{Fe}=\text{O}\cdot+$) intermediates, compound I, to be oxidized. Furthermore, 2-(methylthio)thiophene with this orientation can produce the corresponding R(+)-enantiomer of sulfoxide by displacing the oxygen from the heme iron. Unexpectedly, this orientation is inconsistent with the distances between the protons and the heme iron obtained in my relaxation experiments (Table 3.3), in which the 4-proton of 2-(methylthio)thiophene is closer to the heme iron than other protons. On the other hand, another docked 2-(methylthio)thiophene (-18.2 kJ/mol) that has its thiophene ring facing towards the heme is consistent in orientation with that of NMR experiments. However, in such orientation, the sulfur group is located far away to the heme iron and would be hardly oxidized by compound I in sulfoxidation. Moreover, the distances observed between the protons of the sulfide and the heme iron is much shorter than that of NMR results. Therefore, 2-(methylthio)thiophene should bind to CPO in multiple orientations, which explains both the experimental and theoretical results. This is also supported by the fact that the outcome of NMR experiments reflects an ensemble of multiple orientation of substrates in the active site [174]. The difference between NMR results and that of docking may be also attributed to the solvent effect, as the additional co-solvent was used in the relaxation experiments.

Figure 3.12: Ribbon and stick representations of the docking model of (A) 2-(methylthio)thiophene and (B) 2-(methylthio)pyridine in the active site of CPO. Three different orientations of 2-(methylthio)thiophene and 2-(methylthio)pyridine are shown. For the sake of clarity, the similar orientation of the docked substrates in the same cluster are all omitted. The lowest-energy docked substrates are colored green. The docked substrates with the orientation that is consistent with NMR experiments are colored blue. Heme is shown as red sticks. The side chain of Glu 183 is shown as yellow sticks.

For docking of 2-(methylthio)pyridine, all the conformations were ranked in two cluster with mean binding energies of -19.2 kJ/mol and -18.3 kJ/mol. These binding energies are comparable to that of 2-(methylthio)thiophene, indicating two substrates has similar binding affinity to CPO. The binding energies (in the magnitude) of these sulfides are lower than that of indole (Chapter 2), indicating that sulfides binding to CPO is also weak. This is consistent with the dissociation constant of CPO-2-(methylthio)thiophene complex (~22 mM) obtained from NMR experiments. As shown in Figure 3.12B, the orientation of lowest-energy docked 2-(methylthio)pyridine are productive that sulfur is located near the heme iron, whereas the orientation of a different docked 2-(methylthio)pyridine is consistent with that reported by NMR results [81]. This indicates multiple binding orientations of substrate in CPO-2-(methylthio)pyridine complex. Therefore, the multiple orientations of sulfides binding to CPO could be a general feature of CPO-sulfides complexes.

3.3.5.2 The substituent effects on sulfides binding to CPO

To understand the effect of substituents (electronic donating or withdrawing groups) on the sulfides binding to CPO, 2-(methylthio)toluene and 1-(methylthio)-4-nitrobenzne were

docked in the active site of CPO (Figure 3.13). As expected, these sulfides show multiple and distinct binding orientations. The sulfide 2-(methylthio)toluene binds to CPO in orientations that sulfur are located either far or close to the heme iron (Figure 3.13A). This is consistent with the orientations of 2-(methylthio)thiophene and 2-(methylthio)pyridine observed in previous docking (Figure 3.13). In the contrast, with the strong electronic-withdrawing nitro group on the benzene ring of 1-(methylthio)-4-nitrobenzne, all the orientations observed seem to be unproductive, i.e., sulfur are always far away to the heme center. Furthermore, the mean binding energies (in the magnitude) of 1-(methylthio)-4-nitrobenzne (-16.0, -16.4, -18.4, and -19.2 kJ/mol, 4 clusters) are much lower than that of 2-(methylthio)toluene (-20.1 and -21.7 kJ/mol). This indicates that the electronic withdrawing group is highly unfavorable for the sulfides binding to CPO. This also agrees with the previous report that electronic withdrawing group dramatically decrease the product conversion of CPO-catalyzed sulfoxidation [41].

Figure 3.13: Ribbon and stick representations of the docking model of (A) 2-(methylthio)-toluene and (B) 1-(methylthio)-4-nitrobenzne in the active site of CPO. The lowest-energy docked substrates are colored green.

3.3.5.3 The steric effects on the sulfides binding to CPO

To understand the steric effects on the sulfides binding to CPO, (propylthio)benzene and 1-methoxy-2-(methylthio)benzene were docked in CPO's active center. The sulfur group of (propylthio)benzene is far away from the heme iron in all the docked orientations

(Figure 3.14A). Therefore, the low optical and chemical yield of (propylthio)benzene [41] is attributed to the inaccessibility of sulfur to approach the heme center. This is also consistent with the finding (Chapter 2) that the approaching of 2-proton of indole to the heme iron is the prerequisite of the regioselective oxidation of indole catalyzed by CPO. It is clear that the long propyl group hinders the approaching of sulfur atom to compound I. This agrees with the report that the increase of aliphatic length of sulfides dramatically decreases the *ee*% and yield of sulfoxidation catalyzed by CPO [41].

Figure 3.14: Ribbon and stick representations of the docking model of (A) (propylthio)-benzene and (B) 1-methoxy-2-(methylthio)benzene in the active site of CPO. The lowest-energy docked substrates are colored green.

