ABSTRACT

Protein Film Voltammetry of Heme Oxygenase

Azaan Altaf Ramani

Director: Patrick J. Farmer, Ph.D.

Heme Oxygenase (HO), a heme-degrading enzyme, is responsible for many physiological functions including heme catabolism, O_2 sensing, cellular signaling, iron homeostasis, and antioxidant defense. In vivo, HO is responsible for the catabolism of heme to free iron, CO, and biliverdin. The conversion of heme by HO proceeds through three successive oxygenation reactions forming α-meso-hydroxyheme, verdoheme and biliverdin using seven electrons donated through NADPH-cytochromeP450 reductase. HO enzyme was isolated and reconstituted with Fe(III)-Protoporphyrin. Purity and activity of the enzyme was tested using SDS-PAGE gel and UV-Vis Spectroscopy. Protein Film Voltammetry was carried out under anaerobic and aerobic conditions using basal and edge plane pyrolytic graphite (PG) electrode with films containing Nafion and HO. Under anaerobic conditions, a reduction peak is observed indicating redox activity of the Fe-metal in the heme. Under aerobic conditions a reduction peak is observed suggesting a possible turnover of the enzyme.

APPROVED BY DIRECTOR OF HONORS THESIS

_____

Dr. Patrick J. Farmer, Department of Chemistry

APPROVED BY THE HONORS PROGRAM

_____

Dr. Andrew Wisely, Director

DATE: _____

PROTEIN FILM VOLTAMMETRY OF HEME OXYGENASE

A Thesis Submitted to the Faculty of

Baylor University

In Partial Fulfillment of the Requirements for the

Honors Program

By

Azaan Altaf Ramani

Waco, Texas

May 2013

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ACKNOWLEDGMENT

Through my years at Baylor, I have had the opportunity to work with numerous remarkable individuals. First and foremost, I would like to express my deepest and sincere gratitude to Dr. Patrick J. Farmer for giving me an opportunity to work in his lab and learn from him. Without his constant support, guidance, and encouragement, this thesis would not have been possible and I would not be where I am today.

I would also like to thank all the graduate, post-graduate and undergraduate students in the Farmer lab for always giving me different ideas to improve my project and answering all my questions. In particular, I would like to thank Tara Clover who taught me everything I know about protein expression and purification. Without her constant suggestions and motivation, it would have been a very difficult journey.

Lastly, I would like to thank my parents who always worried about me when I spent many late nights in the lab and constantly called to check if I was okay. I will always be greatly indebted to them.

To my parents, Altaf and Shabina Ramani, for their constant support, guidance, love, and encouragement.

CHAPTER ONE

Introduction

Redox reactions, which involve the transfer of electrons, are one of the most important aspects of biochemical systems, especially proteins. The transfer of electrons is responsible for many functions of proteins including respiration, photosynthesis, cell defense, and gene control.[1] The malfunctioning of the redox reactions are responsible for many diseases in humans. The understanding of redox biochemistry is of essential medical significance.

Heme proteins, containing iron protoporphyrin IX, are one of the most extensively studied classes of proteins due to their various biological functions. Heme proteins such as hemoglobin, myoglobin, and cytochrome *c* have been extensively studied using various voltammetry methods. Over the past decades, protein film voltammetry techniques have been developed to study the electron transport mechanism. *Shen et al.* studied electrochemical behavior of four heme protein-polyamidoamine (PAMAM), which include hemoglobin (Hb), myoglobin (Mb), horseradish peroxidase (HRP), and catalase.[2] The heme proteins were studied using cyclic voltammetry and demonstrated well-defined quasi-reversible peaks. The peaks for Hb-, Mb-, and HRP-PAMAM films were observed at -0.37 V vs. SCE and -0.47 V vs. SCE for catalase-PAMAM film (Figure 1). [2]

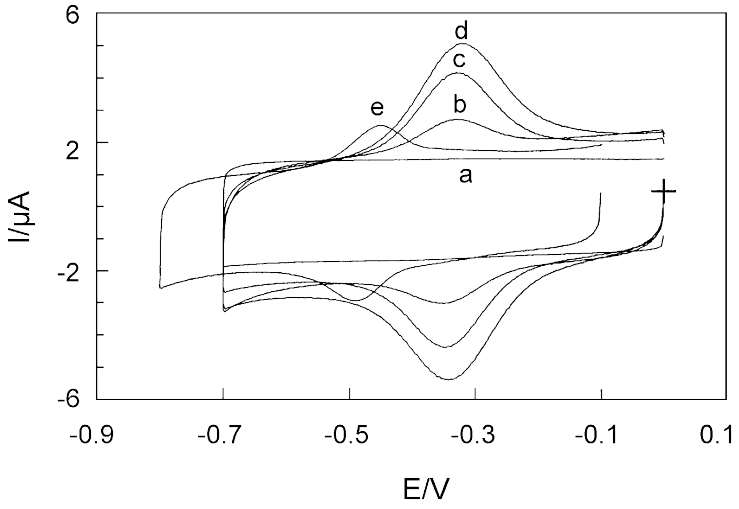

Figure 1. Cyclic voltammograms at 0.2 V s^{-1} in pH 7.0 buffers for (a) 0.025 mM Hb in buffers at bare PG electrodes, and for (b) HRP-PAMAM, (c) Mb-PAMAM, (d) Hb-PAMAM, and (e) Cat-PAMAM film electrodes.

