ABSTRACT

BARRIOS, DAVID ARLEN. Probing the Mechanism and Reactivity of Biomacromolecular and Small Molecule Catalytic Systems with H₂O₂ and O₂. (Under the direction of Dr. Reza A. Ghiladi).

Dehaloperoxidase (DHP) provides both a biologically-relevant globin and peroxidase function for the marine terebellid polychaete, *Amphitrite ornata*. The peroxidase activity imparts a necessary detoxification pathway to combat biogenically synthesized toxins in the benthic ecosystems inhabited by *A. ornata*. Previous studies have shown that halogenated phenols are oxidized utilizing this peroxidase mechanism. In these studies, we explore a novel peroxygenase reactivity for DHP with hydrogen peroxide in the presence of a proposed native substrate, halogenated indoles. The existence of the peroxygenase pathway was confirmed using labeled oxygen studies and analysis by LC-MS. Characterization of reactive intermediates were performed using stopped-flow UV-visible spectroscopy and resonance Raman. Further, the reactivity of DHP with a non-native substrate, nitrophenol, which also exhibits peroxygenase activity, is explored using labeled oxygen studies and LC-MS as well as stopped-flow UV-visible spectroscopy. Additionally, the reactivity of DHP and H₂O₂ with the native substrate 2,4-dichlorophenol is explored and was discovered to exhibit both peroxidase and peroxygenase activity as well as an independent dehalogenation step. Lastly, the reactivity of various iridium complexes with substituted dibenzyl ethers was explored under O₂ in dimethyl sulfoxide.
Probing the Mechanism and Reactivity of Biomacromolecular and Small Molecule Catalytic Systems with H₂O₂ and O₂

by
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A dissertation submitted to the Graduate Faculty of North Carolina State University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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DEDICATION

I dedicate this thesis and the accomplishment of this degree to my family and friends. Each has played a positive role and provided the love and support necessary for this endeavor to be successful.
BIOGRAPHY

On October 10, 1974, David A. Barrios was born to Sandra and Rito Barrios. Upon their divorce, he was cooperatively raised by both parents and his step-mother, Elena Barrios. He graduated from Langham Creek High School in Houston, Texas in 1993. After serving in the United States Navy, David began a career in Information Technology until he found himself unemployed in 2001. After the death of a close cousin, Arthur L. Barrios, David was inspired to attend college and was subsequently accepted to the University of Illinois at Urbana-Champaign. After a rather mediocre performance in general chemistry, he was encouraged to remain in the advanced chemistry curriculum by Gretchen Adams of the U of I, and discovered success in organic chemistry the following year. She would also recommend David for the first of his two summer research internships funded by the Howard Hughes Medical Institute, which he performed under the guidance of Dr. Yi Lu.

Under Dr. Yi Lu and Dr. Dewain Garner, David worked on work involving the incorporation of non-native organometallic cofactors into apo-myoglobin. His work was credited with two coauthorships. It was during this undergraduate research experience that David realized he wanted to continue to explore research and applied to graduate school. Upon being accepted to North Carolina State University, he was immediately drawn to the research being performed by Dr. Reza Ghiladi.
ACKNOWLEDGMENTS

As with many accomplishments in life, an individual is rarely successful without assistance and support from family, friends, and coworkers. First, I would extend my gratitude to Dr. Reza Ghiladi for his leadership and temperament. Reza fosters an environment of exploration and learning that is very individual for each graduate student. He provided the freedom to explore research avenues, but also provided the structure necessary to succeed and remain focused. He does this by being a resource for knowledge, and even when he did not know the answer, he was wise enough to steer me in the right direction to acquire it. I would also like to thank Dr. Elon Ison for his help and expertise. He possesses a wisdom beyond his years and will continue to be successful in his research endeavors. His dedication and passion to our science is infectious and serves as an inspiration to everyone who collaborates with or works for him. I would also like to thank the past and current members of the Ghiladi group: Drs. Elke Feese, Rania Dumarieh, and Jennifer D’Antonio; and Brad Carpenter and Xingci Situ for the help and assistance over the years and making the years pleasant at NC State. Further, I would like to thank the members of the Ison group for their help, support, and friendship as well.

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Chapter 1 – Probing the Mechanism and Reactivity of Biomacromolecular and Small Molecule Catalytic Systems with $\text{H}_2\text{O}_2$ and $\text{O}_2$

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Introduction

The population of the planet is expected to reach 8 billion people in just over a decade and to reach 9 billion by the early 2040s. Considering this ever-growing population, the finite abundance of natural resources necessitates the development and utilization of new and innovative methods for performing chemical transformations. This “new” chemistry cannot be limited to production and manufacturing alone, but must also include comprehensive methods for reclamation of necessary and relevant chemical building blocks from all forms of refuse in order to maintain these needed chemical feedstocks (1, 2). Bringing about reformation of this type is likely to occur via small, incremental and complimentary strategies involving multiple scientific disciplines. While this has been the normal course of progress through scientific advancement, an unprecedented urgency towards sustainability and waste minimization has arisen due to a global realization that the resources necessary to support a growing and ever more industrialized society are already strained. Catalysis provides a promising strategy for efficiently performing these chemical conversions.

Paul Anastas first described the rather nebulous concept of “green chemistry,” and later quantified these guidelines in his “12 Principles of Green Chemistry” (2-4). These guidelines provide a framework on which to build sustainable or renewable methods in industrial chemical manufacturing, energy production, and waste management with the overarching concept of minimization. It is logical that finding ever more efficient synthetic techniques for chemical transformations minimizes byproducts and thus, excess waste. Developing highly
selective catalysts that generate desired products and eliminate stoichiometric processes provides a significant amount of promise towards these ends (5-7).

For billions of years, Nature has been utilizing transition metals to efficiently perform and catalyze the biosynthesis of the building blocks of life, and through the process of evolution, it has developed intricate enzymatic systems for performing these chemical modifications with high efficiency and specificity. While it has achieved this by employing several strategies, many employ the iron-centered porphyrin, heme, enclosed within a protein scaffold as a common motif. The most widely utilized is the heme $b$ form also known as protoporphyrin IX and is illustrated in Figure 1.1 (8). The heme is generally stabilized in the scaffold through coordinate covalent bonds between the iron and amino acid side chains such as histidine or cysteine. The heme iron provides the reactivity of a transition metal center while the protein scaffold provides protection and imparts tuning of the heme iron reduction potential, substrate specificity and selectivity, channels for directing cosubstrates (proton, water), and stabilization of reaction intermediates.
In order to exploit the head start that Nature possesses in chemical transformation, understanding the mechanism and key features involved in these extensive transformation processes may lead to the rational design of novel synthetic systems capable of cost effectively providing the necessary chemical transformations of the future.

**Dehaloperoxidase-hemoglobin from *Amphitrite ornata***

Dehaloperoxidase-hemoglobin (DHP) is a multifunctional enzyme isolated from the marine terebellid polychaete, *Amphitrite ornata* (Figure 1.1) (9). This ancient organism resides in coastal estuaries and other benthic ecosystems. The main body of the organism is contained within a sand-encrusted tube, but utilizes tentacles to extract nutrients from its environment. Competing for the nutrient resources in the ecosystem habituated by *A. ornata* are other organisms, such as *Notomastus lobatus* and *Saccoglossus kowalewskyi*, that excrete biosynthesized bromophenols, bromoindoles, and bromopyrroles as chemical deterrents.

![Figure 1.1. The heme b prosthetic group](image-url)
against other nutrient competitors and predators \((10, 11)\). This evolutionary pressure has forced \textit{A. ornata} to combat these toxins by evolving dehaloperoxidase, which provides a necessary \(O_2\) transport and storage function as a globin, to also perform a necessary detoxification function via a well-defined peroxidase mechanism \((10-26)\).

\textbf{Figure 1.2.} The marine organism, \textit{Amphitrite ornata}

There are two reported isoenzymes for dehaloperoxidase, DHP A and DHP B. Each is transcribed from two distinct genes \((\textit{dhpa} \text{ and } \textit{dhp}b\), respectively) \((27)\). They are different by only five amino acids in their 127 amino acid sequence (I9L, R32K, Y34N, N81S, and S91G) \((10)\). With DHP, oxidation of 2,4,6-trihalogenated phenol (TCP) occurs via two distinct one electron oxidations coupled with dehalogenation at the \textit{para}- position to evolve
the respective 2,6-dihaloquinone as illustrated in Figure 1.2. This stepwise one-electron chemistry is typical for a peroxidase. However, we have recently identified a novel peroxygenase oxidation pathway for DHP with halogenated indoles and nitrophenols, which will be investigated in these studies (Chapter 2 and 3).

![Figure 1.3. The oxidative dehalogenation of trihalogenated phenols with DHP in H_2O_2](image)

In most organisms, these distinctly different enzymatic functions are performed by separate enzymes. Distinct forms of the heme iron and enzyme are illustrated in Figure 1.3. Globins require the ferrous form of the enzyme to reversibly bind O_2. Peroxidases and other oxidoreductases such as peroxygenases prefer ferric as the resting state for catalytic initiation. To discuss the ability of DHP to perform this diverse chemistry in a single enzyme, it is necessary to understand some conserved structural and electronic characteristic of each class and how this relates to the observed functionality in DHP.
Globins: Structure Function Relationships and Electrochemical Properties

Globins, in some form or another, are found in most forms of life on the planet and span the major kingdoms: animal, plant and bacteria. These iron-centered heme containing proteins are utilized as \( \text{O}_2 \) transport and storage in aerobic respiring organisms. They are also known to act as oxygen scavengers as in leghemoglobin to prevent fouling of the nitrogen fixation process by nitrogenases in plant roots (8).

Myoglobin (Mb) is one of the most well studied enzymes of the globin family. Many of its structural features found in Mb are conserved in other globins, and thus, Mb will serve as a basis for our discussion on the structure-function relationships found within the globin family. Myoglobin is a globular, single chain protein containing a necessary heme protoporphyrin IX cofactor. X-ray crystal structures have elucidated eight conserved alpha
helices (A – H) that maintain a common three-dimensional structure, referred to as the “globin fold.” Remarkably, the globin fold is also found in dehaloperoxidase (28-30), but exhibits little sequence homology to known hemoglobins. The heme prosthetic group in Mb is held between helices E and F through a coordinate covalent bond between a conserved histidine residue and the heme iron center. This interaction is through the Nε of the imidazole side chain and is termed the proximal histidine (8). This five-coordinate system allows for the binding of axial ligands such as O2 or water on the distal side which are stabilized through hydrogen bonding with an additional histidine residue on the distal side. X-ray crystallographic studies have elucidated that the distal histidine of Mb is flexible and can be found in two conformations depending on the axial ligand: a “closed” conformation, which stabilizes ligands bound axially to the heme iron, and “open” confirmation, which is a more solvent exposed conformation. This flexibility of the distal histidine has also been observed in DHP (20, 23, 31). In X-ray crystallographic studies with DHP, para-halophenols were found to induce the open conformation of the histidine and inhibit peroxidase activity. In Chapter 2, a perturbation of the Soret band for DHP, indicative of the open conformation, in the presence of 5-haloindoles is also observed, yet catalytic turnover in the presence of H2O2 is still readily observed with this substrate. The distal pocket of Mb contains hydrophobic residues.
The reduction potential of the heme iron in Mb is controlled through interactions between the proximal His, the iron, and the carbonyl oxygen of a nearby leucine residue. This provides for reduction potentials of the Fe$^{III}$/Fe$^{II}$ couple in globins are necessarily positive because reversible O$_2$ binding can only occur with the Fe$^{II}$ form as shown in Figure 1.4. Similar to other globins, DHP also has positive reduction potentials for the ferric/ferrous couple (DHP A - 204 mV and DHP B – 206 mV). Myoglobin and other globins exhibit limited reactivity with H$_2$O$_2$, unlike peroxidases.

**Peroxidases: Structure and Function**

Peroxidases comprise another class of heme-containing proteins that can oxidize a variety of substrates while reducing hydrogen peroxide (32, 33). The general reaction is illustrated in Scheme 1.1. The overall structure consists of ten alpha helical groups (A-J) with the heme group binding between helices B and F. The heme, as with globins, is stabilized by coordinate covalent binding of the heme iron through the N$_e$ of a conserved histidine residue.
on the proximal side (excluding chloroperoxidase). Further, peroxidases also utilize a distal histidine for stabilizing ligand binding at the axial position and assist in activation of $H_2O_2$.

**Scheme 1.1**

$$AH_2 + H_2O_2 \xrightarrow{\text{Peroxidase}} A + 2H_2O$$

The peroxidases are divided into mammalian and plant groups with the latter being further divided into three classes (I-III). Class I consists of yeast cytochrome c peroxidase and ascorbate peroxidase and contain a conserved tryptophan residue on the proximal side. Class II is comprised of peroxidase enzymes, which contain two calcium ions and conserved disulfide bridges, and are excreted by fungal organisms. These include lignin peroxidase and manganese peroxidase. Class III is composed of plant excretory peroxidases such as horseradish peroxidase (HRP) and peanut peroxidase. This class also possesses two calcium ions and 4 four disulfide bridges but in different locations than those found in Class II.

HRP is one of the most well studied of the peroxidases. The classic peroxidase cycle arises from a resting ferric form of the enzyme and is illustrated in Figure 1.5. Hydrogen peroxide oxidizes HRP by two electrons. $H_2O_2$ is heterolytically cleaved to evolve water and the enzyme takes on an Fe(IV) oxo with a radical delocalized in the now cationic porphyrin
known as Compound I. Through stepwise one electron reductions by substrate, the ferric form is restored. The ferryl oxygen is lost as a water molecule and not incorporated into the substrate.

The activation of H$_2$O$_2$ by HRP and other peroxidases is assisted by conserved histidine and arginine residues on the distal side, as well as the proximal histidine. The distal histidine accepts a proton from H$_2$O$_2$ as the associated oxygen atom binds to the iron center. The O-O bond is then heterolytically cleaved and the leaving \( ^{-} \text{OH} \) accepts the proton from the distal histidine to evolve water and Compound I. The heterolytic cleavage of H$_2$O$_2$ is also assisted by an aspartic acid side group on the proximal side. The combined effect of the Asp and His residues on the proximal side makes the reduction potential negative for the Fe$^{III}$/Fe$^{II}$

Figure 1.6. The classic peroxidase cycle with hydrogen peroxide and phenol
couple in HRP (-266 mV) (34), and allows for the ferric form to be preferred under physiological conditions. While DHP has a positive reduction potential, it still exhibits a relevant peroxidase mechanism, with the rate of peroxidase reactivity for DHP falling between that observed for HRP and Mb (34).

**Peroxygenase Chemistry and Cytochrome P450**

One of the most well studied enzymes is cytochrome P450. This is due to the broad spectrum of substrates that can be functionalized by the enzyme including aliphatic C-H bonds. This enzyme family also uses a heme iron center to catalyze the oxidation of various substrates. The protein fold observed from a number of X-ray crystal structures of several of the P450 family is conserved and is unlike any other enzyme yet discovered. Unlike globins and peroxidases, the heme cofactor is stabilized by a conserved cysteine residue on the proximal side. The necessity of the cysteine residue for activity has been demonstrated through mutation studies, in which the Cys357His of P450<sub>cam</sub> exhibits very low catalytic activity, but did provide an enhancement in peroxidase activity (35, 36).

With P450, O<sub>2</sub> serves as the sacrificial reductant rather than H<sub>2</sub>O<sub>2</sub>. P450 is activated by substrate binding to the enzyme (not the active site), which is in the ferric form. This substrate binding induces reduction of P450 by a reductase to the ferrous form, which binds O<sub>2</sub> to form oxyferrous. After another reducing equivalent and a proton, water is evolved and Compound I is formed as in the peroxidase mechanism. However, unlike most peroxidases (excluding chloroperoxidase), the resting ferric form of the enzyme is restored through direct incorporation of the ferryl O-atom into the C-H bond of the substrate. This classifies P450 as
a monooxygenase; however, P450 is capable of accessing Compound I using H$_2$O$_2$ via the termed “peroxide shunt.” The O-atom that is incorporated into substrate is now derived from hydrogen peroxide via the ferryl oxo to provide a peroxygenase mechanism. Both reactivities have been confirmed by labeled oxygen studies and demonstrate this O-atom incorporation is derived from the ferryl oxygen. We will investigate a novel and biologically relevant peroxygenase activity for DHP with halogenated indoles in Chapter 2. Also we demonstrate that some substrates of DHP are capable of being oxidized by both the peroxidase and peroxygenase mechanisms in Chapter 3.

This type of reactivity necessarily requires that the substrate can access the active site of the enzyme to allow for this type of O-atom transfer. The process of incorporation of the O-atom into the substrate is a two electron process and is referred to as the oxygen rebound step. The exact details of the mechanism for O-atom insertion are not completely known for all systems, but several models exist depending on the nature of the substrate. These proposed models may serve as models for the peroxygenase activity observed with DHP and H$_2$O$_2$.