As shown in Figure 3.14B, 1-methoxy-2-(methylthio)benzene to CPO binds to CPO in multiple orientations that seem to rotate freely in the active site of CPO. Surprisingly, the orientation of the lowest-energy docked 1-methoxy-2-(methylthio)benzene is similar to that of 2-(methylthio)pyridine that has sulfur group located near the heme iron. Therefore, the low reaction yield of 1-methoxy-2-(methylthio)benzene may be attributed to the ortho-methoxy group that hinders the approaching of oxygen to sulfur group.

3.4 Conclusion

In this chapter, *cis*- β -methylstyrene and styrene were chosen to study CPO-catalyzed enantioselective epoxidation using NMR spectroscopy. The preliminary results indicates that the solubility of *cis*- β -methylstyrene and styrene is lower than 1 mM in water. To

enhance the solubility of *cis*- β -methylstyrene/styrene, a series of common solvents including hydrophobic solvents, hydrophilic solvents, and the surfactants were examined in the *cis*- β -methylstyrene-CPO system. The results showed that all the tested solvents were unsatisfied for the NMR experiment due to a limited solubility of *cis*- β -methylstyrene and styrene. Nevertheless, it was found that *tert*-butanol was a preferred co-solvent.

The hydrophilic styrene derivatives including 4-aminostyrene, 3-aminostyrene, styrene-4-carboxylic acid, and 4-styrene sulfonic acid were chosen as the alternative substrate for studying epoxidation. The solubility and the relaxation time (T_1) of these four compounds were measured using UV and NMR spectroscopy, respectively. Although all the compounds have a high solubility in water up to 100 mM, T_1 obtained cannot be used to fit to Equation 2.2. This is attributed to the fact that 4-aminostyrene and 3-aminostyrene were polymerized in acidic pH, while styrene-4-carboxylic acid and 4-styrene sulfonic acid were not the substrate of CPO.

It was found that CPO activity can maintain at a constant level when using *tert*-butanol as a co-solvent, while the solubility of 2-(methylthio)thiophene is at least up to 15 mM in 25% (V/V) *tert*-butanol. Thus, the structure of CPO-2-(methylthio)thiophene complex was investigated using paramagnetic relaxation in 25% (V/V) *tert*-butanol. The results showed that the sulfide binding to CPO is quite weak (~ 22 mM).

From the docking study, the multiple orientations of sulfides binding to CPO was confirmed. It was demonstrated that the attachment of a strong electronic-withdrawing substituent can dramatically change the binding orientation of sulfides. On the other side, the steric effects of substituent may hinder the approaching of the active sulfur to the oxygen atom of CPO compound I, leading to a low ee% and yield of sulfoxide. Therefore, the binding orientation of sulfides may induce the enantioselectivity in the way how easily oxygen can transfer to the sulfur group.

CHAPTER 4

**SPECTROSCOPIC INVESTIGATIONS OF THE MECHANISM OF
HALIDE-DEPENDENT DEGRADATION OF DYES CATALYZED BY
CHLOROPEROXIDASE**

4.1 Introduction

Dyes are substances that impart color to materials and are synthetically produced nowadays [202]. The applications of synthetic dyes are widespread in textile, paper, food, and other industries that cause environmental pollution and potential hazards to human health [202, 203, 204]. Unfortunately, as for daily use, these dyes are usually designed with high chemical and photolytic stability. That is, dyes are not readily degradable under mild condition. Moreover, azo dyes, characterized by the presence of azo bonds ($-N=N-$), are resistant to the microbial attack. This recalcitrance to biodegradation is attributed to the xenobiotic feature of azo bonds and sulphonate groups [205, 206]. In decades, azo dyes have been the largest class of synthetic dyes that constitute 60-80% of all organic colorants [202, 207]. To effectively degrade/decolorize dyes, especially azo dyes, much effort has been made on developing physical [202, 208], chemical [202, 208], and biological treatments [202, 208, 209]. However, the outcome of physical approaches such as adsorption is greatly affected by environmental conditions [210]. The chemical approaches such as fenton process [211, 212, 213] are also limited, for instance, the complete degradation of 47 μ M Direct blue 15 (or 50 μ M Mordant red 73) is achieved around 50 min. Alternatively, the use of enzyme is promising in terms of environmental friend and high efficiency [204, 214]. Many enzymes were reported to catalyze such degradation. Muralikrishna et al. reported phenolic azo dye oxidation by Laccase from *Pyricularia oryzae* [215]; Ollikka, et al. reported oxidation of crocein orange G by lignin peroxidase isoenzymes [216]; Lopez et al. reported oxidative degradation of azo dyes by manganese peroxidase [217]. Recently, Jiang's group reported a high efficient decolorization of azo dyes, orange G and

Sunset yellow, using a small concentration of chloroperoxidase (CPO) [218]. However, the mechanism of CPO-catalyzed decolorization is not clear.

Chloroperoxidase (CPO), a heme protein secreted by the marine fungus *Caldariomyces fumago* [23], is one of the most versatile biocatalyst that catalyzes halogenation [23] in the presence of halide ion (except fluoride) and oxidation of a variety of substrates such as sulfides [41, 197], alcohols [34], and arylamine [130]. This enzyme can be highly produced up to 1-1.5 g/liter using Blanke's method [111]. Furthermore, the pure CPO is stable for weeks at room temperature [109], and CPO is significantly robust that can function under harsher reaction conditions than other peroxidases [39]. Therefore, CPO has a great potential for applications such as decolorization. Tremendous efforts have been made during the past few decades to understand the mechanism of CPO-catalyzed reactions [66, 168]. In particular, CPO-catalyzed reactions in the absence and presence of halide proceed with distinct mechanism. For instance, the oxidation of sulfides is enantioselective in the presence of CPO and H_2O_2 , whereas in the presence of chloride, this enantioselectivity is diminished [219]. In the presence of halide, the pathway for CPO-catalyzed halogenations is proposed to involve a ferric hypohalite intermediate known as Compound X [64, 66, 70]. However, the mechanism of CPO-catalyzed halogenation is still under investigation.

The aim of this chapter is to explore the mechanism of CPO-catalyzed biodegradation of synthetic dyes, especially azo dyes, using hydrogen peroxide (H_2O_2) in the presence of halide ion. A variety of dyes including orange G, methyl orange, nuclear fast red, gentian violet, and azure B were examined (Figure 4.1). The decolorization conditions regarding pH, chloride/bromide concentrations, and H_2O_2 concentrations were investigated. It was found that the presence of chloride/bromide ion significantly enhanced the decolorization rate and efficiency of dyes, indicating the involvement of a hypohalite intermediates ($Fe-OX^-$) during decolorization. In addition, the degradation products of orange G in the CPO- H_2O_2 - Cl^- system were clearly assigned by NMR spectroscopy. This work provides, for

the first time, the experimental evidence for the important role of halide in degradation of dyes catalyzed by CPO, and will shed light on designing the novel biochemical degradation system.

Figure 4.1: The structure of synthetic dyes. The chemical shifts (/ppm) of orange G at pH 2.7 is shown in blue number.

4.2 Material and Method

4.2.1 Enzyme and chemicals

Caldariomyces fumago (ATCC number: 16373) was purchased from ATCC (Manassas, VA). CPO was isolated and purified according to published protocols [147, 148] with slight modifications. CPO with Reinheitszahl value (R_z , $A_{398} \text{ nm}/A_{278} \text{ nm}$) of 1.3 or higher was used in all experiments. Horseradish peroxidase (HRP) was purchased from Sigma-Aldrich (St. Louis, MO) as lyophilized powder ($\sim 150 \text{ U/mg}$). The concentration of CPO was determined by measuring the absorbance at 398 nm using a molar extinction coefficient of $91,200 \text{ M}^{-1}\text{cm}^{-1}$ [243], while the concentration of HRP was determined at 402 nm using a molar extinction coefficient of $102,000 \text{ M}^{-1}\text{cm}^{-1}$ [220].

Orange G, methyl orange, and nuclear fast red were purchased from Fisher (Pittsburgh, PA) with biological stain grade. Azure B and gentian violet were obtained from MP Biomedicals (Solon, OH) and were of biological stain grade. Hydrogen peroxide (30 wt.

% in H₂O) and hypochlorite (reagent grade, chlorine 10-15%) was obtained from Sigma-Aldrich (St. Louis, MO). Unless otherwise specified, all the chemicals were of analytical grade and were purchased from Sigma-Aldrich (St. Louis, MO). Millipore Milli-Q water (18.2 MΩ) was used throughout.

4.2.2 UV-Vis spectroscopic measurement of decolorization of synthetic dyes

To investigate the decolorization process of synthetic dyes, UV-Vis spectra were recorded on CaryBio-300 UV-Vis spectrometer. The decolorization efficiency of dyes was calculated using Equation 4.1, where A_0 is the absorbance of the dye monitored at the wavelength of maximum absorbance (λ_{max}) before addition of H₂O₂, and A_t is the absorbance that reaches a stable value after addition of H₂O₂.

$$Efficiency(\%) = \frac{A_0 - A_t}{A_0} \times 100 \quad (4.1)$$

To determine the reaction rate of decolorization of orange G, the absorbance at 477 nm (λ_{max}) was continually monitored by CaryBio-300 UV-Vis spectrometer. The decolorization rate was calculated according to Equation 4.2, where A_0 is the absorbance monitored at time t_0 , A_t is the absorbance monitored at time t_t , and 20.7 (mM⁻¹cm⁻¹) is the reported extinction coefficient of orange G [221].

$$Rate(\mu/s) = \frac{A_0 - A_t}{20.7 \times (t_0 - t_t)} \times 1000 \quad (4.2)$$

Kinetic assay for decolorization of orange G was carried out in the concentration range from 0.01 to 0.40 mM. I assumed the decolorization reaction follows the Michaelis-Menten kinetics. The values of kinetic parameters, Michaelis constant (K_m) and maximum rate (V_{max}), were obtained by linear regression analysis of the double reciprocal Lineweaver-Burk plots, according to Equation 4.3, where S is the concentration of the substrate orange G, V is the initial reaction rate measured in the linear range of decolorization in 5-10 s.

$$\frac{1}{V} = \frac{K_m}{V_{max}} \cdot \frac{1}{[S]} + \frac{1}{V_{max}} \quad (4.3)$$

The turnover number, k_{cat} , was calculated according to Equation 4.4, where E_0 is the concentration of the enzyme CPO.

$$V_{max} = k_{cat}[E_0] \quad (4.4)$$

4.2.3 NMR spectroscopic investigations of degradation of orange G

The NMR experiments were carried out on a Bruker 600 MHz NMR spectrometer operated with TopSpin software, version 2.1. All spectra were recorded at 300 K and two-dimensional data were processed using NMRPipe [222]. The COSY experiment was performed using the standard Bruker pulse sequence (cosyprqf) with presaturation during the relaxation delay of 2 s on the HOD signal, 4096 data points in the F2 dimension, and 512 increments in F1. The data matrix was processed to give a matrix of 2048×512 points, and a sine bell apodization function was applied before Fourier transformation.