**Catalysis with Small Molecule Iridium Complexes**

While enzymes offer renewable sources of catalytic systems and may provide insight into rational catalytic design for novel synthetic systems, progress continues to be made using small molecule transition metal systems (37-40). The group 9 metals have shown the ability to oxygenate alkenes, benzyl carbons, ethers and alcohols (41-45). Cobalt and rhodium have exhibited radical mechanisms for regioselective oxidation of ether substrates to
form esters (41, 46). Cobalt was found to also induce cleavage of dibenzyl ethers at ambient temperatures rather than evolving the ester.

The ether C-O bond is one of the strongest in organic molecules (47). They are a key component of lignocellulosic biomass derived from plant materials, which has received attention as a novel feedstock for fuels and fine chemicals due to its abundance and renewability (48-55). This type of biomass contains energy-rich hexoses and pentoses encased in a complex heteropolymer of interconnected phenolic monomers. The complex structure and variations in the covalent cross-linking of lignocellulosic biomass has been essential for the continued evolutionary survival of plants. The extensive, tenacious, and integral carbon-carbon and ether bonds present in lignin have made plants resistant to facile degradation by insects, pathogens, and scientists. However, the paper industry uses industrial processes to degrade the lignin polymer and access the crystalline cellulose within, which is essential for bulk paper production. Industrial degradation processes like those used by the pulp and paper industry also utilize a series of radical reactions to oxidatively delignify plant materials and access the desired cellulose (54, 56, 57). Using oxygen-variants (i.e. O2, O3, H2O2), acids, bases, heat, and various chlorinated reagents, they can supply cellulose on an industrial scale sufficient for our current pulp and paper needs. (57-59) Unfortunately, many of these processes are not completely in keeping with the previously outlined tenets of green chemistry. Therefore, identifying catalytic systems capable of activating and utilizing O2 to cleave the ether C-O bond presents a desirable goal. In chapter 4, we investigate the novel cleavage of various substituted dibenzyl ethers using Ir(III) complexes with O2.
References


Chapter 2 - Investigations of a Novel Peroxygenase and Oxidase Reactivity for Dehaloperoxidase B

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Abstract

Brominated indoles are known to be prevalent in marine ecosystems, and may present a natural substrate for detoxification enzymes isolated from marine organisms. Dehaloperoxidase from the marine annelid, *Amphitrite ornata*, has shown to exhibit diverse chemistry as both a globin for oxygen transport and a peroxidase for the degradation of halogenated phenols. Herein, we describe the discovery of a novel peroxygenase activity for DHP with various indole substrates. This peroxygenase oxidation is confirmed with isotopically labeled experiments. A novel oxidase mechanism was also discovered for DHP in the presence of a suitable reducing substrate.

Introduction

The enzyme, dehaloperoxidase (DHP), originally isolated from the marine worm, *Amphitrite ornata*, exists in two isoforms, DHP A and DHP B (1, 2). This enzyme offers a relevant globin function for *A. ornata*, but also provides a necessary peroxidase function (3). The peroxidase mechanism for DHP A and DHP B has been extensively studied (4-26), and is believed to have arisen due to evolutionary pressure to provide a detoxification pathway for *A. ornata*. In the benthic coastal ecosystems, the natural habitat for *A. ornata*, other organisms secrete biogenically-synthesized halometabolites as chemical deterrents against other nutrient competitors. DHP has been shown to utilize the well-studied peroxidase mechanism to oxidize one of these halometabolites, 2,4,6-trihalogenated phenol (TXP), to the respective 2,6-dihaloquinone by two discreet, one electron redox reactions (25) in the
presence of \( \text{H}_2\text{O}_2 \). Another reported halometabolite found in considerable amounts in marine ecosystems are brominated indoles and seemed a viable candidate as a substrate for DHP.

Indoles and their derivatives continue to gain interest. Brominated indoles and biosynthesized chemical derivatives continue to be isolated from marine organisms. One such natural product derivative, meridianins, have been found to inhibit some protein kinases, which are key to the proper phosphorylation of proteins utilized in metabolism and normal cell function. Abnormal phosphorylation is linked to disease states in humans so derivatives of these compounds may find use in novel drug therapeutics. Indole signaling is also a known regulator of bacterial behavior \((27, 28)\). Fascaplysin, a pigment isolated from a marine sponge and an indole derivative, has found to have efficacy as an antimicrobial and as an anticancer agent \((29-32)\). Further, brominated indoles have been isolated from several species
of the Hemichordata phylum, which cohabitate with *A. ornata* in coastal estuaries, and may represent another family of chemical deterrents evolutionary targeted by *A. ornata* and DHP.

DHP adopts similar high valent oxidation states as other peroxidases in the presence of H$_2$O$_2$, but unlike other peroxidases, DHP is necessarily stable in the ferrous form to facilitate globin function. Cytochrome P450, an oxygenase, can also adopt similar oxidized intermediates utilizing O$_2$ or H$_2$O$_2$. Commonly with the P450 family, a thiol-stabilized ferrous heme species reduces O$_2$ in the presence of a reductase to evolve water and form a
ferryl-oxo (Fe^{IV}=O) with a porphyrin-based, π-cation radical, commonly referred to as Compound I. P450 can also access Compound I from the ferric form in the presence of H$_2$O$_2$ via the “peroxide shunt” also mechanism while evolving an equivalent of water. By definition, the ferryl oxygen of Compound I in P450 is incorporated into the oxidized substrate, whether it is formed by the reduction of O$_2$ (oxygenase) or H$_2$O$_2$ (peroxygenase).

For DHP and most other peroxidases, a proximal histidine is utilized rather than a cysteine to stabilize the heme iron. Similar to the peroxide shunt of P450 and consistent with a typical peroxidase oxidation, ferric DHP reacts with H$_2$O$_2$ to form a similar two-electron oxidized species known as Compound ES. An equivalent of water is evolved as well as a Fe^{IV}=O, but with a protein-based (tyrosyl) radical rather than one localized on the porphyrin. Studies utilizing site directed mutagenesis have shown that replacing the naturally occurring tyrosines closest to the active site in DHP B (Y28 and Y38) with redox inactive phenylalanines inhibits the formation of the amino acid radical, and instead produces the Compound I analog possessing an oxidized Fe^{IV}=O and a radical that is “trapped” on the porphyrin cofactor, and due to the similarity has also been termed Compound I. With the extensive scope of substrates that have shown reactivity P450, it was logical to conclude that DHP, which is capable of forming analogous redox active intermediates and has already demonstrated the ability to oxidize diverse substrates, may also be promiscuous towards a broader range of substrates including indoles (33).

Thus, a series of various substituted indoles were screened for reactivity with DHP in the presence of H$_2$O$_2$. These studies showed that singly oxygenated, 2- and 3-oxindoles derivatives were the major products observed. The latter was quantitatively oxidized to the 3-
oxindolenine species by DHP \textit{in situ}. Minor amounts of the dioxygenated species were also observed (Scheme 2.1). Isotopically-labeled oxygen LC-MS studies demonstrated a novel peroxygenase pathway for this indole oxidation by DHP and \( \text{H}_2\text{O}_2 \). The highest turnover was exhibited by the double mutant (Y28/38F) of DHP enzymes used. Stopped-flow experiments showed a spectral shift in the Soret of ferric \( \text{w.t.} \) DHP B indicative of protein-ligand binding affinity and also showed that ferric DHP is reduced to oxyferrous over longer time periods. Resonance Raman and ligand binding studies confirmed an affinity between DHP and the 5-haloindoles (I > Br > Cl > F), but no correlation to overall turnover was observed. The ferric to oxyferrous reduction was explored using 5-bromo-3-oxindole (\( 3\text{a} \)) and revealed novel oxidase functionality for DHP.

\section*{Experimental}

\textit{Materials and Methods}. Isotopically labeled \( \text{H}_2\text{O}^{18} \text{O} \) (90\% \( ^{18}\text{O} \)-enriched) and \( \text{H}_2\text{O}^{18} \) (98\% \( ^{18}\text{O} \)-enriched) were purchased from Icon Isotopes (Summit, NJ). Acetonitrile (MeCN) was HPLC grade and all other chemicals were purchased in the highest quality from VWR, Sigma-Aldrich or Fisher Scientific and used without further purification. UV-visible spectroscopy was performed on a Cary 50 UV-visible spectrophotometer. Stock solutions (2 mM) of substrates (all indoles) were prepared in MeOH stored in the dark at \(-20\) °C until needed and were periodically screened to ensure that they had not degraded. Aliquots were thawed and stored on ice during use. Solutions of \( \text{H}_2\text{O}_2 \) were prepared fresh daily and kept on ice until needed. The concentration was determined by UV-vis \( (\varepsilon_{240} = 46 \text{ mM}^{-1}\text{cm}^{-1}) \). For DHP B wild type, DHP B (Y28/38F), and DHP A, expression and purification were
performed as previously reported (7, 12). Lyophilized horseradish peroxidase and horse heart myoglobin were purchased from Sigma-Aldrich and stored at -20 °C until utilized. They were reconstituted in 100 mM KP\textsubscript{i} at pH 6 and concentrations determined using accepted values.

\textit{Preparation of Ferric Enzyme.} An aliquot of DHP (A, B or Y28/38F) in 100 mM KP\textsubscript{i} (pH 7) and 40\% glycerol (v/v) was thawed and charged with an excess of potassium ferricyanide (~2 min) to allow for quantitative conversion to ferric. Excess oxidizing agent was removed using gel filtration chromatography (Sephadex G-25) pre-equilibrated with 100 mM KP\textsubscript{i} at pH 7. The enzyme-containing eluent fraction was collected and subjected to successive buffer exchanges (3x) via centrifugation using an Amicon Ultra centrifugal filter (10,000 KDa MW). Enzyme concentration was determined spectrophotometrically ($\varepsilon_{\text{Soret}} = 116,400$ mM\textsuperscript{-1}cm\textsuperscript{-1}) for all isoenzymes (7).

\textit{Preparation of Oxyferrous DHP B.} An aliquot of DHP B in 100 mM KP\textsubscript{i} (pH 7) and 40\% glycerol (v/v) was thawed and charged with an excess of sodium ascorbate, and incubated for 30 min at 4 °C to quantitatively reduce the enzyme to the ferrous form. The binding of atmospheric O\textsubscript{2} provided oxyferrous DHP B (418 (Soret); 542, 578 nm) (26). Excess ascorbate salt was removed utilizing the same protocol as that for potassium ferricyanide removal during ferric preparation (vide supra). The presence and concentration of oxyferrous was determined spectrophotometrically ($\varepsilon_{\text{Soret}} = 116,400$ mM\textsuperscript{-1}cm\textsuperscript{-1}) (26).

\textit{Enzyme Assay Protocol.} Reactions were performed in triplicate at pH 7 in 100 mM KP\textsubscript{i} at 25 °C. Buffered solutions (total reaction volume 250 μL) of DHP (10 μM final concentration) and nitrophenol (500 μM final concentration) were pre-mixed, and then the
reaction was initiated upon addition of $\text{H}_2\text{O}_2$ (500 $\mu$M final concentration). After 5 minutes, reactions were quenched with excess catalase. Reaction samples were analyzed by reverse-phase HPLC coupled with a photodiode array detector as described below.

Anaerobic studies were performed as described above in an MBraun Lab Master 130 nitrogen-filled glove box (<1 ppm $\text{O}_2$, <1 ppm $\text{H}_2\text{O}$) using argon degassed solutions of buffer, peroxide, substrate and enzyme. Caution was used in degassing enzyme to avoid enzyme precipitation. Using similar protocols, triplicate experiments were performed in the presence of D-mannitol (500 $\mu$M), superoxide dismutase (SOD), DMSO (10% v/v). For the studies with sodium formate, a buffered solution (100 mM KPi; 500 $\mu$M sodium formate) at pH 7 was utilized.

$[\text{H}_2\text{O}_2]$ Studies with 1a and 1b in the Presence of w.t. DHP B. Reactions were performed in triplicate using a protocol similar to that utilized for the enzyme assays (vide infra). Fresh peroxide solutions were prepared in 100 mM KPi at pH 7 and concentration determined as previously described. Reactions with final concentrations of 50, 100, 250, 500, and 1000 $\mu$M $\text{H}_2\text{O}_2$ (enzyme – 10 $\mu$M; substrate – 500 $\mu$M) were run for 5 min and quenched with catalase, then analyzed by HPLC.

Analysis of Sample by HPLC. A 100 $\mu$L aliquot of reaction sample was diluted 10-fold with 900 $\mu$L of 100 mM KPi (pH 7). Diluted samples were analyzed using a Waters 2796 Bioseparation Module coupled with a Waters 2996 Photodiode Array Detector, and equipped with a Thermo-Scientific ODS Hypersil (150 mm x 4.6 mm) 5 $\mu$m particle size C18 column. Separation of observed analytes was performed using a linear gradient of binary solvents (solvent A - $\text{H}_2\text{O}$ containing 1% trifluoroacetic acid; solvent B - MeCN). Elution
was performed using the following conditions: (1.5 mL/min A:B) 95:5 to 5:95 linearly over 10 minutes; 5:95 isocratic for 2 minute; 5:95 to 95:5 linearly over 1 minute, then isocratic for 4 minutes. Data analysis was performed using the Empower software package (Waters Corp.). Calibration curves for all indoles and available products were performed using serial dilutions of commercially available analytes and utilized to determine the amount of substrate conversion.

**LC-MS Studies with Indoles.** Experiments were analyzed using a 6210 LC-TOF mass spectrometer (Agilent Technologies, Santa Clara, CA). Analyte separation was performed using the same conditions and column as HPLC studies with the exception of solvent A (water with 0.1 % formic acid). Samples were analyzed using electrospray ionization in negative mode to provide observation of the [M+H]⁺ species. Spectra were collected each second while scanning in the range from 100 – 1000 m/z. Data analysis was performed using Agilent software. Quantitation of the amount of ¹⁸O-labeled incorporated was performed using previously established methods (34). In labeled H₂O₂ experiments, an aliquot of 2.15% (w/w) solution of 90% enriched H₂¹⁸O₂ was diluted 3-fold to provide a peroxide solution of ~20 mM. A final reaction volume (100 mM KP₃ pH 7) of 250 μL containing 10 μM enzyme, 50 eq. of substrate, and ~50 eq. of labeled peroxide was allowed to react for 5 min before quenching with catalase enzyme. Then, 20 μL of undiluted reaction mixture was injected for LC-MS analysis.

For labeled water studies, stock solutions of reactants ([enzyme] = 120 μM; [H₂O₂] = ~12.5 mM) in unlabeled water were kept at sufficiently high concentrations to allow for the 98% enriched H₂O to be diluted to ~89% in the final reaction mixture. Labeled water (207.5
μL) was charged with 21 μL enzyme, 10 μL H₂O₂, and 12.5 μL substrate (in MeOH) and reacted for 5 min, then quenched with catalase and 20 μL of undiluted reaction mixture was injected for LC-MS analysis.

Stopped-Flow UV-visible Studies with DHP and Nitrophenols. Experiments were performed using a Bio-Logic SFM-400 triple mixing stopped flow instrument coupled to a rapid scan diode array UV-visible spectrophotometer. Experiments were performed at room temperature and all solutions were prepared in 100 mM KP₁ (pH 7). Single mixing experiments reacting 10 μM enzyme with 50 μM H₂O₂ were performed to determine the time of maximum formation of Compound ES (wild type DHP) or Compound I (DHP B (Y28/38F)). Double mixing experiments were then performed with enzyme and H₂O₂ using the appropriate delay times to observe Compound ES / Compound I reactivity with 1a (10, 25, or 50 eq.). Spectra (900 scans) were collected over short (2, 25 and 250 ms; 300 scans each) and long acquisitions. For substrate pre-incubation studies with DHP, single mixing conditions were utilized and observed over 83 s. Specifically, a solution of DHP (10 μM final concentration) and 4-NP (10 equiv.) was prepared and then mixed with H₂O₂ (10 equiv.). Data were evaluated using Specfit Global Analysis System software (Spectrum Software Associates) and fit by SVD analysis as either one-step, two species or two-step, three species irreversible mechanisms, where applicable.

Resonance Raman Studies with DHP and Indole Substrates. Samples were prepared prior to analysis with final concentrations of 50 μM wild type DHP B and 500 μM indole substrate in 100 mM KP₁ at pH 7 and 10% MeOH (v/v) then transferred to a 5 mm diameter glass NMR tubes and immediately measured. Resonance Raman spectra were obtained by
Soret band excitation using a Coherent Mira 900 titanium sapphire (Ti:sapphire) laser. The Ti:sapphire laser was pumped using a Coherent Verdi 10 frequency doubled diode pumped Nd:vanadate (Nd:VO₄) laser producing 10 W at 532 nm. The beam generated was sent through a Coherent 5-050 doubler to generate a normal working range of 400-430 nm for Soret band excitation of both DHP only and the DHP / indole complexes. The beam was collimated and cylindrically focused to a vertical line of ~0.5 mm on the sample. Laser power at the sample was 60 mW. Scattered light was collected with a Spex 1877 triple spectrometer equipped with a liquid nitrogen-cooled CCD detector controlled by Spectramax software.