4.3 Results and Discussion

4.3.1 UV spectra of decolorization in the CPO-H₂O₂-Cl⁻ system

To investigate decolorization of dyes in the CPO-H₂O₂-Cl⁻ system, UV-vis spectra of orange G (0.01 mM) were recorded in the presence of 10 nM CPO and 20 mM chloride ion (Cl⁻) at pH 2.7. Figure 4.2A illustrates the typical UV-Vis spectra of orange G during the decolorization process. Before addition of hydrogen peroxide (H₂O₂), the spectrum of orange G showed a strong absorption at 477 nm (λ_{max}) with a shoulder at 419 nm (Figure 4.2A). These absorption bands originate from the azo bond (-N=N-) of orange G that undergoes azo-hydrazone tautomerism [223]. Additionally, the weak absorption at 329 nm (Figure 4.2A) is ascribed to the $\pi \rightarrow \pi^*$ transition in the naphthalene structure [224]. Five minutes after addition of H₂O₂, the absorption at 477 nm significantly decreased (Figure 4.2A), indicating the cleavage of azo bond of orange G in the CPO-H₂O₂-Cl⁻ system. Furthermore, the spectrum after addition of H₂O₂ exhibited the same absorption band as the spectrum of orange G with addition of hypochlorous acid (HClO) (Figure 4.2A). The similarity of the spectra indicates the degradation products of orange G in the CPO-H₂O₂-

Cl^- system are essentially the same as that in the HClO system. This is consistent with the reports that the products of CPO-catalyzed chlorination reactions are the same as those produced by the corresponding HClO reactions [225, 226, 227]. More importantly, this suggests the mechanism of degradation of azo dyes is related to CPO-catalyzed chlorination reactions that involve a ferric hypochlorite-adduct intermediate (Fe-OX), compound X [66, 70]. It is worth mentioning that decolorization of orange G is negligible in the presence of CPO but absence of Cl^- (Figure 4.2A). This clearly indicates that decolorization of dyes is affected by the presence of Cl^- .

Figure 4.2: UV-Vis spectra during decolorization of orange G (0.01 mM) at pH 2.7. (A) The red solid line represents the spectrum of orange G; the gray solid line represents the spectrum of orange G in the absence of Cl^- with addition of a final concentration of 0.5 mM H_2O_2 ; the green dash line represents the spectrum of orange G in the presence of 20 mM Cl^- with addition of a final concentration of 0.5 mM H_2O_2 ; and the violet dash line represents the spectrum of orange G with addition of a final concentration of 0.5 mM HClO . All the spectra were obtained in 50 mM phosphate buffer (pH 2.7) containing 0.01 μM CPO. (B) The changes of absorbance at 477 nm after addition of 0.5 mM H_2O_2 . Reaction conditions are: 0.01 mM orange G, 0.01 μM CPO, 20 mM Cl^- , and a final concentration of 0.5 mM H_2O_2 in 50 mM phosphate buffer at pH 2.7.

Figure 4.2B shows the change of the absorbance of orange G (0.01 mM) at 477 nm during the time. After addition of H_2O_2 . The absorbance of orange G decreased continually and reached a constant value in 70 s (Figure 4.2B). This short time indicates that decolorization of orange G is efficient and fast in the CPO- H_2O_2 - Cl^- system. Moreover, using Equation 4.2, the reaction rate of decolorization was calculated to be 0.21 $\mu\text{M/s}$ in first 10

Table 4.1: Decolorization efficiencies of the synthetic dyes in the CPO-H₂O₂-Cl⁻ system

Dyes	λ_{max} (nm)	Times (s)	Efficiency ^{a,b}
Orange G	477	<80	99.3±1.2%
Methyl orange	506	<10	98.1±5.7%
Gentian violet	593	<40	96.5±0.8%
Nuclear fast red	518	<5	96.7±3.1%
Azure B	648	<180	70.4±2.2%

^aThe decolorization time and efficiency of dyes were evaluated from the visible absorbance at λ_{max} .

^bReaction condition: 50 mM phosphate buffer (pH=2.7) containing 0.01 mM synthetic dyes, 0.01 μ M CPO, and 20 mM Cl⁻, with addition of H₂O₂ (to a final concentration of 0.5 mM), T=20°C.

s, 0.30 μ M/s in 10-30 s, and 0.04 μ M/s in 30-70 s. The decrease of the absorbance was linear in a short time (10-30 s). Thus, the reaction rate was calculated within such linear ranges and discussed below unless otherwise specified.