**Protein-Ligand Binding Studies with DHP and Indole Substrates.** These studies were performed in triplicated for each measurement and utilizing similar to previously published protocols (35). Stock solutions of 2 mM indole (Cl-, Br- and I-) in MeOH were prepared. The UV-visible spectrophotomer was blanked with 10 μM ferric *w.t.* DHP B in 100 mM KP₄ at pH 7 and 10% MeOH. While maintaining these concentrations for enzyme and MeOH, spectra were acquired in the presence of 2.5, 5, 10, 25, 50 and 100 eq. of indoles. Analysis by nonlinear regression using the Grafit software package (Erithacus Software Ltd.) provided a calculated $A_{max}$, which was in turn used to calculate $\alpha$ for the average ΔA for each indole concentration. A nonlinear regression plot provided the reported apparent $K_d$ values.

**Studies with Ferric wild type DHP B and 3-oxindole (7a.).** In these UV-visible studies, ~250 μM 3-acetoxy-5-bromoindole were incubated in the presence or absence of 10 μM ferric *w.t.* DHP B in 100 mM KP₄ at pH 7 (< 1 min) in a quartz cuvette. Reactions were initiated by the addition of porcine liver esterase and spectra collected at the times indicated.
Anaerobic studies were performed in the glove box, sealed and then measured in the atmosphere.

Results

Table 2.1. Substrate conversion observed for various indoles in the presence of ferric w.t. DHP B and H₂O₂

<table>
<thead>
<tr>
<th>Enzyme + Substrate</th>
<th>Conversion (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DHP B Ferric</strong></td>
<td></td>
</tr>
<tr>
<td>+Indole</td>
<td>24.1 (±2.3)</td>
</tr>
<tr>
<td>+4Br-indole</td>
<td>41.1 (±2.0)</td>
</tr>
<tr>
<td>+5Br-indole</td>
<td>48.1 (±2.3)</td>
</tr>
<tr>
<td>+6Br-indole</td>
<td>34.4 (±0.6)</td>
</tr>
<tr>
<td>+7Br-indole</td>
<td>46.1 (±1.7)</td>
</tr>
<tr>
<td>+5F-indole</td>
<td>26.0 (±1.3)</td>
</tr>
<tr>
<td>+5Cl-indole</td>
<td>37.5 (±1.6)</td>
</tr>
<tr>
<td>+5I-indole</td>
<td>34.0 (±2.0)</td>
</tr>
<tr>
<td>+tryptophan</td>
<td>n.r.</td>
</tr>
</tbody>
</table>

*Reaction conditions: 10 μM ferric w.t. DHP B; 500 μM indole; 500 μM H₂O₂ in 100 mM KP₁ at pH 7 (5 min. at r.t.)

**Catalytic Oxidation of Various Indoles with DHP B.** Due to the limited commercial availability of the reaction products or their characteristic spectral values in the literature,
only conversion based on substrate loss can be reported for most of the indoles studied with DHP and H\textsubscript{2}O\textsubscript{2}. The reactions were run under the non-optimized conditions of 50 equivalents of substrate and oxidant with respect to the enzyme (500, 500 and 10 μM, respectively), and the results presented in Table 2.1. The reactions with 1a and 7Br-indole exhibited the highest turnover of the indoles studied and were only slightly more reactive than those with 4Br-indole. While there was a more considerable decrease in reactivity observed with 6Br-indole, it is evident by these studies that bromination at any of the arene positions enhances reactivity considerably over that of the unsubstituted indole (1b). Additionally, the halogen series of commercially available 5-substituted indoles (5X-indole where X = F, Cl, or I) were also screened. The 5Cl-indole and 5I-indole exhibited only slightly reduced reactivity as compared to that of 1a, but the 5F-Ind showed a significant decrease and was experimentally equivalent to the reactivity of the unsubstituted indole. Additionally, tryptophan exhibited no reactivity under these same conditions, and reactions run in the absence of either enzyme or H\textsubscript{2}O\textsubscript{2} also exhibited no turnover of the indole substrate.
Figure 2.1. (A) Typical HPLC UV-vis trace at 242 nm for oxidation of 1a with DHP in the presence of H₂O₂; (B) Results of [H₂O₂] studies with 1 and Ferric DHP B w.t. (● / 2a; ♦ / 4a; ▲ / 5a; ■ / 6a); (C) Typical HPLC UV-vis trace at 242 nm for oxidation of 1b with DHP in the presence of H₂O₂; (D) Results of [H₂O₂] studies with 1b and Ferric DHP B w.t. (● / 2b; ♦ / 4b; ▲ / 5b; ■ / 6b)

The availability of some of the oxidation products for 1a and 1b afforded more conclusive identification of the reaction products and their relative quantities. A typical HPLC trace at 242 nm is shown in Figure 2.1.A for studies performed with 1a. The major product was determined to be 5-bromo-2-oxindole (2a). The other products observed were 5-
bromo-3-oxindolenine (7a), 5-bromo-2,3-dioxindole (4a), 5-bromo-3-hydroxy-2-oxindole (5a), and \(N\)-(4-bromo-2-formylphenyl) formamide (6a), and \((E)\)-5,5'-dibromo-[2,2'-biindolylidene]-3,3'-dione (9a). A similar product distribution was observed for studies performed with 1b (Figure 2.1.C). Products were identified by their characteristic retention time and UV-vis spectra as compared to authentic samples or by similarity of their UV-visible spectra with those of their respective unsubstituted analogue.

Unlike other studies with indole oxidation (33, 34), the respective 3-oxindole species (3a and 3b) were not observed directly as major products; rather, it appears that they are completely converted to the two-electron oxidized, 3-oxindolenine species (7a and 7b). This oxidation is also coupled with the loss of two protons. It is likely that Compound ES is reduced by 3a and 3b to evolve 7a and 7b, respectively, as well as an equivalent of \(H_2O\) to restore the ferric form of DHP B. Unfortunately, the amount of 7a and 7b formed could not be quantified; however, it was the only species of sufficient concentration, other than 2a and 2b, that could be observed in our subsequent LC-MS studies (\textit{vida infra}); therefore, 7a and 7b are believed to be major products of indole oxidation by DHP B. It is apparent that halide substitutions on the arene ring of the indole enhanced reactivity considerably.

\([H_2O_2]\) \textit{studies with 5-bromoindole (1a) and indole (1b)}. The concentration of \([H_2O_2]\) was varied to observe any insightful trends. The results are presented in Figure 2.1.B for 1a and 2.1.D for 1b. As stated, the major product of the oxidation of 1a is 2a. It appears to reach a maximum formation \textit{ca.} 500 \(\mu\)M \(H_2O_2\) and remains constant. This may be the result of reaching a saturation in \(H_2O_2\) for DHP B, which is known to form Compound RH, an unreactive oxidized species in the presence of excess \(H_2O_2\) (72). The other minor products
were also quantified and appear in the insert. These minor products contain an additional incorporated O-atom and this secondary oxidation is consistent with other indole studies (33, 34). They are believed to arise from oxidation by exogenous H₂O₂ in solution, but may be partially derived from an enzyme-dependent pathway. However, studies with 2a as the substrate under the same reaction conditions showed no secondary oxidation and thus it was determined to be a terminal product of indole oxidation; however, the doubly oxygenated products may arise from a separate reaction pathway involving the 3-oxindole intermediate. Due to their low concentration, the origins of the minor products were not explored further at this time.

**Enzyme variation studies with 1a and 1b.** To complement the studies with ferric DHP B, experiments were also performed with some variants - oxyferrous DHP B, ferric DHP B (Y28/38F), and ferric w.t. DHP A, as well as horseradish peroxidase (HRP) and horse heart myoglobin (Mb). The results are provided in Table 2.2. The double mutant exhibited the highest conversion observed in these studies (62.2%) with 1a. DHP B (Y28/38F) has been previously shown to be incapable of forming Compound ES and is halted at Compound I. The proximity of the two oxidizing equivalents in Compound I of Y28/38F may allow for faster substrate oxidation compared to that of the wild type. In studies starting from oxyferrous w.t. DHP B, there was no significant difference in reactivity over the ferric, which is consistent with previous peroxidase studies (26). Oxyferrous w.t. DHP B can react with H₂O₂ to form Compound II - a ferryl oxo species without the radical present on the porphyrin
Table 2.2. Substrate conversion observed for various indoles in the presence of isoenzymes of DHP, HRP, Mb and H₂O₂

<table>
<thead>
<tr>
<th>Enzyme + Substrate</th>
<th>Conversion (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DHP B Oxyferrous</td>
<td></td>
</tr>
<tr>
<td>+5Br-Indole</td>
<td>44.9 (±4.8)</td>
</tr>
<tr>
<td>DHP B (Y28/38F) Ferric</td>
<td></td>
</tr>
<tr>
<td>+5Br-Indole</td>
<td>62.2 (±2.5)</td>
</tr>
<tr>
<td>DHP A Ferric</td>
<td></td>
</tr>
<tr>
<td>+4Br-Indole</td>
<td>15.7 (±0.7)</td>
</tr>
<tr>
<td></td>
<td>20.3 (±1.7)</td>
</tr>
<tr>
<td>horseradish peroxidase</td>
<td></td>
</tr>
<tr>
<td>+5Br-Indole</td>
<td>n. r.</td>
</tr>
<tr>
<td>horse heart myoglobin</td>
<td></td>
</tr>
<tr>
<td>+5Br-Indole</td>
<td>n. r.</td>
</tr>
</tbody>
</table>

*Reaction conditions: 10 μM enzyme; 500 μM indole; 500 μM H₂O₂ in 100 mM KP_i at pH 7 (5 min. at r.t.)*

or protein via a two electron oxidation by H₂O₂. In peroxidase studies with DHP, Compound II is reduced by substrate to access ferric, which then allows for the enzyme to proceed through the standard peroxidase cycle for further catalytic oxidation (26). The peroxygenase mechanism for oxyferrous reactivity will need to be explored further, but may occur through a similar pathway involving enzyme reduction by a reaction product.
Studies with DHP A exhibited attenuated activity as compared to ferric DHP B and is consistent with the literature for DHP, in that isoform A has been shown to exhibit a lower turnover than DHP B for peroxidase activity (7). The previously reported reduction potentials for DHP A and DHP B are nearly identical (202 ± 6 mV and 206 ± 6 mV, respectively) (7); therefore, similar to the hypothesis proposed in the peroxidase studies, the marked difference in the observed peroxygenase reactivity for the two Isoenzymes cannot seemingly be the result of their electrochemical differences. Overlaid X-ray crystal structures of both forms of DHP show minimal differences in their tertiary structure, and thus, the differences in reactivity may arise to the structural alteration imparted by the variance in their primary amino acid sequences. Finally, studies were also performed with horseradish peroxidase (HRP) and horse heart myoglobin (Mb) in the presence \( \text{H}_2\text{O}_2 \) with neither exhibiting conversion of substrate. HRP has previously been shown to be reactive towards \( \text{1b} \) and towards 3-acetoxy indole. This likely occur through a series of one electron oxidations as is common for peroxidases, however, it is not understood why an analogous reactivity is not observed for \( \text{1a} \) considering its reduction potential has been reported to be the same as \( \text{1b} \) (36), but may arise from the increased steric bulk of \( \text{1a} \), as many peroxidases are known to limit access to the distal pocket by the presence of a conserved Arg residue involved in hydrogen bonding in the distal pocket.

**Reaction Studies with Radical and Activated Oxygen Scavengers.** Some additional studies designed at probing the mechanism were performed with the most active indole, \( \text{1a} \). Reactions were performed in the presence of DMSO, sodium formate, D-mannitol and superoxide dismutase (SOD) with the results presented in Table 2.3. All showed nearly
equivalent reactivity to reactions run in their absence. Turnovers in DMSO, formate and D- mannitol were only slightly lower, but did not inhibit the reactivity significantly. Also, there was no considerable fluctuation in the ratio of the product peak areas observed by HPLC, and we conclude that these results together imply that the reaction mechanism does not involve freely diffusible radical intermediates. Similarly, reactivity in the presence of SOD showed no alteration in substrate turnover and seemingly eliminates this chemistry being performed

Table 2.3. Substrate conversion observed for various indoles in the presence of ferric w.t. DHP, H$_2$O$_2$, and additives

<table>
<thead>
<tr>
<th>Enzyme/Substrate + Additive / Control</th>
<th>Conversion (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DHP B Ferric / 4Br-Indole</strong></td>
<td></td>
</tr>
<tr>
<td>Anaerobic</td>
<td>40.1 (±0.9)</td>
</tr>
<tr>
<td><strong>DHP B Ferric / 5Br-Indole</strong></td>
<td></td>
</tr>
<tr>
<td>+500 μM mannitol</td>
<td>46.2 (±1.6)</td>
</tr>
<tr>
<td>+500 mM formate</td>
<td>45.8 (±1.1)</td>
</tr>
<tr>
<td>+SOD</td>
<td>46.1 (±0.7)</td>
</tr>
<tr>
<td>+DMSO (10% v/v)</td>
<td>45.0 (±4.4)</td>
</tr>
<tr>
<td>+500 μM 4-BP</td>
<td>23.2 (±5.1)</td>
</tr>
</tbody>
</table>

*Reaction conditions: 10 μM ferric w.t. DHP B; 500 μM indole; 500 μM H$_2$O$_2$; and additive in 100 mM KP$_4$ at pH 7 (5 min. at r.t.)
by an exogenous enzyme-reduced oxygen species like superoxide or •OH. Reactions were also performed anaerobically under N₂ and showed no significant decrease in reactivity. This is consistent with previous studies with DHP B under peroxidase conditions (26), and rules out an oxygenase mechanism for the observed reactivity.

Experiments were also performed in the presence of a known inhibitor of DHP, 4-bromophenol (4-BP). It has been shown to have a binding affinity for the enzyme and has recently been shown by X-ray crystallography experiments to bind in the distal pocket and has been experimentally shown to slow the formation of Compound ES by disturbing the hydrogen-bonding network necessary for the reduction of H₂O₂ by ferric. In fact, a 50% attenuation in reactivity was observed for the 1a and 7Br-indole. A complete attenuation was not expected as subsequent binding studies will show that 1a has an ~10x higher affinity for ferric DHP. However, some additional mechanistic insight was necessary to determine whether indole was simply a new substrate that was susceptible to oxidation by the well-established peroxidase cycle of DHP or if oxygenation was occurring through a novel mechanistic pathway.
Figure 2.2. (A) TIC of 2a from reactions run in H$_2^{18}$O; (B) TIC of 2a from reactions run in H$_2$O; (C) TIC of 7a from reactions run in H$_2^{18}$O; (D) TIC of 7a from reactions run in H$_2$O

Isotopically-labeled Oxygen Studies. As the reactivity was determined to be O$_2$ independent, the various experimental permutations of labeled and unlabeled H$_2^{18}$O and H$_2$O (90% and 98% O-atom enriched, respectively) were performed with 1a and 1b and
subsequently analyzed by LC-MS. The resulting isotopic distributions for the major products with the various $^18$O-atom sources (2a and 7a) are shown in Figure 3.2. Figure 2.2.A and 2.2.C show the background subtracted TIC of experiments run in H$_2^{18}$O and exhibit minimal label incorporation. Figure 2.2.B and 2.2.D show these same experiments in H$_2^{18}$O$_2$ and

<table>
<thead>
<tr>
<th>Product</th>
<th>18O-atom source</th>
<th>% relative abundance [M+H]$^+$ ion</th>
<th>%$^{18}$O incorporated$^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>2a</td>
<td>none</td>
<td>m/z 210 100 0 n.a. n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td></td>
<td>H$_2^{18}$O$_2$</td>
<td>4.7 100 89.5 n.a. 94.2 (104)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>H$_2^{18}$O</td>
<td>100 99.4 0 n.a. 99.4</td>
<td></td>
</tr>
<tr>
<td>7a</td>
<td>none</td>
<td>n.a. 99.2 100 - n.a. n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td></td>
<td>H$_2^{18}$O$_2$</td>
<td>n.a. 6.1 100 75.8 88.1 (97.9)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>H$_2^{18}$O</td>
<td>n.a. 99.8 100 1.3 0.8 (0.9)</td>
<td></td>
</tr>
</tbody>
</table>

* number in parenthesis represents normalized incorporation as a function of $^{18}$O enrichment from source

illustrate a clear isotopic shift of both 2a and 7a by +2 Da, and clearly demonstrates that the oxygen atom incorporated is derived from H$_2$O$_2$ indicative of a peroxygenase mechanism. The labeled water studies offer confirmation of this observation and eliminate oxidation by a
peroxidase mechanism. The relative isotopic abundances and normalized $^{18}$O-incorporation are presented in Table 3.4. Quantitative incorporation was determined using previously established methods (34). Products were identified by the respective $m/z$ of the (M+H)$^+$ ion, and retention time as compared to available standards. The minor dioxygenated products were of low concentration and their origins were not pursued further at this time. In studies with 1b and 4Br-indole (1c), the oxygen incorporated into the respective 2-oxindole product was derived exclusively via the peroxygenase mechanism (Appendix Table A.1 and Appendix A.2). However, the results for the respective 3-oxindolenine product were not as straightforward. Experiments run in H$_2^{18}$O with 1b and 1c showed a mix of labeled and unlabeled 3-oxindolenine product possibly indicative of a mix of peroxidase and peroxyxygenase product. This effect was more pronounced in 2b than in 4-bromooxindolenine. Experiments with 1b run in only labeled water exhibited minimal label incorporation (~5.5%), and the doubly-labeled experiments with 1b also failed to show complete label incorporation (60.1%). The reasons for this anomaly are still unclear especially when one considers that the 2a showed complete label incorporation from H$_2^{18}$O as did 7a and 4-bromooxindole. The dead time between reaction quenching and sample injection / analysis were constant for all samples so O-atom exchange with any unlabeled species in solution is unlikely. Resonance Raman experiments and binding affinity studies performed (vide infra) show that 1a and other 5-haloindoles possess a binding affinity for DHP and may direct the orientation and approach of these substrates to the active site that is optimal for peroxygenase reactivity. Thus, indoles lacking an affinity may have more flexibility in the active site allowing for less discriminate chemistry. Additional labeled studies with other indole
substrates may better illuminate the source of this variance, but there is unequivocal evidence for a novel peroxygenase pathway in DHP.