Table 4.1 lists the decolorization efficiency of a variety of synthetic dyes including orange G, methyl orange, nuclear fast red, gentian violet, and azure B in the CPO-H₂O₂-Cl⁻ system. The decolorization for these dyes (0.01 mM) proceed with high efficiencies of 70.4-99% in a short time (<180 s). This result demonstrates that the CPO-H₂O₂-Cl⁻ system is very efficient and fast for the decolorization of the tested dyes. This also suggests a great potential for utilization of the CPO-H₂O₂-Cl⁻ system in biodegradation of synthetic dyes. Additionally, in a control experiment, decolorization of these dyes was not observed in the absence of enzyme CPO.

4.3.2 Effect of chloride and bromide on decolorization of orange G

To examine the effect of chloride (Cl⁻) and bromide (Br⁻) on decolorization of azo dyes, the reaction rate of decolorization of orange G were measured in the presence of Cl⁻ or Br⁻ (0-100 mM). Figure 3A shows the dependence of the reaction rate of decolorization of orange G on the concentration of Cl⁻/Br⁻. As the concentration of Cl⁻ increased from 0 to 20 mM, the decolorization rate increased 400-fold from 0.00075 to 0.30 μ M/s (Figure 4.3A). This agrees with my finding that the decolorization efficiency of orange G is merely 6% for the CPO-H₂O₂ system in the absence of Cl⁻ (Figure 4.2A). This also suggests that Cl⁻

(or Br^-) participates in the decolorization reactions presumably by forming an intermediate, compound X, with CPO. Furthermore, the participation of Cl^- (or Br^-) is also seen in myeloperoxidase (MPO) that catalyzes the formation of hypochlorous acid (HClO) in the presence of Cl^- and H_2O_2 [13, 228, 229]. Therefore, the decolorization of orange G is most likely caused by the formation of an enzyme-bound HClO in the CPO- H_2O_2 - Cl^- system. In addition, the decolorization rate gradually decreased down to $\sim 30\%$ of the maximum, as the concentration of chloride was increased from 20 to 100 mM, (Figure 4.3A). This may be attributed to that HClO reacts with excess of Cl^- forming molecular chlorine (Cl_2) that competes with decolorization of orange G [53].

Figure 4.3: The effect of chloride and bromide on decolorization of orange G. (A) The effect of the concentration of chloride (rectangular, Cl^-) and bromide (triangular, Br^-) on the decolorization rate of orange G (10 μM). (B) the reaction rate of orange G (10-100 μM) in the presence of 20 mM chloride (rectangular, Cl^-) or bromide (triangular, Br^-). The data with uncertainty (95%) were obtained from three repetitive experiments in 50 mM phosphate buffer (pH 2.7) containing 0.01 μM CPO, 20 mM Cl^-/Br^- , and with addition of a final concentration of 0.5 mM H_2O_2 .

Table 4.2 lists the Michaelis constant (K_m) and the turnover number (k_{cat}) obtained in the presence of Cl^-/Br^- . These values are consistent in magnitude with those reported by Zhang et al. [218]. It was found that the K_m value obtained in the presence of Br^- is higher than that of Cl^- (Table 4.2). On the other side, the k_{cat} value obtained in the presence of Cl^- is higher than that of Br^- . The overall reaction rates of decolorization of orange G in the presence of Br^- are much higher than that of Cl^- (Figure 4.3B), suggesting that HBrO

Table 4.2: The kinetic parameters of decolorization of orange G.^a

	K_m (μM)	V_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu\text{M}^{-1}\text{S}^{-1}$)	R^2
Cl^-	174	9.12	911	5.24	0.989
Br^-	131	9.12	911	5.24	0.989

^aThe parameters were obtained in 50 mM phosphate buffer (pH 2.7) containing 0.01-0.1 mM orange G, 10 μM CPO, and 20 mM Cl^-/Br^- by addition of H_2O_2 (to a final concentration of 0.5 mM).

is produced more efficient than HClO . The high decolorization rate in the presence of Br^- is consistent with the relative rates of CPO-catalyzed halogenation, $\text{Br}^- > \text{Cl}^-$ [28], which were rationalized by the relative rates of the nucleophilic attack of halide ion on compound I [53]. Moreover, this high rate could also be attributed to that the dissociation constant of CPO-bromide complex is higher than that of CPO-chloride complex [100]. Although the chloride binding site was not found in the crystal structure of CPO [25], the bromide binding sites were identified in the crystal structure at the active site of CPO [26], and Cl^- was reported as a competitive inhibitor for cyanide binding to CPO [230]. Thus, the latter explanation seems more likely to me. Therefore, this work provides the evidence that halide binding to CPO is an essential step in decolorization of azo dyes. In addition, The decolorization of orange G in the presence of iodide (I^-) were complicated and thus not discussed herein, because I^- itself is a strong reductive reagent that consumes H_2O_2 [28].

4.3.3 Effect of pH on the decolorization of orange G

To probe pH effect on the decolorization of azo dyes, the decolorization rate and efficiency were determined in the pH range of 2.7-5.0. In the presence of 20 mM Cl^- , the decolorization rate gradually decreased as pH increased from 2.7 to 5.0 (Figure 4.4A). The dependence of the decolorization rate on pH could be attributed to the varying of binding affinity of CPO- Cl^- complex at different pH [100]. This pH dependence also indicates that the protonation of the enzyme-activated ClO^- (to HClO), compound X, is a prerequisite of the decolorization of orange G. This notion is supported by the report that protonated iron porphyrin- HClO complex catalyzed the chlorination of monochlorodimedone, whereas the unprotonated ClO^- complex was unreactive towards monochlorodimedone [65]. Further-

more, Glu 183, a postulated acid-base catalyst in compound I formation [72, 170], is the only polar group close to the heme iron, and may have a low $pK_a < 4.5$. Thus, the low pH blocks the proton delivery from HClO ($pK_a=7.5$ [231]) to Glu 183 so that it maintains the protonation of HClO.

Figure 4.4: pH dependence of decolorization of orange G (0.01 mM). (A) The decolorization rate of 0.01 mM orange G in the presence of 20 mM Cl⁻ (rectangular) and 100 mM Cl⁻ (triangular) at pH range of 2.7-5.0. (B) The decolorization efficiency of 0.01 mM orange G in the presence of 20 mM Cl⁻ at pH range of 2.7-5.0. The data with uncertainty (95%) were obtained from three repetitive experiments in 50 mM phosphate buffer (pH 2.7-5.0) containing 0.01 μM CPO, 20/100 mM Cl⁻, and with addition of a final concentration of 0.5 mM H₂O₂.

It is found that the decolorization is best performed with an optimum pH of 2.7 in the presence of 20 mM Cl⁻ (Figure 4.4A). This optimum pH is generally consistent with that found in CPO-catalyzed halogenation [31], indicating the decolorization mechanism share the same feature of CPO-catalyzed halogenation such as the presence of compound X. It should be also noted that CPO is completely denatured at pH lower than 2.0 [232]. In the contrast, the optimum pH of the decolorization rate increased to pH 3.0 in the presence of 100 mM Cl⁻. This shift of optimum pH is also seen in myeloperoxidase, where optimal pH for chlorination is dependent on the [H₂O₂]/[Cl⁻] ratio [228, 233, 234].

The decolorization efficiency was decreased at pH 5.0 (Figure 4.4B), where the decolorization took approximately an hours to complete. This low efficiency is consistent with the decreasing of the decolorization rate at high pH (Figure 4.4A). The decolorization effi-

ciency was still relatively high at pH 4.0, as the decolorization took shorter time (~10 min) to complete. Therefore, the recommended reaction pH is at pH 2.7-5.

4.3.4 Effect of hydrogen peroxide on decolorization of orange G

To examine the effect of H_2O_2 on the decolorization of azo dyes, the decolorization rate of orange G (0.01 mM) was determined in the presence of CPO (0.01 μM) and Cl^- with the addition of H_2O_2 (0.1-5.0 mM). As expected, the decolorization rate was greatly enhanced, as the concentration of H_2O_2 was increased up to 0.5 mM (Figure 4.5). However, further increasing the H_2O_2 concentration from 0.5 to 5.0 mM decreases the decolorization rate. This could be attributed to that the excess of H_2O_2 compete with Cl^- to react with compound I [235, 236]. It is also possible that CPO is inactivated at the high concentration of H_2O_2 due to the formation of a highly reactive iron peroxy species (compound III) that leads to heme degradation [237]. Nevertheless, CPO is still a stable enzyme in the presence of H_2O_2 , as the half-life time of CPO is reported at least five-fold higher than that of other peroxidases such horseradish peroxidase (HRP), lignin peroxidase, and lactoperoxidase [238]. Thus, the H_2O_2 concentration is crucial to the optimization of CPO-catalyzed degradation.

4.3.5 The comparison of CPO- H_2O_2 - Cl^- with HRP- H_2O_2 - Cl^- system

Horseradish peroxidase (HRP) is a heme-containing enzyme that catalyzes peroxide-dependent reactions in a catalytic cycle similar to CPO [239]. To understand the mechanism of decolorization, HRP (1 μM) were used for the decolorization of orange G (0.01 mM) in the presence of H_2O_2 and Cl^- at pH 2.7. Remarkably, the decolorization of orange G was observed in the HRP- H_2O_2 - Cl^- system (Figure 4.6), although not to the same extent of CPO- H_2O_2 - Cl^- system. This indicates the decolorization mechanism is directly related to the presence of compound I. The low decolorization efficiency may be attributed to the fact that HRP is irreversibly denatured at acidic condition (pH 2.7) [240], leading to an incomplete decolorization. It is also worth noting that the active-site structure of CPO

Figure 4.5: The effect of H_2O_2 on the decolorization rates of orange G. Reaction conditions are: 50 mM phosphate buffer (pH 2.7) containing 0.01 mM orange G, 0.01 μM CPO, and 20 mM (rectangular) or 100 mM (triangular) Cl^- with addition of various (final) concentrations of H_2O_2 .

and HRP are different [25, 69, 241]. In the absence of Cl^- , the decolorization of orange G in the HRP- H_2O_2 system was not observed (Figure 4.6). This is consistent with my finding that the presence of Cl^- is necessary for CPO-catalyzed decolorization of orange G (Figure 4.3A).