**Stopped-Flow Studies with DHP B wild type and (Y28/38F) mutant.** To complement the intriguing results observed in the isotopically labeled oxygen studies, a series of single and double mixing experiments were performed using stopped-flow UV-visible spectroscopy. Single mixing experiments were performed with DHP B w.t and (Y28/38F) with 5 equiv. of H$_2$O$_2$ to determine the times of optimal formation of Compound ES or
Figure 2.3. (A) Stopped-flow UV-visible spectroscopic monitoring (900 scans, 83 s) of the double-mixing reaction between preformed w.t. DHP B Compound ES and 10 eq. 1a at pH 7; (B) Stopped-flow UV-visible spectroscopic monitoring (900 scans, 83 s) of the double-mixing reaction between preformed w.t. DHP B Compound ES and 25 eq. 1a at pH 7; (C) Stopped-flow UV-visible spectroscopic monitoring (900 scans, 83 s) of the double-mixing reaction between preformed w.t. DHP B Compound ES and 50 eq. 1a at pH 7
Compound I, respectively. Subsequently, double mixing experiments were performed utilizing these delay times to mix substrate at these maxima. Three concentrations of 1a were used (10, 25, and 50 equiv. wrt DHP B). The UV–vis spectra were recorded of the various reactive intermediates over 83 seconds (Figure 2.3). In the studies with *wild type*, Compound ES ($\lambda = 418$, 545, and 584 nm) was converted rapidly to what appears to be predominately Ferric ($\lambda = 402$, 508, and 633 nm). The conversion was fast enough with the 25 and 50 equiv. studies such that Compound ES was not observed on the timescale of the instrument. The apparent ferric species proceeded to persist over these shorter observation times. The Soret peak of ferric was slightly broadened and blue-shifted ($\lambda \approx 400$ nm) from reported values ($\lambda = 407$ nm) (7). This was subsequently attributed to a binding affinity between ferric DHP B
and 1a. When these experiments were repeated over longer observation time (833 sec) (Figure 2.4), similar results were observed initially, but the ferric form was observed to eventually be reduced to oxyferrous DHP B ($\lambda = 418, 542, 578$) after two minutes, and continued to
Figure 2.5. (A) Stopped-flow UV-visible spectroscopic monitoring (900 scans, 83 s) of the double-mixing reaction between preformed (Y28/38F) DHP B Compound I and 10 eq. 1a at pH 7; (B) Stopped-flow UV-visible spectroscopic monitoring (900 scans, 83 s) of the double-mixing reaction between preformed (Y28/38F) DHP B Compound I and 25 eq. 1a at pH 7; (C) Stopped-flow UV-visible spectroscopic monitoring (900 scans, 83 s) of the double-mixing reaction between preformed w.r.t. DHP B Compound ES and 50 eq. 1a at pH 7
increase in concentration over the 833 s observation time. In the studies performed with the (Y28/38F) mutant, formation of the Oxyferrous analogue was observed to form faster and could be seen in the shorter 83 s observation studies (Figure 2.5). Y28/38F is known to be kinetically faster for peroxidase reactivity with trihalogenated phenols, and also exhibited the highest turnover of 1a. It is likely faster than w.t. because of the proximity of the two oxidizing equivalents. Also of note, the Soret of oxyferrous species observed in the presence of 1a is in good agreement with reported values (26) implying that there is no binding affinity between oxyferrous DHP B and 1a.

With H\textsubscript{2}O\textsubscript{2} being a limiting reagent, ferric is expected to be the final resting state of the enzyme, but 3-oxindole is known to be readily oxidized by O\textsubscript{2} so it is conceivable the ferric may also be reduced by \(\frac{1}{2}\) molar equivalent of 3a to form 7a, and following O\textsubscript{2} binding, provide oxyferrous species observed. Reduction of ferric to ferrous / oxyferrous by a
Figure 2.6. Proposed peroxygenase mechanism for indole oxidation by DHP in the presence of H$_2$O$_2$.

reaction product has been previously observed with DHP and the peroxidase reaction product, dihaloquinone. This chemistry is revisited in the subsequent DHP studies with 3-oxindole. The two main observations from these stopped flow studies were 1) the perturbation of the Soret of ferric w.t. DHP B implying a potential binding affinity for 1a; and 2) the apparent reduction of ferric w.t. DHP to ferrous / oxyferrous by one of the primary products of indoles oxidation. A tentative reaction mechanism is presented in Figure 2.3 and is supported by the stopped-flow UV-visible studies with 1a.
Resonance Raman studies with DHP B and various indoles substrates. Resonance Raman spectra were collected of ferric w.t. DHP B in the presence of 50 eq. of various indoles in 10% MeOH (v/v) / 100 mM KP$_i$ at pH 7 to ascertain which, if any, of the indoles

**Figure 2.7.** Resonance Raman spectra of various indoles (500 μM) with ferric w.t. DHP B (50 μM) in 100 mM KP$_i$ at pH 7 and 10% MeOH (v/v)
studied exhibited a binding affinity for ferric w.t. DHP B. Specifically, altering the coordination sphere of the heme iron induces known spectral shifts in the high frequency heme vibrational modes. The results are illustrated in Figure 2.7. Panel A shows the Raman spectra collected for the brominated indoles and 1b, which clearly shows that only 1a, the with bromine in the 5-position, was capable of perturbing the heme iron from primarily 6cHS to 5cHS Ferric DHP B. This is indicated by a loss of the 6cHS heme population ($\nu_3 = 1477$ cm$^{-1}$; $\nu_2 = 1563$ cm$^{-1}$) and an increase in the 5cHS heme population ($\nu_3 = 1494$ cm$^{-1}$; $\nu_2 = 1569$ cm$^{-1}$; $\nu_{10} = 1630$ cm$^{-1}$). This shift to 5cHS is believed to be indicative of the distal-His$^{55}$-stabilized aqua ligand in ferric being displaced, and opening of the distal coordination site of the heme iron. Previous reports have shown a similar phenomenon for with ferric DHP B in the presence of 4-BP, a known inhibitor of DHP, where X-ray crystallography studies have shown that 4-BP binds in the distal pocket and “pushes” His$^{55}$ into an “open” or solvent exposed confirmation (11, 22). This limits the availability of His$^{55}$ to stabilize the water ligand through hydrogen bonding, thus lowering the population of 6cHS heme. This same observation for 1a implies a similar phenomenon, and would also rule out the exchange of the distal H$_2$O for 1a coordinating to the heme iron as a cause for the observed spectral shift in the Soret. In Figure 2.7.B, the spectra for the 5-substituted halogen series are presented. There was an obvious decrease in the 6cHS heme population and increase in the population of 5cHS that follows the halogen series (I > Br > Cl > F) indicative of a binding preference for 5-haloindoles with ferric DHP B. To correlate this phenomenon and allow for quantitative assessment, binding affinity studies were performed to determine the $K_d$ value for those
indoles that exhibited a perturbation in the heme coordination environment by resonance Raman.

**Protein-Ligand Binding Studies with DHP B w.t.** A series of studies to determine the dissociation constant \( (K_d) \) for relevant indoles was performed using UV-visible spectroscopy and previous reported methods. Figure 2.8 shows the UV-visible spectra of ferric DHP B w.t. in 10% MeOH (v/v) / 100 mM KPi at pH 7 only and in the presence of 50 eq. of 1a. Consistent with the stopped-flow results, a broadening and a blue shift of the Soret was observed to ca. 402 nm and attenuation of the Soret consistent with ligand dissociation from the heme iron was also observed. The visible bands remained consistent with the exception

![Figure 2.8](image)

**Figure 2.8.** (A) UV-visible spectrum of 10 μM ferric w.t. DHP B (black) and 10 μM ferric w.t. DHP B in the presence of 50 eq. 1a; (B) UV-visible spectra of the change in absorbance at various concentrations of 1a
of a new broad shoulder at 540 nm and a new peak at 585 nm (Figure 2.8.A insert). To ascertain the $K_d$ for 1a, the spectrophotometer was baselined using 10 µM Ferric DHP B w.t. in 10% MeOH and the spectra were recorded in the presence of various concentrations of the by nonlinear regression provides a calculated $A_{max}$ of 0.51, which was in turn used to calculate $\alpha$ for the average $\Delta A$ for each [1a]. A second nonlinear regression plot provided an apparent $K_d$ of 150.1 ± 9.8 µM for 1. This is almost a 10-fold higher affinity than what is observed with 4-BP. The $K_d$ values were experimentally determined for the other 5-substituted halogens (5Cl-Ind: 418.5 ± 35.4 µM; 5I-Ind: 57.7 ± 8.3 µM), and there is an apparent trend in $K_d$ that correlates with the halogen series. In that, 5I-indole had the highest binding affinity and 5-Cl-indole had the lowest. The 5F-indole was too low to measure due to poor solubility at the higher concentrations necessary to induce Soret perturbation, and therefore, its binding affinity is lower than 5Cl-indole. Of note, all three of the quantified indoles exhibited higher affinity for DHP than any of the 4-monohalophenols, a known inhibitor of the enzyme. X-ray crystal studies are underway to attempt to reveal where these 5-haloindoles bind in the distal cavity and how they are oriented with respect to the active site.
Figure 2.9. UV-visible spectra of 3-oxindole (~250 μM) after addition of LE in 100 mM KP\textsubscript{i} (pH 7) scanned at times indicated (aerobic); B) UV-visible spectra of 3-acetoxyindole (~250 μM) and ferric \textit{w.t.} DHP B (10 μM) only in 100 mM KP\textsubscript{i} (pH 7) (black), and after the addition of LE for time = 0 – 5 minutes (aerobic); C) UV-visible spectra of 3-oxindole (~250 μM) and ferric \textit{w.t.} DHP B (10 μM) after the addition of LE for time = 3 – 6 minutes, 10 and 12 minutes (anaerobic)
Studies of 3-oxindole with Ferric DHP B w.t. The observed reduction of ferric DHP B to oxyferrous in the stopped-flow UV-visible studies performed necessitated additional scrutiny. It is known that 3a can be oxidized to 7a upon exposure to O₂. Subsequently, 7a can react with an equivalent of 3a to form the indigo intermediate, 8a, which is subsequently oxidized to form the indigo chromophore, 9a. To observe the rate of formation due to O₂ only, 3-acetoxyindole was reacted with porcine liver esterase (LE) to form 3a in situ and the UV-visible spectrum was recorded every minute for 5 minutes (Figure 2.9.A). There was minimal change observed with only a slight increase in the spectra ca. 289 and 640 nm. We believe these slight increases are indicative of the formation of minimal amounts of the 5Br-indigo species, 9a. Next, ferric w.t. DHP B was incubated with 3-acetoxyindole and no spectral changes were observed (Figure 2.9.B (black spectrum)). Upon addition of LE, UV-visible spectra were immediately recorded every minute for 5 minutes (Figure 2.9.B). There was an immediate shift from ferric to oxyferrous DHP B - even at t=0. A considerable increase in the spectral features at 289, 370, and 659 nm was also observed corresponding to the formation of 9a. This spectral increase was greater than what would be expected for a stoichiometric conversion. Therefore, ferric DHP B was prepared under anaerobic conditions and LE added to initiate reaction in the glove box (Figure 2.9.C). Though some increase at ~650 nm was initially observed by the time the first spectra (time = 3 min.) could be recorded, it is likely due to stoichiometric reduction of oxyferrous or a minimal amount of unpurged O₂ remaining in solution. Despite this, the spectra collected remained constant over 12 minutes. Additionally, a broad peak at ~558 nm appeared and remained constant over time. This is indicative of the formation of ferrous w.t. DHP B (26). Therefore, the oxidation
of 3a to 7a, and subsequent oxidation to the indigo dimer, 9a, is proposed to proceed through a novel oxidase mechanism (Figure 2.10). Oxyferrous DHP B reacts with one equivalent of 3a to evolve 7a and a water molecule, which likely results in the formation of Compound II. This can subsequently react with another equivalent of 3a to form 7a and H₂O, restoring ferrous DHP B, which can readily rebind O₂, and restart the catalytic cycle. However, control studies eliminating a roll for superoxide or •OH for the observed reactivity need to be performed.

Figure 2.10. Proposed oxidase mechanism for w.t. DHP B with 3-oxindole
Discussion

Dehaloperoxidase from the marine annelid, *A. ornata*, possesses a biologically relevant and well-investigated globin and peroxidase function. These present studies were conclusively able to elucidate novel peroxynase and oxidase pathways for the oxidation of indoles with DHP and H$_2$O$_2$. With these diverse chemistries arising from two resting oxidation states of the enzyme, it is apparent that the nature of the substrate dictates the enzymatic pathway utilized. With Fe$^{II}$, O$_2$ dictates globin function, but the enzyme can also perform oxidase chemistry in the presence of a suitable reducing substrate. The Fe$^{III}$ species on the other hand, in the presence of H$_2$O$_2$ performs exclusively peroxidase chemistry with trihalogenated phenols, but can also perform peroxynase chemistry in the presence of indoles as demonstrated in these studies. Binding affinity for DHP did not exhibit an apparent correlation with selection of the catalytic pathway utilized for oxidation. These studies showed that 5-halophenols exhibit a binding affinity for DHP and exhibited catalytic peroxynase turnover, but 4-halophenols have a known binding affinity for ferric DHP and are an *inhibitor* of DHP. Further, trihalogenated phenols are a natural substrate for DHP and exhibit no binding affinity for ferric DHP. X-ray crystal structures have shown that these monohalophenols “push” the flexible distal His to a solvent exposed conformation, which reduces its ability to bind a water ligand, and also hinders its ability to deprotonate H$_2$O$_2$ in the initial step of ES formation. If the indoles that exhibit binding affinity are also pushing the distal histidine away from the iron center, this raises the question of how these indoles do not cause enzyme inactivation or do not exhibit attenuated reactivity as compared with their
non-binding analogues. Examining the structure of the substrate, indole binding and displacement of the distal His may orient the indole nitrogen such that it can act as the proton acceptor during $H_2O_2$ activation when oxidizing ferric to Compound ES. X-ray crystal studies are currently underway to observe where the indole substrates localize when co-crystallized with DHP. This may also serve to explain the interesting mixed 18O-labeling results observed with the 3-oxindolenine products of 1b and 4Br-indole oxidation.

Binding affinity was also not indicative of the overall turnover for the indoles studied. This is exemplified by the equivalent turnover of 1a and 7Br-indole, but only 1a exhibited an affinity for ferric DHP. However, it is possible that the other halogenated indoles bind in a manner that does not disturb the heme coordination environment but still aligns them for peroxynasal or peroxidase reactivity. Nevertheless, the studies with varying arene substituents on the indole demonstrated that ring substitution considerably enhances peroxynasal reactivity. While these substituted indoles more sterically bulky, the arene halogen may provide a “handle” to facilitate proper molecular orientation for peroxynasal oxidation.

DHP has been characterized as a globin, yet it possesses minimal sequence homology with other globins. Further, it lacks any structural analogy to cytochrome c peroxidase, a model for the heme peroxidase family. The results presented in these current studies further challenge our understanding of the structure-function relationship of heme proteins because DHP has now been shown to also possess both a peroxynasal and oxidase function like P450, without possessing any known structural or sequence homology to the P450 family. The most profound difference between these systems is the proximal ligand coordinated to
the heme iron. P450 and chloroperoxidase utilize a conserved cysteine residue that coordinates to the heme iron. The redox potential of this heme-thiolate conjugate is proposed to be fine-tuned through hydrogen bond interactions of this proximal cysteine with other hydrogen bond acceptors from the protein scaffold (aspartic/glutamic acid residues, peptide backbone). DHP, however, utilizes a histidine in the proximal position, which is a motif common to peroxidases and globins.

References


Chapter 3 - Reactivity and Spectroscopic Investigations of Dehaloperoxidase with Nitrophenols: Peroxygenase Oxidation of a Non-native Substrate

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Abstract

In these studies, we expand on the chemistry of dehaloperoxidase (DHP) with the non-native substrate, 4-nitrophenol. We quantify formally quantify the $^{18}$O enrichment in studies with labeled water and hydrogen peroxide. Further, stopped-flow UV-visible spectroscopy was used to examine reaction intermediates for the enzyme during this peroxygenase oxidation reaction. LC-MS studies with the native substrate of DHP, 2,4,6-trichlorophenol, were conclusively shown to be oxidized by a peroxidase mechanism, but provided only the dichloroquinone product without observed intermediates. However, labeled LC-MS studies with 2,4-dichlorophenol revealed a multi-step oxidation pathway involving both peroxidase and peroxygenase activity as well as an isolated dehalogenation pathway.

Introduction

A number of natural products contain nitro groups (1); however, the bulk of the accumulation of these products in soil and water samples arises from industrially synthesized nitrophenols (NP) and other nitroaromatic compounds that have found uses as polymers, pesticides, dyes and explosives (2-6). They are known carcinogens and considered highly toxic. Further, they are listed as a priority pollutant and present a long-term health risk according to the EPA(7-12). While some methods of biodegradation exist in Nature (13-15), it remains highly desirable to find additional techniques for combating the buildup of these toxic compounds. Extensive studies involving various isoforms of Cytochrome P450 from rat liver microsomes and other eukaryotes have been studied for their ability to metabolize the
The hydroxylation of 4-NP to form 4-nitrocatechol (4-NC). The various isoforms of P450 are known to catalyze a broad scope of substrates. As DHP has shown similar peroxigenase chemistry when compared to P450 for haloindoles, we wanted to investigate if DHP was able to catalyze the hydroxylation of nitroaromatics such as 4-NP. Understanding the method of oxidation for these substrates may provide insight into developing efficient degradation catalysts to combat their continued accumulation in the ecosystem.

**Figure 3.1.** Proposed degradation products for the oxidation of 4-NP with w.t. DHP B and H₂O₂: Number in parenthesis corresponds to the normalized level of ¹⁸O enrichment based on the isotopic abundances observed. Calculations were performed similar to previously published methods (16).
In the initial studies performed in collaboration with Dr. Jennifer D’Antonio, we reported the oxidation of 4-nitrophenol (4-NP) via a newly reported peroxygenase mechanism. Based upon biochemical assays, HPLC, and LC-MS studies, it was proposed that the reaction of 4-NP with DHP in the presence of H₂O₂ proceeded through a series of hydroxylation reactions, and then a subsequent two electron oxidation to form a quinone species as shown in Figure 3.1. Using isotopic labeling studies with H₂¹⁸O₂ and H₂¹⁸O, the first step, i.e. the insertion of the O-atom into 4-NP forming 4-NC, follows a peroxygenase mechanism wherein the O-atom is derived from hydrogen peroxide. However, the reactivity of DHP with this non-native substrate leaves a number of outstanding questions pertaining to the mechanism of 4-NP conversion to 4-NHBQ that still need to be addressed. Further, stopped-flow UV-visible studies were performed with 4-NP to gain mechanistic insight regarding which activated form of DHP is performing this novel reactivity. Finally, we investigated whether a similar peroxygenase activity was observed for the native substrates 2,4-dichlorophenol (DCP) and 2,4,6-trichlorophenol (TCP) using similar ¹⁸O-labeled LC-MS studies. The TCP studies confirm a dehaloperoxidase mechanism for its oxidation to dichloroquinone (DCQ) with DHP B in the presence of H₂O₂, whereas the DCP studies exhibited both peroxidase and peroxygenase chemistry as well as an independent dehalogenation pathway.

**Experimental**

*Materials and Methods.* Isotopically labeled H₂¹⁸O₂ (90% ¹⁸O-enriched) and H₂¹⁸O (98% ¹⁸O-enriched) were purchased from Icon Isotopes (Summit, NJ). Acetonitrile (MeCN)
was HPLC grade and all other chemicals were purchased in the highest quality from VWR, Sigma-Aldrich or Fisher Scientific and used without further purification. UV-visible spectroscopy was performed on a Cary 50 UV-visible spectrophotometer. Stock solutions (2 mM) of substrates (2-NP, 3-NP, 4-NP, DNP, TNP, DCP and TCP) were prepared in 100 mM Kp$_i$ at desired pH and stored at -80 °C until needed and were periodically screened to ensure that they had not degraded. Aliquots were thawed and stored on ice during use. Solutions of H$_2$O$_2$ were prepared fresh daily and kept on ice until needed. The concentration was determined by UV-vis ($\varepsilon_{240} = 46$ mM$^{-1}$cm$^{-1}$). For DHP B wild type, DHP B (Y38F), DHP B (Y28/38F), and DHP A, expression and purification were performed as previously reported (17, 18). Lyophilized horseradish peroxidase and horse heart myoglobin were purchased from Sigma-Aldrich and stored at -20 °C until utilized. They were reconstituted in 100 mM KP$_i$ at pH 6 and concentrations determined using accepted values.

**Preparation of Ferric enzyme.** An aliquot of DHP B in 100 mM KP$_i$ (pH 7) and 40% glycerol (v/v) was thawed and charged with an excess of potassium ferricyanide (~2 min) to allow for quantitative conversion to ferric DHP. Excess oxidizing agent was removed using gel filtration chromatography (Sephadex G-25) pre-equilibrated with 100 mM KP$_i$ at the desired pH. The enzyme-containing eluent fraction was collected and subjected to successive buffer exchanges (3x) via centrifugation using an Amicon Ultra centrifugal filter (10,000 KDa MW). Enzyme concentration was determined spectrophotometrically ($\varepsilon_{\text{Soret}} = 116,400$ mM$^{-1}$1 cm$^{-1}$) for all Isoenzymes (18, 19).

**Preparation of Oxyferrous DHP B.** An aliquot of DHP B in 100 mM KP$_i$ (pH 7) and 40% glycerol (v/v) was thawed and charged with an excess of sodium ascorbate, and
incubated for 30 min at 4 °C to quantitatively reduce the enzyme to the ferrous form. The binding of atmospheric O₂ provided oxyferrous DHP B (418 (Soret); 542, 578 nm) (19). Excess ascorbate salt was removed utilizing the same protocol as that for potassium ferricyanide removal during ferric preparation (vide supra). The presence and concentration of oxyferrous was determined spectrophotometrically (19).

**Enzyme Assay Protocol.** Reactions were performed at various pH values in 100 mM KP₁ at 25 °C. Buffered solutions (total reaction volume 250 μL) of DHP (10 μM final concentration) and nitrophenol (500 μM final concentration) were pre-mixed, and then the reaction was initiated upon addition of H₂O₂ (500 μM final concentration). After 5 minutes, reactions were quenched with excess catalase. Reaction samples were analyzed by reverse-phase HPLC coupled with a photodiode array detector as described below. Anaerobic studies were performed as described above in an MBraun Lab Master 130 nitrogen-filled glove box (<1 ppm O₂, <1 ppm H₂O) using argon degassed solutions of buffer, peroxide, substrate and enzyme. Caution was used in degassing enzyme to avoid enzyme precipitation.

**Analysis of Sample by HPLC.** A 100 μL aliquot of reaction sample was diluted 10-fold with 900 μL of 100 mM KP₁ at reaction pH. Diluted samples were analyzed using a Waters 2796 Bioseparation Module with an in-tandem Waters 2996 Photodiode Array Detector equipped with a Thermo-Scientific ODS Hypersil (150 mm x 4.6 mm) 5 μm particle size C18 column. Separation of observed analytes was performed using a linear gradient of binary solvents (solvent A - H₂O containing 1% trifluoroacetic acid: solvent B - MeCN). Elution was performed using the following conditions: (1.5 mL/min A:B) 95:5 to 5:95 linearly over 4 minutes; 5:95 isocratic for 1 minute; 5:95 to 95:5 linearly over 1 minute, then
isocratic for 7 minutes. Data analysis was performed using the Empower software package (Waters corp.). Calibration curves for 2-NP, 3-NP, 4-NP, DNP, and TNP were performed using serial dilutions of commercially available analytes and utilized to determine the amount of substrate conversion.

**LC-MS Studies with Nitrophenols.** Experiments were analyzed using a 6210LC-TOF mass spectrometer (Agilent Technologies, Santa Clara, CA). Analyte separation was performed using the same conditions and column as HPLC studies with the exception of solvent A (water with 0.1 % formic acid). Samples were analyzed using electrospray ionization in negative mode to provide observation of the \([M-H]\) species. Spectra were collected each second while scanning in the range from 100 – 1000 m/z. Data analysis was performed using Agilent software. Quantitation of the amount of \(^{18}\text{O}\)-labeled incorporated was performed using previously established methods (16). In labeled \(\text{H}_2\text{O}_2\) experiments, an aliquot of 2.15% (w/w) solution of 90% enriched \(\text{H}_2^{18}\text{O}_2\) was diluted 3-fold to provide a peroxide solution of \(~20\) mM. A final reaction volume (100 mM KP, pH 7) of 250 \(\mu\)L containing 10 \(\mu\)M enzyme, 50 eq. of substrate, and \(~50\) eq. of labeled peroxide was allowed to react for 5 min before quenching with catalase enzyme. Then, 20 \(\mu\)L of undiluted reaction mixture was injected for LC-MS analysis.

For labeled water studies, stock solutions of reactants ([enzyme] = 120 \(\mu\)M; \([\text{H}_2\text{O}_2]\) = \(~12.5\) mM) in unlabeled water were kept at sufficiently high concentrations to allow for the 98% enriched \(\text{H}_2\text{O}\) to be diluted to \(~89\)% in the final reaction mixture. Labeled water (207.5 \(\mu\)L) was charged with 21 \(\mu\)L enzyme, 10 \(\mu\)L \(\text{H}_2\text{O}_2\), and 12.5 \(\mu\)L substrate (in MeOH) and
reacted for 5 min. Reactions were quenched with catalase and 20 μL of undiluted reaction mixture was injected for LC-MS analysis.

**Stopped-Flow UV-visible Studies with DHP and Nitrophenols.** Experiments were performed using a Bio-Logic SFM-400 triple mixing stopped flow instrument coupled to a rapid scan diode array UV-visible spectrophotometer. Experiments were performed at room temperature and all solutions were prepared in 100 mM KP1 (varying pH). Single mixing experiments reacting 10 μM enzyme with 100 μM H2O2 were performed to determine the time of maximum formation of Compound ES (wild type DHP) or Compound I (DHP B (Y28/38F)). Double mixing experiments were then performed with enzyme and H2O2 using the appropriate delay times to observe Compound ES / Compound I reactivity with 4-NP (10 equiv.). Spectra (900 scans) were collected over short (2, 25 and 250 ms; 300 scans each) and long acquisitions. For substrate pre-incubation studies with DHP, single mixing conditions were utilized and observed over 83 s. Specifically, a solution of DHP (10 μM final concentration) and 4-NP (10 equiv.) was prepared and then mixed with H2O2 (10 equiv.). Data were evaluated using Specfit Global Analysis System software (Spectrum Software Associates) and fit by SVD analysis as either one-step, two species or two-step, three species irreversible mechanisms, where applicable.

**Results and Discussion**

**Reactivity of Nitrophenols with DHP and H2O2.** A series of substituted nitrophenols were screened for reactivity with DHP in the presence of H2O2 (Table 3.1). The reactions that
were performed in conjunction with Dr. Jennifer D’Antonio are noted below. Reactions were performed at pH 6 using a final concentration of 10 μM enzyme with 50 equiv. of substrate,

### Table 3.1. Reactivity of DHP and Isoenzymes with 4-NP under various conditions

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>Conversion (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DHP B Ferric</strong></td>
<td>+2-nitrophenol</td>
<td>49.6 (±0.8)</td>
</tr>
<tr>
<td></td>
<td>+3-nitrophenol</td>
<td>43.7 (±1.3)</td>
</tr>
<tr>
<td></td>
<td>+4-nitrophenol</td>
<td>39.4 (±0.7)*</td>
</tr>
<tr>
<td></td>
<td>+2,4-dinitrophenol</td>
<td>16.2 (±1.6)*</td>
</tr>
<tr>
<td></td>
<td>+2,4,6-trinitrophenol</td>
<td>n. r.*</td>
</tr>
<tr>
<td><strong>DHP B Oxyferrous</strong></td>
<td>+4-nitrophenol</td>
<td>37.6 (±1.7)</td>
</tr>
<tr>
<td><strong>DHP B Ferric / 4-nitrophenol</strong></td>
<td>+500 μM D-mannitol</td>
<td>37.6 (±0.6)*</td>
</tr>
<tr>
<td></td>
<td>+500 mM formate</td>
<td>35.6 (±0.2)*</td>
</tr>
<tr>
<td></td>
<td>+SOD</td>
<td>37.8 (±4.2)*</td>
</tr>
<tr>
<td></td>
<td>+DMSO</td>
<td>38.3 (±0.4)</td>
</tr>
<tr>
<td></td>
<td>+anaerobic</td>
<td>43.2 (±0.4)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>Conversion (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DHP B (Y38F) Ferric</strong></td>
<td>+4-nitrophenol</td>
<td>37.1 (±1.4)*</td>
</tr>
<tr>
<td></td>
<td>+2,4-dinitrophenol</td>
<td>16.6 (±1.2)*</td>
</tr>
<tr>
<td></td>
<td>+2,4,6-trinitrophenol</td>
<td>n. r.*</td>
</tr>
<tr>
<td><strong>DHP B (Y28/38F) Ferric</strong></td>
<td>+4-nitrophenol</td>
<td>37.2 (±1.0)*</td>
</tr>
<tr>
<td></td>
<td>+2,4-dinitrophenol</td>
<td>17.2 (±0.6)*</td>
</tr>
<tr>
<td></td>
<td>+2,4,6-trinitrophenol</td>
<td>n. r.*</td>
</tr>
<tr>
<td><strong>DHP A Ferric</strong></td>
<td>+4-nitrophenol</td>
<td>21.6 (±0.1)</td>
</tr>
<tr>
<td></td>
<td>+2,4-dinitrophenol</td>
<td>4.1 (±1.3)*</td>
</tr>
<tr>
<td></td>
<td>+2,4,6-trinitrophenol</td>
<td>n. r.*</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>Conversion (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Other Enzymes</strong></td>
<td>horseradish peroxidase</td>
<td>+4-nitrophenol</td>
</tr>
<tr>
<td></td>
<td>horse heart myoglobin</td>
<td>+4-nitrophenol</td>
</tr>
</tbody>
</table>
initiated by the addition of 50 equiv. of H₂O₂, incubated at room temperature and finally quenched with catalase after 5 minutes. Reactions were then subsequently analyzed by HPLC. The mononitrophenols exhibited the highest reactivity (~40–50% conversion). Conversely, reactivity was ~3-fold lower for 2,4-dinitrophenol (DNP) and completely absent with 2,4,6-trinitrophenol (TNP).

After observing conversion with DHP B, a sampling of the nitrophenol substrates was also screened for reactivity with DHP A and two mutants of DHP B. Consistent with previous studies with DHP A involving the peroxidase oxidation of trihalogenated phenols (18) and the peroxxygenase oxidation of indoles, lower overall reactivity was observed with DHP A. When studies employed the mutants of DHP B [(Y38F) and (Y28/38F)] that have been shown to form Compound I (an Fe⁴⁺-oxo with porphyrin π-cation radical) rather than the Compound ES intermediate found in WT DHP, nearly identical results to those of the wild type enzyme were observed.

*Labeled Isotope Studies with 4-NP with DHP.* Isotopically labeled studies were performed using H₂¹⁸O₂ with wild type DHP B and 4-NP. The results of these studies are summarized in Figure 3.1. It can be seen that the formation of 4-NC occurs via a peroxxygenase mechanism because the studies with H₂¹⁸O₂ exclusively exhibited a +2 Da shift, which is indicative of this type of hydroxylation. Catechols are known to cleave and form the ring-opened products; however, these were not observed in either the HPLC studies performed by Dr. D’Antonio or in the mass spectrometric analysis performed here. Additional labeled H₂O and H₂O₂ studies optimized to more clearly observe the secondary
oxidation products (4-NPg and 4NQ) are needed to determine the mechanistic origin of the second O-atom insertion.