4.3.6 NMR spectra of orange G in CPO-catalyzed decolorization

To identify the degradation products of azo dyes, proton NMR spectra of orange G (1.0 mM) were obtained in the presence of CPO. Before addition of H_2O_2 , seven main peaks were observed in the spectrum (Figure 4.7A) and are assigned, based on their splitting pattern and shifts position, to the protons of orange G (see Figure 4.1). With addition of H_2O_2 , all the main peaks were shifted due to the cleavage of the azo bond of orange G (Figure 4.7B). More importantly, only one cleavage of azo bond is predominate, as numbers of peaks shown in the spectrum. The cleavage could be at the position between naphthalene and azo bond. This cleavage is also seen in degradation of orange G by hypochlorite [242]. Six hours after the initial addition of H_2O_2 , three additional peaks appeared at 6.92, 6.98,

Figure 4.6: UV-vis spectra of horseradish peroxidase-catalyzed decolorization at pH 2.7. The red solid line represents the spectrum of 12 nM orange G obtained in 50 mM phosphate buffer (pH 2.7) containing 1 μ M HRP and 20 mM CeCl_3 ; the blue dash line represents the spectrum obtained in 50 mM phosphate buffer (pH 2.7) containing 12 nM orange G, 1 μ M HRP, and 20 mM Cl^- with addition of a final concentration of 0.5 mM H_2O_2 ; and the gray solid line represents the spectrum obtained in 50 mM phosphate buffer (pH 2.7) containing 12 nM orange G and 1 μ M HRP in the absence of Cl^- with addition of a final concentration of 0.5 mM H_2O_2 . The increase of absorbance at 398 nm shown on gray line is attributed to the presence of compound I.

and 7.31 ppm (Figure 4.7C) that are assigned to the protons of phenol. This assignment was further confirmed by comparison with NMR spectrum of phenol at pH 2.7.

To further clarify the peaks assignment, the two-dimensional ^1H - ^1H NMR correlation spectrum of orange G were also recorded in the $\text{CPO-H}_2\text{O}_2\text{-Cl}^-$ system (Figure 4.8). Specifically, three peaks at 7.95, 8.28, and 8.56 ppm are strongly correlated, indicating that the corresponding protons are on the same aromatic ring. These peaks are assigned to a diazonium structure that is probably the precursor of phenol. The presence of diazonium explains a transient absorbance observed at 397 nm during the decolorization of orange G. Two peaks at 6.63 and 7.88 ppm are assigned to 5- and 6- protons of dioxonaphthalene based on their correlation and the splitting pattern. In addition, the halogenated compounds such as 4-chlorophenol were not found in the $\text{CPO-H}_2\text{O}_2\text{-Cl}^-$ system, as shown by the main peaks on the NMR spectra. This is consistent with the report of degradation

Figure 4.7: NMR proton spectra of orange G (1.0 mM) during decolorization. The NMR sample of orange G (1.0 mM) was prepared in 50 mM deuterated phosphate buffer containing 2.0 μM CPO and 20 mM Cl^- at pH 2.7 (A) without and (B) with addition of a final concentration of 4 mM H_2O_2 . (C) The same tested solution of (B) was measured at 6 hours after the initial addition of H_2O_2 . Herein, DCl solution (1.0 M) prepared dilution of DCl (35% wt.% in D_2O) with D_2O was used to adjust pH. To obtain a clear NMR spectrum, H_2O_2 solution (0.1 M) prepared by dilution of H_2O_2 (35% wt. % in H_2O) in D_2O was used to initialize reactions, and CPO stock solution (final 1.0 μM) was added before and after additional of H_2O_2 to better complete degradation.

products determined by liquid chromatography-mass spectrometry (LC-MS) [218]. The non-halogenated degradation may be attributed that CPO is also a dehalogenating enzyme in the presence of H_2O_2 and Cl^- [39]. The absence of halogenation reactions is desirable and indicates a great potential of utilization of the CPO- H_2O_2 - Cl^- system, as the toxicity of chlorophenol may constitute an additional hazard [243, 244].

4.3.7 Mechanism of CPO-catalyzed degradation of orange G

On the basis of NMR and UV-Vis spectroscopic study, a mechanism of CPO-catalyzed degradation of orange G is proposed (Figure 4.9). As for all CPO-catalyzed reactions, the ferric state of CPO (I) reacts with a equivalent of H_2O_2 to produce an oxoferryl π -cation

Figure 4.8: Two-dimensional ^1H - ^1H NMR correlation spectroscopy of orange G in decolorization. Two-dimensional COSY of orange G (1.0 mM) was obtained in 50 mM deuterated phosphate buffer (pH 2.7) containing $2.0\ \mu\text{M}$ CPO and 20 mM Cl^- with addition of 4 mM H_2O_2 . The data were plotted by NMRPipe.

radical intermediate ($\text{Fe}=\text{O}\cdot+$, II), compound I [94, 245]. Compound I subsequently captures Cl^- or Br^- that binds to CPO [169], and forms a hypochlorite adduct ($\text{Fe}-\text{OX}^-$, III), compound X [70]. The different binding affinity of Cl^- or Br^- to CPO may account for the distinct decolorization rate of orange G in the presence of Cl^-/Br^- (Figure 4.3). In the acid environment, compound X is protonated to form hypochlorous/hypobromous acid (HClO/HBrO). The hypothesis of the protonation of compound X is supported by my pH experiments in this work. Furthermore, HClO/HBrO is likely trapped in the active-site pocket of CPO, as several experimental results suggest that CPO chlorinating intermediate cannot escape from the active-site channel [168, 227]. Consequently, the trapped HClO/HBrO forms chlorinium/bromonium ion (Cl^+/Br^+) that breaks the azo bond of or-

ange G, meanwhile the initial ferric resting state of CPO is regenerated. The formation of Cl^+ from HClO has been demonstrated in free solution [246, 247] and in myeloperoxidase-catalyzed reactions [248]. my result clearly indicates the presence of Cl^-/Br^- is essential for CPO-catalyzed decolorization. Therefore, the decolorization of orange G involves compound X and relative CPO intermediates.