**Stopped-Flow UV-visible Studies with 4-NP and DHP B.** Experiments were performed at pH 6 and 7. Single mixing experiments reacting 10 μM enzyme with 10 equivalents of
were employed to determine the time of maximum formation of Compound ES with wild type (418 (Soret), 545, 584 nm) or Compound I with (Y28/38F) (406 (Soret), 545, 584 nm). These times were subsequently used to perform double-mixing experiments to observe the reactivity of the activated enzyme with 10 equiv. of 4-NP. In (Figure 3.2), Compound ES was observed to readily be reduced to ferric (406 (Soret), ~507 nm) over the 83 s observation time. This would be consistent with an overall two electron process for the hydroxylation of 4-NP via a peroxygenase pathway. Interestingly, the pH 7 studies under the same conditions (Figure 3.3.A) exhibited no reactivity between the ferryl intermediate (417 (Soret); 545, 584 nm) and 4-NP over the 83 s observation time. The ferryl species was seen to return to the ferric state slowly over longer observation times (data not reported). Because Compound ES (Fe$^{IV}$-oxo with tyrosyl radical) and Compound II (Fe$^{IV}$-oxo) are indistinguishable by UV-visible spectroscopy under the conditions employed, we cannot conclusively assign which is present as the observed ferryl species; However, Compound ES is known to decay to Compound RH in the presence of excess H$_2$O$_2$ or in the absence of a reducing substrate within the same amount of time, and the lack of formation of Compound RH over the time course of the reaction seems to support a Compound II species as the origin of the ferryl intermediate rather than a Compound ES species.

The formation of Compound II could arise from one electron chemistry wherein the tyrosyl radical of the preformed Compound ES intermediate is reduced by one electron upon oxidation of the phenol substrate. Dunford and coworkers have investigated the effects of the electron donating ability of ring substituents of phenolic substrates on the kinetics of Compound I reduction with HRP (20). They have demonstrated that more electron
Figure 3.3. (A) Stopped-flow UV-visible spectroscopic monitoring (900 scans, 83 s) of the double-mixing reaction between preformed DHP B w.t. Compound ES and 10 eq. 4-NP at pH 7; (B) Stopped-flow UV-visible spectroscopic monitoring (900 scans, 83 s) of the double-mixing reaction between preformed DHP B (Y28/38F) Compound I and 10 eq. 4-NP at pH 7
withdrawing substituents reacted slower with Compound I. This supports the proposed first step of Compound I reduction of HRP with phenols, which is believed to be the abstraction of the phenolic hydrogen (proton and electron) to form Compound II (Fe^{IV}-OH) and a phenolic radical that would be delocalized in the ring. In peroxidase chemistry, this phenolic radical could subsequently react with solvent water to provide the oxidized hydroxylated product. By analogy, in a peroxynasme mechanism for the hydroxylation of 4-NP by DHP, it holds that if the first step is also abstraction of the phenolic hydrogen, the strongly withdrawing nitrophenols would react more slowly than the halogens and may also be a factor for decreasing reactivity as nitro- substitution is increased (4-NP > DNP >> TNP) as seen in Table 3.1.

Continuing with the supposition that hydrogen atom abstraction is the initial step for peroxynasme hydroxylation with DHP as is the consensus for P450 systems, the pK\textsubscript{a} of the substrates may also play a role in the chemistry. At pH 7, 4-NP (pK\textsubscript{a} ~7.2) would be slightly less than 50% in the phenolate form, which would be able to provide only an electron and no proton. Therefore, this may serve to slow a final hydroxylation step where an Fe^{IV}-OH (Compound II) of DHP reacts with the 4-NP radical to provide 4-NC and restore the ferric form of the enzyme by an “oxygen rebound” mechanism (Figure 3.4). Thus, the phenolate form would provide an electron and “lock up” the enzyme at an Fe^{IV}=O (Compound II) that would still require protonation in order to hydroxylate the substrate via the peroxynasme pathway. This may be supported by the observation of the minimal reactivity of DHP towards DNP (pK\textsubscript{a} ~4.2) and the complete lack of turnover for TNP (pK\textsubscript{a} ~0.4) at even pH 6 as both species would also be primarily in the phenolate form.
Independent of the pKₐ of the substrate, the ability to form an Fe⁴⁺-OH is more dependent on the pKₐ of Compound II for an enzyme. CPO, a proposed model for P450, is believed to form an Fe⁴⁺-OH. Compound II species based on resonance Raman and EXAFS studies by Gray and Dawson (21). It is believed to have a pKₐ ≥ 8.2, so that at neutral pH, it would be expected to be found in the protonated form, facilitating the rebound step for hydroxylation of the substrate radical. CPO, unlike other peroxidases, readily performs this two-electron chemistry similar to P450. Peroxidases, on the other hand, execute peroxygenase chemistry poorly, but excel at stepwise one-electron oxidations. HRP, a model peroxidase, is believed to have a pKₐ ≤ 3.2, and thus, would be deprotonated at physiological pH (confirmed by EXAFS) (21). Therefore, ignoring substrate sterics or access to the active site, enzymatic systems that possess a more basic form of Compound II, and allow for the formation of an Fe⁴⁺-OH would be better suited for hydroxylation of substrate via a peroxygenase mechanism. Consequently, the variation in the reactivity observed in these
stopped flow studies between pH 6 and 7 for w.t. DHP B with 4-NP may be attributable to the pKa of the substrate, but it would also depend on the pK\textsubscript{a} of the ferryl oxo species for Compound II of DHP B, such that switching between pH 6 and 7 may alter the structure of Compound II. At pH 6, Compound II of DHP may be protonated or at least more protonated than it would be at pH 7, and allow for more facile hydroxylation via a peroxygenase mechanism. Therefore, regardless of the form of the substrate, Fe\textsuperscript{IV}-OH may be the

**Figure 3.4.** Possible mechanisms for peroxygenase hydroxylation of 4-NP with DHP and H\textsubscript{2}O\textsubscript{2}
predominant form, allowing for peroxygenase chemistry. This would also seem to be supported by the indole studies with DHP and \( \text{H}_2\text{O}_2 \), where peroxygenase chemistry readily occurred at pH 7.

**Figure 3.5.** (A) Stopped-flow UV-visible spectroscopic monitoring (900 scans, 83 s) of the single-mixing reaction between 10 \( \mu \text{M} \) Compound ES preincubated with 10 eq. 4-NP and 10 eq. \( \text{H}_2\text{O}_2 \) at pH 6; (B) Calculated UV-visible spectra for ferric DHP + 4-NP (black), Compound ES (red), and the return to the ferric state (blue) based on a two-step, three-species irreversible fit; (C) Calculated concentration vs. time plot; (D) Plot of raw data vs. fit at 406.5 nm
In studies with DHP B (Y28/38F), the Compound I intermediate reacts faster than the time scale of the instrument because double mixing studies with Compound I and 4-NP resulted in the observation of only the ferric form of the enzyme followed by a decay to a Compound RH-like species (Figure 3.3.B). The spatial proximity of the two oxidizing equivalents would allow for more facile peroxygenase chemistry for this mutant that forms Compound I. Thus, 4-NP being converted via a peroxygenase pathway exhibiting kinetically faster reactivity with DHP B (Y28/38F) is not surprising. Remarkably at pH 7, complete conversion of Compound I to ferric was also observed to occur very rapidly (Figure 3.3.B), which is in contrast to the wild type studies. In that, Compound I rapidly reacts despite the increased presence of the phenolate form. This would seem to contradict our theory on the protonation state of the substrate slowing peroxygenase turnover at pH 7, but Compound II of (Y28/38F) DHP may have a higher pKa than that of wild type allowing for the rapid conversion of Compound I to ferric as observed. Thus, at pH 7, the population of FeIV-OH for (Y28/38F) would be greater than that of wild type and facilitate rapid hydroxylation.

Experiments were also performed where 4-NP was preincubated with ferric DHP B w.t. and subsequently mixed with 10 eq. of H$_2$O$_2$ (Figure 3.5), which allowed for comparison of the kinetics of Compound ES reduction to ferric between the two experimental conditions (preformed ES vs. preincubated 4-NP). Panel A shows the raw data of the spectra and clearly shows ferric DHP evolving to Compound ES, with the enzyme subsequently returning to ferric oxidation state. This is shown more clearly in the SVD analysis fit (panel B). When
comparing panel C from Figure 3.2 and 3.5, the preincubation studies exhibit a more rapid return to ferric. This is even more considerable given that in Figure 3.5A, Compound ES is not preformed, but still provides a higher concentration of ferric over the observation time. In the preformed Compound ES studies, a first order fit provided a $k_{obs} = 2.73 \pm 0.046 \times 10^{-2}$. In the preincubation studies, a second order fit gave $k_2 = 5.43 \pm 0.042 \times 10^{-2}$. Both of these rate constants correspond to the reduction of Compound ES to ferric DHP, and it is apparent that the preincubation studies react about twice as fast. An explanation may be that preincubation allows for the uncharged 4-NP to enter that distal cavity of ferric DHP and subsequently react more rapidly with Compound ES as it is formed in situ. Another possibility is that preformed Compound ES may begin to deactivate by forming Compound RH and limit the availability of the reactive catalyst for substrate turnover.

It is also important to compare these stopped-flow results with those performed with halogenated phenols and halogenated indoles, which are both believed to likely be native substrates of this enzyme. Stopped-flow studies with both TCP and 5-bromoindole demonstrated that DHP is not only reduced to the ferric oxidation state during substrate turnover, but is ultimately reduced further to evolve the ferrous / oxyferrous form of DHP, restoring the necessary globin function. While the exact mechanism of how the enzyme further reacts with the products of oxidation to do this is not yet fully understood, studies with DCQ (19) and 5-bromo-3-oxindole both showed reduction of ferric DHP to the ferrous / oxyferrous forms. This is even more remarkable considering each is degraded by two different oxidization pathways. Yet, the non-native substrate, 4-NP, or its oxidation products did not exhibit the ability to restore ferrous / oxyferrous and globin function despite the fact it
likely evolves a quinone species. This implies that nitrophenol and its enzyme-catalyzed oxidation products can only be oxidized by the higher valent iron species of DHP, i.e. Compound ES or Compound II, but not the ferric form of the enzyme.

**LC-MS Studies with 2,6-dichlorophenol and w.t. DHP B.** Experiments were performed with the various permutations of labeled H$_2$O and H$_2$O$_2$ to ascertain whether DCP underwent either peroxidase or peroxygenase chemistry or a combination. In the unlabeled studies, three peaks corresponding to the empirical formulas C$_6$H$_4$Cl$_2$O$_2$ (t$_R$ ~4.2 min.), C$_6$H$_5$ClO$_2$ (t$_R$ ~2.9 min.), and C$_6$H$_3$ClO$_3$ (t$_R$ ~3.0 min.) were observed. Based on these, we tentatively assign these formulas to the structures shown in Figure 3.6. The first species (m/z 177 Da) is
tentatively believed to be a dichlorophenyl diol (DCPD). This proposed initial hydroxylation step to form DCPD occurs via a peroxidase mechanism because the $^{18}\text{O}$ is only observed to be incorporated into this species when $\text{H}_2^{18}\text{O}$ was the solvent. Further, it appears that exchange of the original unlabeled O-atom in the phenol with solvent occurs during this hydroxylation process, as is evidenced by the high abundance of the +4 Da species in all of the studies for this product species with labeled water as the solvent. This type of $-\text{OH}$ substitution was also observed in $\text{H}_2^{18}\text{O}$ LC-MS studies with TCP, where the dichloroquinone product was seen to be shifted considerably to a +4 Da species. Regardless, it is clear that the first O-atom insertion to form DCPD does not arise from a peroxygenase mechanism or exogenous $\text{H}_2\text{O}_2$ because little to no incorporation is observed for the reactions utilizing labeled peroxide as the oxidant.

A second product species with a molecular mass of 143 Da is shown in Figure 3.6, and is proposed to be a monochlorophenyl diol (CPD), which likely arises from the dehalogenation of DCPD. This latter hypothesis is supported by the consistency in $^{18}\text{O}$-incorporation for both of these products in that a lack of label incorporation with $\text{H}_2^{18}\text{O}_2$ was observed for both species, while a similar incorporation profile of labeled oxygen was observed in the studies with $\text{H}_2^{18}\text{O}$. This dehalogenation step is likely enzyme assisted given the precedence for dehalogenation with this enzyme.

Lastly, the process by which CPD is converted to the chlorohydroxyquinone (CHQ) cannot be completely explained as yet and requires further investigation; however, if they are sequential reaction products, then we propose that the DCD product undergoes an additional O-atom insertion step via a peroxygenase pathway as evidenced by the +2 Da shift in the
labeled peroxide studies and is concomitantly or subsequently oxidized. However, at this
time, we cannot rule out the possibility that the quinone forms first and is then hydroxylated
to provide the same species. It is clear though that O-atom incorporation is occurring via the
peroxygenase mechanism because it is the only product species that exhibits a +2 Da shift
when labeled peroxide is used in either the single-labeled or double-labeled studies. Also, it
is unlikely that the +2 Da shift observed is result of a mixture of the chlorohydroxyquinone
and monochlorotriol (not observed in these studies) because a similar peak distribution would
be expected in the completely unlabeled reactions as well (note that the spectra are all
extracted over the same elution time).

Unlike the studies with 4-NP, we cannot completely rule out the possibility of ring-
opened products, and as such future HPLC UV-visible analysis and NMR studies of product
fractions may provide a better picture. Further, previous studies with 2,6-dichlorophenol
(2,6-DCP) with DHP A and H₂O₂ showed the major product to be 2,6-dichloro-3-hydroxy-
1,4-benzoquinone by NMR studies. Contrary to the observations here, this structural isomer
of 2,4-DCP did not undergo dehalogenation, but did undergo a hydroxylation step similar to
that seen with 2,4-DCP and also two-electron oxidation to the quinone. These differences in
the chemistry observed seems to continue to support the theory that the structure of the
substrate dictates the interaction with DHP and thus the mechanism utilized for oxidation.

While the acidic nature and charged vs. uncharged form of the substrate has been put
forward as a possibility for selection of the mechanistic pathway utilized for oxidation,
portions of these studies would seem to contradict this hypothesis. It is possible that charge
neutral species prefer the peroxygenase mechanism because they are better able to enter the
distal pocket and approach the active site more easily due to the hydrophobic nature of the distal cavity. The pKₐ values of 2-NP (~7.2), 3-NP (~8.3), and 4-NP (~7.2) imply that these substrates would be almost completely in the phenol form for these reactions performed at pH 6, and therefore, uncharged. The lower reactivity observed for DNP (pKₐ ~4.1) and lack of reactivity for TNP (pKₐ ~0.4) would seem to support this hypothesis as well. Although the LC-MS studies at pH 7 with DCP (pKₐ ~7.9) were shown to exhibit both peroxygenase and peroxidase chemistry, the initial hydroxylation step for DCP is consistent with peroxidase chemistry (DCPD), which is then proposed to undergo dehalogenation (CPD) and another O-atom insertion by a peroxygenase step, followed by oxidation to the quinone (CHQ). Therefore, rather than the acidic nature of the substrate dictating reactivity, it is more likely that the structure of the substrate ultimately determines the interaction and orientation within the distal pocket of DHP. Also, the acidity of Compound II for wild type DHP B or (Y28/38F) is likely to strongly influence the chemistry observed in these studies.

Trihalogenated phenols are believed to be a native substrate of DHP, and X-ray crystallographic studies have recently shown TCP readily binds in the distal pocket of DHP B. The trinitrophenols, on the other hand, were completely unreactive. The nitro substituents are sterically similar to the larger halogens (Br- and I-). However, the nitro groups are also more electron withdrawing than the halogens, and this may be a factor in the observed reactivity differences. In other X-ray crystallographic studies by Franzen and coworkers, it was shown that 4-halophenols bind in the distal pocket and were found to be inhibitors of peroxidase activity. The 4-XP substrates are believed to force the distal histidine to a solvent exposed conformation and limit its availability for hydrogen bonding during the activation of
H$_2$O$_2$ by DHP. In contrast, 4-nitrophenols exhibit no distortion of the Soret, which is indicative of alteration to the coordination environment of the heme iron, and are readily hydroxylated via the peroxygenase mechanism and necessarily must enter the distal pocket. We therefore, believe that it is highly unlikely that they bind coincident with 4-XPs in the distal pocket, and lends strength to the theory that substrate structure determines its interaction with the DHP and subsequently its ultimate pathway for oxidation. Additional X-ray crystallographic studies saturated with these various oxidative substrates may provide a better understanding of the selection criteria for orientation and reactivity with DHP and H$_2$O$_2$.

**Conclusions**

It has been demonstrated in this manuscript that DHP was capable of initially oxidizing nitrophenols via a peroxygenase mechanism. While nitrophenols are more structurally similar to the halogenated phenols, which undergo oxidation via a peroxidase cycle, these studies demonstrate that the ring substituents may play a role in the oxidative pathway selected. Additionally, the reactivity with 4NP broadens the substrate scope of DHP, and demonstrates its continued chemical promiscuity. Also, the LC-MS studies with DCP demonstrate a unique chemistry for this substrate when compared to that observed for TCP, which is remarkable considering their similarity in structure and ring substitution.

Considering that DHP has provided an evolutionary advantage for *A. ornata* by providing a both a globin function and a necessary detoxification pathway, its true asset to the enzyme may lie in its promiscuous and indiscriminate reactivity towards several different
types of chemical substrates. A one-size-fits-all enzyme would consume less of the organism’s resources and would be more advantageous than utilizing a different enzyme for each biosynthesized, toxic chemical species it may encounter in its natural habitat.

References


Chapter 4 - Oxidative Cleavage of Dibenzyl Ethers Using Cp*Ir(III) Complex and Oxygen in DMSO

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Abstract

Efficient catalytic systems capable of activating C-H bonds are needed to access potential untapped chemical feedstocks and to minimize chemical waste. Iridium catalysts have shown to be versatile catalyst and exhibit C-H activation. In these studies, we report on the ability of a cationic Ir(III) catalyst to cleave dibenzyl ethers bonds. While a complete mechanism has not been determined, studies suggest the possibility for a radical-based reaction involving O₂ in DMSO solvent.

Introduction

The activation and subsequent functionalization of C-H bonds continues to be a relevant endeavor in modern research (1-4). Several systems utilizing transition metals have been reported over the years. A major challenge for many of these catalytic systems is the efficient functionalization of a metal carbon bond after C-H bond activation by the transition metal catalyst. However, catalytic turnover has been achieved for systems using rhodium or palladium (5-9).

Systems utilizing iridium complexes, another Group 9 metal like rhodium, have demonstrated the ability to activate a variety of C-H bonds (10-20). Many of these catalysts have seen considerable enhancement in catalyst turnover with the addition of electron donating N-heterocyclic carbene (NHC) ligands (12-14, 21). The dimeric complex [(Cp*)IrCl(µ-Cl)]₂, was also shown to catalyze the oxidation of alcohols utilizing O₂ in CH₂Cl₂ instead of a sacrificial organic species (e.g. isopropyl alcohol) (22). These systems
are believed to go through a proposed two-electron mechanism avoiding any radical formation while evolving \( \text{H}_2\text{O}_2 \), which is believed to quickly disproportionate to \( \text{H}_2\text{O} \) and \( \frac{1}{2} \text{O}_2 \). Recently, Heiden et al. showed that \( \text{Cp}^*\text{Ir}(\text{III}) \) complexes with a TsDPEN ligand (TsDPEN = racemic \( \text{H}_2\text{NCHPhCHPhNTs}^\text{–} \)) were capable of heterolytically cleaving \( \text{H}_2 \) to form an \( \text{Ir} \) hydride ([Ir-H]\(^{n+}\)) and a proton bound to the TsDPEN ligand (23-29). This [Ir-H] species was also shown to be formed from other hydrogen donors such as alcohols and amine-boranes. Further, this [Ir-H]\(^{n+}\) species is believed to insert \( \text{O}_2 \) into the iridium-hydrogen bond to form an iridium hydroperoxo species ([Ir-O\(_2\text{H}\)]\(^{n+}\)), which can subsequently be reduced with two equivalent of \( \text{H}_2 \) or other dihydrogen donor to evolve two molecules of water. This is proposed to occur through a two-electron process that also avoids radical formation, and believed to not alter the oxidation state of the Ir(III) catalyst, reminiscent of transfer hydrogenation with these systems. It is conceivable then that a [Ir-O\(_2\text{H}\)] could exhibit chemistry like hydrogen peroxide but avoid the indiscriminate radical nature usually observed with \( \text{H}_2\text{O}_2 \) chemistry.

The ether C-O bond is considered one of the strongest organic bonds in chemistry, and generally requires extreme conditions to induce cleavage. Therefore, it is desirable to find catalytic systems capable of circumventing these issues. Previously, dibenzyl ether has been shown to be cleaved by Co(II) complexes in diglyme by Alper et al via a proposed one-electron pathway (30). The focus of their study was C-H bond activation via the catalytic aerobic oxidation of ethers to the respective ester or lactone; however, they found that with dibenzyl ether there was an overwhelming preponderance for ether cleavage and formation of the respective carboxylic acid as a major product (~85%) with some aldehyde product also
observed. The proposed mechanism involves hydrogen atom abstraction from the ether substrate by an oxygen-bound cobalt species to induce catalytic turnover and initiate the radical chemistry observed.

There are some examples of radical chemistry with iridium in the literature involving Ir(II) porphyrin complexes and Ir(III) complexes as photocatalysts (31-39). Recently, Crabtree et al. demonstrated that Cp*Ir(III) complexes with an electron-rich N-heterocyclic carbene (NHC) ligand are capable of forming stable Ir(IV) complexes (40, 41).

Scheme 4.1.
It is also desirable for catalysts to function in more environmentally innocuous solvents like water or DMSO rather than harsher volatile organics. Although some reactivity with aqua-bound iridium complexes has been previously studied in a multitude of solvent systems, catalytic activity in DMSO has remained largely unexplored.

Incorporating all of these factors, we have utilized \([\text{Cp}^*\text{Ir(NHC)}(\text{OH}_2)_2]\text{OTf}_2\), where \(\text{Cp}^* = \text{tetramethylcyclopentadienyl}; \text{NHC} = 1,3\text{-dimethylimidazole-2-ylidene}; \) and \(\text{OTf} = \text{triflate anion}\) (Scheme 4.1) to catalytically cleave various substituted dibenzyl ethers. Herein, we describe studies on the aerobic catalytic activity of complex 1 in DMSO towards these benzyl ethers to provide the respective carboxylic acid and aldehyde as the major products. GC-MS and isotopically labeled O\(_2\) studies provide some mechanistic insight. We also report on the potential dual role of DMSO in the reaction mechanism – as an apparent oxygen atom acceptor as well as donor.

**Experimental**

*Materials and Methods.* All chemicals were purchased from commercial sources (VWR, Sigma-Aldrich, Fisher) with the exception of those reported synthesized herein. \(^{18}\text{O}_2\) (98%) was purchased from Icon Isotopes (New Jersey). Water utilized in experiments was deionized and passed through a Barnstead EasyPure II UV/UF Ultrapure Water System.

High performance liquid chromatography was performed on a Waters 2796 Bioseparation Module coupled with a Waters 2996 Photodiode Array Detector with a ODS Hypersil (150 mm x 4.6 mm) 5\(\mu\)m particle size column from Thermo Electron Corp. NMR
data was collected utilizing a Varian Mercury 300 or 400 MHz NMR Spectrometer. Chemical shifts are reported as δ, which were calibrated to the natural abundance of the deuterated solvent and referenced downfield from TMS (1H). IR spectra were collected using KBr thin film on a JASCO FT/IR-4100 instrument. GC-MS analysis was performed using an Agilent Technologies 5975 GC-MS EI.

_Synthesis of \([\text{Cp}^*\text{IrCl}_2]_2\) precursor and 1._ The iridium catalyst precursor, \([\text{Cp}^*\text{IrCl}_2]_2\), was synthesized and purified in accordance with previously published methods.(42) Subsequently, catalyst 1 was then synthesized from the dimer as previously reported.(43)

_Synthesis of 2a, 2b and 2d._ The synthesized ethers were produced using a variation of previously reported protocol.(44) A 250 mL round bottom flask was charged with the substituted alcohol (35 mmol), toluene (50 mL) and potassium metal (40 mmol). Solution was refluxed for 1 hour after which an additional 10 mmol of alcohol dissolved in 20mL of toluene was added and refluxing continued for an additional 30 minutes. The respective benzyl chloride (40 mmol), dissolved in 25 mL of toluene were added to the refluxing mixture. After refluxing for an additional hour, the solution was allowed to cool to room temperature and stirred overnight. The mixture was then washed with 1 M HCl (3x100 mL). The organic fraction was collected and dried over Na\(_2\)SO\(_4\). After filtration, solvent was removed by rotary evaporation. Product was purified by flash silica gel column chromatography (20:1 \(n\)-hexanes:ethyl acetate). After removing the eluting solvent by rotary evaporation, the product was recrystallized several times from warm methanol to obtain pure
product. Each was characterized by $^1$H and $^{13}$C NMR spectroscopy, GC-MS, and melting temperature. Compound 2a: $^1$H NMR (300 MHz, CDCl$_3$, δ) 3.83 (s, 6H, -OCH$_3$), 4.50 (s, 4H, -CH$_2$OCH$_2$-), 6.93 (d, J=8.7 Hz, CH$_3$OArH, 4H), 7.32 (d, J=8.7 Hz, -OCH$_2$ArH, 4H). $^{13}$C NMR (75.46 MHz, CDCl$_3$, δ) 21.53 (s, Ar-CH$_3$), 72.10 (s, -H$_2$C-O-CH$_2$-), 128.24 (s, Ar[C-2]), 128.41 (s, Ar[C-3]), 135.66 (s, Ar[C-1]), 137.56 (s, Ar[C-4]). GC-MS: 258 Da (M$^+$); Compound 2b: $^1$H NMR (300 MHz, CDCl$_3$, δ) 2.43 (s, 6H, Ar-CH$_3$), 4.59 (s, 4H, -CH$_2$O-CH$_2$-), 7.30 (q, J=38.7 Hz, 8H, ArH). $^{13}$C NMR (75.46 MHz, CDCl$_3$, δ) 21.54 (s, -CH$_3$), 72.12 (s, -H$_2$C-O-CH$_2$-), 128.25 (s, -OCH$_2$Ar[C-2,6], 128.42 (s, -Ar[C-3,5]CH$_3$), 135.67 (s, -OCH$_2$Ar[C-1]), 137.58 (s, -Ar[C-4]CH$_3$). GC-MS: 226 (M$^+$); Compound 2d: $^1$H NMR (300 MHz, CDCl$_3$, δ) 4.51 (s, 4H, -CH$_2$OCH$_2$-), (m, 4H, CH$_2$-O-CH$_2$), 7.30 (m, 8H, ArH). $^{13}$C NMR (75.46 MHz, CDCl$_3$, δ) 21.53 (s, Ar-CH$_3$), 72.10 (s, -H$_2$C-O-CH$_2$-), 128.24 (s, Ar[C-2]), 128.41 (s, Ar[C-3]), 135.66 (s, Ar[C-1]), 137.56 (s, Ar[C-4]). GC-MS m/z (ion): 226 (M$^+$).

**Standard Reaction Conditions.** Reactions were carried out in 25 mL Chemglass AIRFREE Cajon storage flasks. Vials were charged with 1 (4 µmol), ether substrate (80 µmol), and 800 µL anhydrous DMSO in a N$_2$-filled glove box. After sealing, samples were placed on a vacuum line to remove inert atmosphere and a balloon containing oxygen was attached and each reaction was heated to 100 °C (±5 °C) while stirring for the reported times. For each ether substrate, triplicate control reactions without catalyst were run to provide a baseline for background reactivity. Upon completion, an 800-fold dilution was analyzed by HPLC. All reported conversions and product selectivity yields are based on calibration curves obtained from pure samples of each respective compound.
Isotopically labeled oxygen studies. Reaction was prepared as previously described for a standard reaction except a glass bulb with 98% $^{18}$O$_2$ was attached. The reaction was run for 24 hours at 100 °C (±5 °C). The sample was then analyzed by GC-MS EI.

Reaction workup and analysis. All aforementioned reactions underwent the following workup and analysis: after cooling to room temperature, MeCN (800 μL) was added to each reaction vial. 200 μL of the homogenous solution was diluted 100-fold with 65:35% v/v H$_2$O:MeCN. The final dilutions were analyzed by HPLC [ODS Hypersil (Thermo Electron Corp.) C$_{18}$ (150 mm x 4.6 mm) 5 μm particle size; H$_2$O:MeCN (v/v) from 65:35 to 15:85 for 5 min, a linear gradient return to 65:35 over 1 min, and isocratic 65:35 for 6 minutes; flow rate of 1.5 mL/min; UV-visible spectroscopic monitoring (diode array), 210-500 nm]. Products were determined by their characteristic retention time and with comparison to the UV-visible spectrum of authentic materials. Pure samples of all identified substrates and products were used to prepare calibration curves to enable quantitation. For calibration curves, a linear regression was determined using six 2-fold serial dilutions of 120 mM stock solutions of pure samples. Subsequently, each dilution was diluted further 100-fold and analyzed by HPLC.

Results and Discussion

Reactions with catalyst 1 and ethers 2a - 2d under a variety of conditions were performed and analyzed using HPLC and the results are summarized in Table 1. The highest conversion over 24 h was observed for substrate 2a (Entries 1-3). The major product was benzoic acid derivatives for all ethers studied. In addition, substituted benzaldehydes were also detected, as well as, minor amounts of 4-methoxybenzyl alcohol for the cleavage of 2a.
(Entries 1-3). High conversions were also observed for substrates 2b (Entry 5) and 2d (Entry 7), but no alcohol product was observed for these substrates. The lowest conversion was observed for substrate 2c; equivalent amounts of benzoic acid and benzaldehyde were observed and no benzyl alcohol was detected for this substrate (Entry 6). When anaerobic studies (under N₂) were performed with substrate 2a only trace cleavage (<1%) was observed. Under these conditions, the reaction mixture turned a deep red/brown color over the time course of the reaction. While a deep red color has been observed with other Ir(III) systems, where the presence of Ir-hydrides has been suggested, anaerobic NMR experiments

<table>
<thead>
<tr>
<th>Entry</th>
<th>Substrate</th>
<th>Catalyst Load (mol %)</th>
<th>Reaction Conditions (1 atm)</th>
<th>Subst. Conv. (%)</th>
<th>TON¹</th>
<th>Product selectivity (%) b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Carb. Acid Aldehyde Alcohol</td>
</tr>
<tr>
<td>1</td>
<td>2a</td>
<td>5</td>
<td>O₂</td>
<td>94.0 (+ 5.4)</td>
<td>37.6</td>
<td>62 35 5</td>
</tr>
<tr>
<td>2</td>
<td>2a</td>
<td>2.5</td>
<td>O₂</td>
<td>93.8 (+ 2.5)</td>
<td>37.6</td>
<td>62 32 7</td>
</tr>
<tr>
<td>3</td>
<td>2a</td>
<td>1</td>
<td>O₂</td>
<td>74.3 (+ 8.8)</td>
<td>74</td>
<td>59 37 3</td>
</tr>
<tr>
<td>4</td>
<td>2b</td>
<td>5</td>
<td>O₂</td>
<td>67.8 (+ 2.0)</td>
<td>13.6</td>
<td>76 20 -</td>
</tr>
<tr>
<td>5</td>
<td>2b</td>
<td>2.5</td>
<td>O₂</td>
<td>68.2 (+ 3.4)</td>
<td>27.3</td>
<td>75 24 -</td>
</tr>
<tr>
<td>6</td>
<td>2c</td>
<td>2.5</td>
<td>O₂</td>
<td>41.8 (+ 1.4)</td>
<td>16.7</td>
<td>54 48 -</td>
</tr>
<tr>
<td>7</td>
<td>2d</td>
<td>2.5</td>
<td>O₂</td>
<td>54 (+ 6.2)</td>
<td>21.6</td>
<td>78 15 -</td>
</tr>
<tr>
<td>8</td>
<td>2a</td>
<td>5</td>
<td>N₂</td>
<td>&lt;1</td>
<td>-</td>
<td>- - -</td>
</tr>
<tr>
<td>9</td>
<td>2b</td>
<td>20</td>
<td>N₂</td>
<td>&lt;1</td>
<td>-</td>
<td>- - -</td>
</tr>
</tbody>
</table>

Reaction conditions: Ether (100 mM) with I (1-20 mol%) in anh. DMSO under O₂ (1 atm) or N₂ (1 atm) at 100 °C for 24 h. a) TON = [Substrate loss]/[I]; b) Product selectivity (%) = ([Product]/(2 × [Substrate loss])) × 100; (-) denotes not observed or detected
with \textbf{1} and \textbf{2b} in d$_6$-DMSO did not exhibit a peak at ~ 15 ppm which is usually indicative of iridium hydrides (23-26).

The absence of stoichiometric conversion under N$_2$ implies that O$_2$ is critical for the ether cleavage reaction. Further, the presence of the alcohol product for \textbf{2a} and the known oxidation of similar benzyl alcohols by other Ir(III) catalysts (10, 12, 13, 22) lead us to investigate whether the carboxylic acid and aldehyde products observed during the ether cleavage studies resulted from the oxidation of a alcohol products derived directly from ether cleavage.