(A)

(B)

Figure 4.9: Proposed mechanism pathway of orange G degradation.

The cleavage of orange G occurs between the naphthalene ring and azo bond to produce 7,8-dioxonaphthalene-1,3-disulfonic acid and benzenediazonium chloride (V). The existence of these products was initially reported by Zhang et al. [218], and was further confirmed by my NMR experiment. Significantly, we found that the decolorization is

highly regioselective that only one type of cleavage could be occurred. This regioselectivity was also reported in the study of degradation of azo dyes by hypochlorite [242, 249]. According to my NMR spectra, by releasing nitrogen gas, the diazonium chloride (V) is decomposed to phenol that is oxidized in air to quinone. These results provide, for the first time, a clear picture of degradation product in post-decolorization process catalyzed by CPO.

4.4 Conclusion

This work demonstrate that in addition to previous reported azo dye, orange G and sunset yellow [218], a variety of dyes including methyl orange, nuclear fast red, gentian violet, and azure B can be decolorized efficiently in the CPO-H₂O₂-Cl⁻ system. The optimum of CPO-catalyzed decolorization is governed by both the chloride/bromide concentration and pH. Contrary to the presumed oxidative mechanism [218], this work clearly indicates degradation of azo dyes is related to CPO-catalyzed halogenation reactions that involve a ferric hypochlorite-adduct intermediate (Fe-OX), compound X. My NMR result reveals that the decolorization of orange G is regioselective that the cleavage is preferably at naphthalene carbon-nitrogen bond.

CHAPTER 5

CONCLUSION

This dissertation describes the characterization of the structure of chloroperoxidase (CPO)-indole complex and CPO-2-(methylthio)thiophene complex using both NMR and computational techniques to understand the mechanism of CPO-catalyzed regio- and enantioselective transformations. The orientations of bound substrates in the heme crevice were defined, providing, for the first time, the experimental and theoretical explanation of the stereoselectivity displayed by CPO. It has been demonstrated that formation of CPO-indole complex is responsible for the escorted delivery of oxygen from CPO compound I directly to the 2-position of indole, leading to the regioselective oxidation of indole. Moreover, the dissociation constants of CPO-indole complex and CPO-2-(methylthio)thiophene complex were found approximately 20 and 22 mM, respectively. It has also been revealed that the van der Waals interactions are much stronger than the Coulomb interactions in indole binding to CPO, indicating that the association of CPO-substrate complex is primarily governed by hydrophobic rather than electrostatic interactions.

My results has provided important information regarding the topology of the heme active center of CPO, the residues involved in the catalytic cycle, and the location/orientation of substrate binding. Such information will serve as a lighthouse for designing more efficient CPO mutants that target specific substrates and produce desired products that are difficult to obtain under mild conditions. In docking a series of sulfides at the active site of CPO, the multiple orientations of sulfides binding to CPO were illustrated. It has been revealed that, in CPO-catalyzed enantioselective sulfoxidation, the enantiomeric excess (ee%) and chemical yield of suloxide is closely related to the accessibility of the sulfur group to the heme center. In particular, the steric effects may hinder the approaching of oxygen from compound I to the active sulfur, resulting in the decrease of ee% and yield. These results should help elucidate mechanisms of CPO-catalyzed enantioselective reactions.

This dissertation also represents the first study on the mechanism of biodegradation of synthetic dyes catalyzed by CPO. It has been demonstrated that a variety of dyes can be degraded/decolorized efficiently in the CPO-H₂O₂-Cl⁻ system. Furthermore, the optimum of CPO-catalyzed decolorization is governed by both the presence of chloride/bromide and pH. My results, for the first time, revealed that this biodegradation of azo dyes involves a ferric hypohalite intermediate (Fe-OX), compound X. Additionally, using NMR spectroscopy, the degradation products has been clearly assigned, revealing that the decolorization of orange G is also a regioselective transformation. This work provides a starting point for unraveling the mechanism of CPO-catalyzed biodegradation of synthetic dyes and will shed light on designing the novel biochemical degradation system.

Although the mechanism of regioselective oxidation of indole has been proposed in this dissertation, the questions such as the types of active intermediates remain to be answered. For instance, it is not clear whether the indole radical intermediate, whose presence is important to determine whether the oxidation is undergoing a direct oxygen insertion or two consecutive electronic transfers, is present in oxidation. This intermediate, hopefully, can be identified using EPR by trapping it at cryogenic temperature. Another mystery remained is what structural features of CPO determines the enantioselectivity in oxidation. This mystery may be solved by molecular dynamic simulation of the CPO-substrate complex with the input from NMR relaxation experiments. This work will be the future direction to continue this research. For understanding regioselective biodegradation of dyes, it is also worth to evaluate the cleavage of azo bond using quantum mechanic methods.

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Rui Zhang and Xiaotang Wang, (2012) Spectroscopic investigations of the mechanism of halide-dependent biodegradation of azo dyes catalyzed by chloroperoxidase. submitted to *PLOS One*.

Rui Zhang, David Chatfield, and Xiaotang Wang, (2012) Structural Basis of the Regio- and Enantio-selective Oxidations Catalyzed by Chloroperoxidase. *Abstracts of papers, 54th ENC conference, Pacific Grove, LA*.

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