\textit{Alcohol oxidation studies with 1 under O$_2$ in DMSO.} Aerobic oxidation experiments were performed with substituted alcohols, aldehydes, and carboxylic acids of each ether according to Scheme 4.2. The aldehydes and carboxylic acids exhibited no conversion under the same reaction conditions utilized for ether cleavage; however, the alcohol derivatives of each ether studied were primarily converted exclusively to the aldehyde at moderate levels (Table 4.2) with the exception of the \textit{para}-methoxy alcohol, which exhibited the highest conversion, but
Scheme 4.2.

![Scheme 4.2.](image)

Table 4.2. Aerobic reactivity of para-substituted benzyl alcohols with 1 in DMSO

<table>
<thead>
<tr>
<th>R</th>
<th>Subst. Conv. (%)</th>
<th>TON&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Aldehyde</th>
<th>Carb. Acid</th>
<th>Ether</th>
<th>Ester</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH₃O</td>
<td>71.3 (±6.4)</td>
<td>28.5</td>
<td>19</td>
<td>37</td>
<td>38</td>
<td>5</td>
</tr>
<tr>
<td>CH₃</td>
<td>54.7 (±3.2)</td>
<td>21.9</td>
<td>94</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>H</td>
<td>12.5 (±2.4)</td>
<td>5.0</td>
<td>93.5</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cl</td>
<td>27.1 (±4.1)</td>
<td>10.8</td>
<td>91</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Reaction conditions: Alcohol (100 mM) with 1 (2.5 mol%) in anh. DMSO under O₂ (1 atm) at 100 °C for 24 h; a – TON = [Substrate loss]/[1]; b – Product selectivity (%) = [Product]/([Substrate loss]x2); (-) denotes not observed or detected;
also produced the carboxylic acid, the bis-ether (2a), and 4,4'-dimethoxybenzyl benzoate. This may likely be due to the more reactive benzylic C-H bond provided by the strongly electron-donating methoxy group. As stated, alcohol oxidation of these substituted benzyl alcohols to the corresponding aldehyde is well known under similar conditions, but the appearance of the other side products has not been observed. Nonetheless, the lack of the corresponding carboxylic acid as a major product of alcohol oxidation for the other alcohols implies that the carboxylic acid product observed during ether cleavage occurs independently and is likely not the result of alcohol oxidation. However, the catalytic intermediates for ether cleavage and alcohol oxidation may be different, and the latter may not provide a reaction pathway to these additional species. Additionally, focusing exclusively on the %conversion / TON results of alcohol oxidation, a trend similar to that observed for ether bond cleavage is apparent. Specifically, the alcohols with a more electron rich or with an available lone pair at the para-position provided the higher overall turnover (MeO > Me > Cl > H).

**Scheme 4.3**
GC-MS Studies with $^{18}$O$_2$ and 2b. As a complete picture of the mechanism for ether cleavage was not immediately apparent from these results, studies involving isotopically-labeled molecular oxygen were performed and analyzed by GC-MS EI to understand the origin of the various oxygen containing products as well as the ultimate role of O$_2$ (Scheme 4.3). Ether cleavage studies were performed under $^{18}$O$_2$ using 2b as a substrate, which exhibited a high turnover and cleanly gave only the respective carboxylic acid and benzaldehyde. The results of these experiments are summarized in Tables 4.3.

**Table 4.3.** Results of studies performed utilizing $^{18}$O$_2$ with 1 and 2b over 24 h at 100 °C

<table>
<thead>
<tr>
<th>Substrate</th>
<th>O-Atom Source</th>
<th>$^{18}$O incorporated$^\dagger$</th>
<th>$^{16}$O$^{18}$O</th>
<th>$^{18}$O$^{18}$O</th>
</tr>
</thead>
<tbody>
<tr>
<td>3b</td>
<td>$^{18}$O$_2$</td>
<td>79.0 (80.6)</td>
<td>8.4 (8.5)</td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>$^{18}$O$_2$</td>
<td>95.4 (97.4)</td>
<td>0.6 (0.6)</td>
<td></td>
</tr>
<tr>
<td>4b*</td>
<td>$^{18}$O$_2$</td>
<td>3.8 (3.9)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Reaction conditions: 2b (100 mM) with 1 (2.5 mol%) in anh. DMSO under O$_2$ (< 1 atm) at 100 °C for 24 h; $^\dagger$ - number in parenthesis corresponds to normalized 18O-atom incorporation based on 98% enriched $^{18}$O$_2$ utilized * -Only one label present because of the nature of the substrate

In the studies with $^{18}$O$_2$, the carboxylic acid product ($m/z$ 136 Da) was primarily the singly labeled species ($m/z$ 138 Da), which implies that only $\frac{1}{2}$ $^{18}$O$_2$ is incorporated, suggesting that the other O-atom may be derived from the ether substrate (the ether O atom).
Dimethyl sulfone (DMSO$_2$) was also observed ($m/z$ 94 Da) for the first time, and consisted almost exclusively of the single-labeled dioxygen product ($m/z$ 96 Da) as displayed in Table 4.3. Sulfone would seem to be produced by an O-atom transfer to DMSO from an activated oxygen species because it also incorporates ½ $^{18}$O$_2$. For the aldehyde product ($m/z$ 120 Da), it was observed to be primarily unlabeled even when $^{18}$O$_2$ was utilized.

*Solvent Variation Studies.* This potential bifunctional role of DMSO was explored further using solvent variation studies. Reactions were run with 2b in varying ratios of DMSO and H$_2$O (Table 4.4). Anhydrous DMSO produced the highest turnover but Entry 2, which possessed minimal amounts of water still showed similar chemistry. This shows that anhydrous DMSO is not a rigorous requirement for clean chemistry. However, as the

<table>
<thead>
<tr>
<th>Entry</th>
<th>DMSO:H$_2$O (%DMSO)</th>
<th>Subst. Conv. (%)</th>
<th>TON$^a$</th>
<th>Product Selectivity (%)$^b$</th>
<th>Carb.</th>
<th>Acid</th>
<th>Aldehyde</th>
<th>Ester</th>
<th>Alcohol</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100</td>
<td>67.8 (±2.1)</td>
<td>13.6</td>
<td>76</td>
<td>20</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>87.5</td>
<td>61.2 (±3.3)</td>
<td>12.2</td>
<td>68</td>
<td>28</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>54.3 (±2.6)</td>
<td>10.9</td>
<td>47</td>
<td>25</td>
<td>20</td>
<td>9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>35.8 (±1.7)</td>
<td>7.2</td>
<td>31</td>
<td>31</td>
<td>21</td>
<td>11</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Reaction conditions: 2b (100 mM) with 1 (2.5 mol%) in various ratios of DMSO:H$_2$O under O$_2$ (1 atm) at 100 °C for 24 h; $a$ – TON = [Substrate loss]/[1]; $b$ – Product selectivity (%) = [Product]/([Substrate loss]x2); (−) denotes not observed or detected.
concentration of DMSO was decreased, additional reaction products became more prevalent including the dibenzyl ester and benzyl alcohol. Further, studies with the similarly substituted esters under the same reaction conditions as ether cleavage exhibited no reactivity.

*Sulfone Formation Studies.* GC-MS EI revealed the presence of DMSO$_2$ as a reaction side-product. To determine its mechanistic origin, a series of control experiments was performed, in which, each of the key chemical species (catalyst, O$_2$, and ether (2b)) were excluded individually and the systems were allowed to react for 24 h then subsequently analyzed by GC-MS. All exclusion experiments resulted in a lack of sulfone formation. These results suggest that the formation of DMSO$_2$ is dependent on the iridium catalyst, the ether, molecular oxygen and DMSO. Of note, molecular oxygen and DMSO in the presence of catalyst did not produce sulfone and the color of the solution did not change as it did under anaerobic conditions.

Experiments were also performed aerobically / anaerobically in the presence of H$_2$O$_2$ (100 mM) with 1 (5 mol%) and 2a (100 mM), then analyzed by GC-MS (data not shown). While no ether cleavage was observed under any of these conditions, all studies resulted in the formation of DMSO$_2$. Of interest, GC-MS analysis of studies with 4-methylbenzyl alcohol also exhibited DMSO$_2$ formation. Hydrogen atom abstraction from the benzylic C-H bond is more facile over other ethers due to resonance stabilization offered by the adjacent phenyl ring. Therefore, it appears that overall ether cleavage reactivity in these systems is seemingly dictated by the nature of the *para*-substituent. In that, electron donating substituents on the ring (through resonance or induction) better facilitate ether cleavage under these conditions.
Formation of a [Ir-H]$^\text{Ir}$ species can arise via the extraction of a hydrogen atom or proton from the ether substrate. Alper and coworkers have reported a radical-based mechanism for the cleavage of dibenzyl ether with a CoCl$_2$ catalyst (30), which exhibited a

Table 4.4. Summary of reactions with catalyst 2b in anhydrous DMSO under O$_2$ (1 atm) at 100 °C for 24 h

<table>
<thead>
<tr>
<th>Catalyst</th>
<th>Subst.</th>
<th>Conv. (%)</th>
<th>TON$^a$</th>
<th>Carb.</th>
<th>Acid</th>
<th>Aldehyde</th>
<th>Alcohol</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Cp*Ir(OH)$_2$]OTf$_2$</td>
<td>2a</td>
<td>91.5 (±7.7)</td>
<td>18.3</td>
<td>63</td>
<td>33</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2b</td>
<td>66.4 (±5.2)</td>
<td>13.3</td>
<td>78</td>
<td>25</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>[Cp*IrCl$_2$]</td>
<td>2a</td>
<td>55.4 (±2.0)</td>
<td>11.1</td>
<td>48</td>
<td>40</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2b</td>
<td>7.8 (±3.0)</td>
<td>1.6</td>
<td>56</td>
<td>44</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>IrCl$_3$•3 H$_2$O</td>
<td>2a</td>
<td>35.8 (±4.7)</td>
<td>7.2</td>
<td>26</td>
<td>30</td>
<td>36</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2b</td>
<td>n.r.</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>CoCl$_2$</td>
<td>2a</td>
<td>69.8 (±1.1)</td>
<td>14.0</td>
<td>48</td>
<td>37</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2b</td>
<td>4.4 (±0.4)</td>
<td>0.9</td>
<td>25</td>
<td>75</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

Reaction conditions: 2b (100 mM) with catalyst (5 mol%) in anh. DMSO under O$_2$ (1 atm) at 100 °C for 24 h; a – TON = [Substrate loss]/[I]; b – Product selectivity (%) = [Product]/([Substrate loss]x2); (-) denotes not observed or detected

similar product distribution to these studies. Although the cobalt and iridium catalysts are in the same group, they are in two different oxidation states and may not utilize similar reaction mechanisms. Yet as stated, Crabtree and coworkers have recently reported the isolation of a stable Cp*Ir(IV) species with an ancillary NHC ligand, which may seemingly allow for the
abstraction of a hydrogen atom to form the benzylic radical on the ether and form the Cp*Ir(IV)-H in an radical initiation step. Whether the Ir-H species is actually Ir(IV) requires further investigation.

Studies with Various Ir(III) Catalysts and CoCl₂. To better understand how the electronic nature of iridium plays a role in ether cleavage, a series of additional iridium catalysts and CoCl₂ were examined. Precursors in the synthesis of catalyst 1 were studied under the same reaction conditions with the results summarized in Table 4.5. The cationic [Cp*Ir(OH₂)₃]OTf₂ showed equivalent reactivity to that of 1 (see Table 4.1). The other iridium systems also showed the ability to induce ether cleavage of 2a with similar product distributions, but exhibited considerably less reactivity towards 2b. The CoCl₂ catalyst used in Alper’s studies also cleaved 2a with carboxylic acid still being the major product. While a limited number of iridium catalysts were screened in these studies, all showed the ability to react with 2a, but were less reactive towards 2b.
To determine the dependence on catalyst concentration for turnover of substrate, a series of 2 hour reactions were performed at various catalyst loads with 2a and 2b (Figure 4.1). Regardless of the substrate utilized, 2.5 mol% appears to be the optimum concentration of iridium catalyst under these conditions. At higher concentrations of catalyst, an attenuation in reactivity was observed. If it is a radical-based mechanism for ether cleavage, a chain termination step involving the catalyst or an intermediate could give rise to this phenomenon. Higher catalyst load would increase the likelihood of this termination step occurring and thus slow or limit overall turnover. It is also important to note that these shorter reactions exhibited a similar product distribution to their 24 h counterparts (carboxylic acid in excess).
Comments on the Possible Mechanism. In these studies, a series of dibenzyl ethers with a variety of para substituents were cleaved using 1 and O₂ to provide the corresponding carboxylic acid and aldehyde as major products. In the aerobic studies, the preference for ether cleavage and carboxylic acid formation is in agreement with observations made by Alper et al. in their studies with cobalt (30). In their studies, they observed that the more stable the radical (at the carbon adjacent to oxygen), the more likely the radical mechanism is to prefer fragmentation, and carboxylic acid production. We believe this phenomenon is viable for the four benzyl ethers studied due to the electronic stability provided the benzylic radical by the adjacent π-system of the phenyl ring. Studies with benzyl alcohols exhibited oxidation primarily to the aldehyde, but carboxylic acid formation was limited/absent, and therefore, is likely formed through reactive intermediates present only during ether cleavage. Also it appears from the product distribution, the isotope labeling, and aerobic oxidation studies that carboxylic acid and aldehyde production appear to occur independently. Supporting this is the fact that the carboxylic is consistently in molar excess over that of the aldehyde even at short reaction times, therefore they cannot both arise from a single step during ether cleavage.

These reactions were performed in DMSO, which appears to play a bifunctional role in this reactivity – both as an O-atom acceptor and donor. Addressing the former, H₂O₂ resulting from hydrogen transfer to O₂ in previous alcohol oxidation studies is believed to quickly disproportionate to water and ½ O₂. Recall from these studies, DMSO₂ was observed in the alcohol oxidation studies and the experiments performed with H₂O₂, and may arise from a side reaction between DMSO and H₂O₂, either formed in situ or exogenous.
Conclusions

In these studies, we have demonstrated the ability of several Ir(III) complexes capable of selectively cleaving substituted dibenzyl ethers to carboxylic acid and aldehyde. Preliminary results (product distribution, isotope labeling, aerobic oxidation studies) appear to suggest that carboxylic acid formation primarily occurs independent from aldehyde formation. Details of the mechanism for this cleavage are still under investigation.

References


Appendix
Appendix A

Table A.1. Relative $^{18}$O-atom incorporation into 2b and 7b for isotopically-labeled studies

<table>
<thead>
<tr>
<th>Product</th>
<th>$^{18}$O-atoms</th>
<th>$^{18}$O-enrichment from source</th>
<th>m/z 132</th>
<th>m/z 134</th>
<th>m/z 136</th>
<th>m/z 138</th>
<th>% relative abundance [M+H]$^+$ ion</th>
<th>% $^{18}$O incorporated *</th>
</tr>
</thead>
<tbody>
<tr>
<td>2b</td>
<td>none</td>
<td>100 0.77 - X N/A</td>
<td>H$_2^{18}$O$_2$</td>
<td>100 88 1.9 X 46.6 (51.8)</td>
<td>H$_2^{18}$O</td>
<td>100 5.9 - X 4.8 (5.5)</td>
<td>H$_2^{18}$O$_2$ / H$_2^{18}$O</td>
<td>90.0 100 - X 52.5 (60.1)</td>
</tr>
<tr>
<td>7b</td>
<td>none</td>
<td>X 100 - - N/A</td>
<td>H$_2^{18}$O$_2$</td>
<td>X 10.1 100 0.8 90.8 (100)</td>
<td>H$_2^{18}$O</td>
<td>X 100 0.83 - &gt;1</td>
<td>H$_2^{18}$O$_2$ / H$_2^{18}$O</td>
<td>X 8.3 100 0.9 92.3 (108)</td>
</tr>
</tbody>
</table>

* number in parenthesis represents normalized incorporation as a function of $^{18}$O enrichment from source
Table A.2. Relative $^{18}$O-atom incorporation into 2c and 7c for isotopically-labeled studies

<table>
<thead>
<tr>
<th>Product</th>
<th>$^{18}$O-atoms</th>
<th>% relative abundance [M+H]$^+$ ion</th>
<th>$^{18}$O incorporated $^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>2c</td>
<td>none</td>
<td>$m/z$ 210 $m/z$ 212 $m/z$ 214 $m/z$ 216</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>$H_2^{18}O_2$</td>
<td>37.7</td>
<td>89.8 X</td>
</tr>
<tr>
<td>7c</td>
<td>none</td>
<td>X</td>
<td>94.2 0.5</td>
</tr>
<tr>
<td></td>
<td>$H_2^{18}O_2$</td>
<td>6.8 X</td>
<td>85.2 91.2 (101)</td>
</tr>
</tbody>
</table>

* number in parenthesis represents normalized incorporation as a function of $^{18}$O enrichment from source