Protein film voltammetry has also been used to examine the electrochemistry of manganese-substituted myoglobin in dimethyldidodecylammonium bromide (ddab) film. *Lin et al.* demonstrated the cyclic voltammograms of MnMb/ddab films at various scan rates and reported reduction potentials at – 0.25 V and - 0.41 V vs SCE (Figure 2).[3]

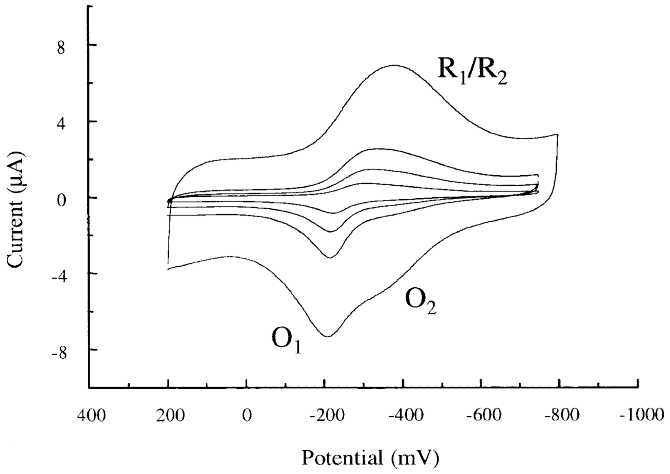

Figure 2. Cyclic voltammograms of MnMb/ddab film at scan rates of 10, 50, 100 and 500 mV/s in pH 7.0, 0.05 M phosphate buffer; 0.05 mM NaBr as the electrolyte

Another heme protein that is involved in a variety of physiological functions including heme catabolism, O_2 sensing, cellular signaling, iron homeostasis, and antioxidant defense is heme oxygenase (HO).[4] The electrochemistry of heme oxygenase trapped in a film has not been reported.

Protein Film Methods

Protein film voltammetry (PFV) developed by Frasier Armstrong, is a widely used technique to study the mechanistic and electrochemical properties of protein. In this technique, the protein is adsorbed as a monolayer allowing for direct electrochemical measurements. Although this technique is useful to measure the transfer of electrons in the protein, the signal response is very low and the protein concentration is limited. An alternative method, developed by Rusling, which uses films of surfactants or other water insoluble materials immobilizes the protein on the surface of the electrode and allows for direct electrochemistry. In this method, the response signal is strong and low concentration of protein is required.

Despite the growing interest in PFV, only about 40 different proteins have been studied which serve many important physiological functions. Some of the well-studied proteins include cytochrome P450, hemoglobin, myoglobin, cytochrome C, several hydrogenases, glucose oxidase, Ps-I And PS-II photosystems, some Mo-containing proteins and Fe-S, and other metalloprotiens (with Ni-Fe, Mn or Cu as

redox centers). [1] Table 1 summarizes kinetics and thermodynamic parameters of various enzymes on using different films and electrodes.

Table 1 Thermodynamic and kinetic features of relevant enzymes studied at various electrodes in a protein film voltammetric scenario

Protein name	Redox-active site(s)	E° vs. Ag/AgCl/V	k_{max}/s^{-1}	Type of electrode
Iso-1-cytochrome *c* (YCC)	Haem-Fe^{3+}/Fe^{2+}	+0.220	1,800	Gold electrode modified with Cys102
Cytochrome P450 BM3 (wild type)	Haem-Fe^{3+}/Fe^{2+}	−0.200	250	Carbon electrode modified with DDAPSS film
Cytochrome P450 BM3 (mutant 1–12G)	Haem-Fe^{3+}/Fe^{2+}	−0.200	30	Carbon electrode modified with DDAPSS film
NiFe hydrogenase (from *Desulfovibrio fructosovorans*)	Ni^{3+}/Ni^{2+} and [Fe–S] clusters	−0.290 and −0.340	/	Edge pyrolytic graphite electrode
Complex I (flavoprotein subcomplex of NADH)	[Fe–S] cluster	−0.320	/	Pyrolytic graphite electrode
Cytochrome *c* nitrite reductase	Haem-Fe^{3+}/Fe^{2+}	−0.325		Pyrolytic graphite electrode
Catalase (Cat)	Haem-Fe^{3+}/Fe^{2+}	−0.420	75	Pyrolytic graphite electrode modified with polyacrylamide hydrogel films
Catalase (Cat)	Haem-Fe^{3+}/Fe^{2+}	−0.470	47	Pyrolytic graphite modified with nanosized polyamidoamine dendrimer film
Hemoglobin	Haem-Fe^{3+}/Fe^{2+}	−0.340	23	Pyrolytic graphite modified with nanosized polyamidoamine dendrimer film
Myoglobin	Haem-Fe^{3+}/Fe^{2+}	−0.340	20	Pyrolytic graphite modified with nanosized polyamidoamine dendrimer film
Horseradish peroxidase	Haem-Fe^{3+}/Fe^{2+}	−0.340	10	Pyrolytic graphite modified with nanosized polyamidoamine dendrimer film
Hemoglobin	Haem-Fe^{3+}/Fe^{2+}	−0.350	50	Pyrolytic graphite electrode modified with collagen film
Catalase	Haem-Fe^{3+}/Fe^{2+}	−0.465	35	Pyrolytic graphite electrode modified with collagen film
Cytochrome P450	Haem-Fe^{3+}/Fe^{2+}	−0.470	/	Pyrolytic graphite electrode modified with genetically enriched CYP1A2 and CYP3A microsomes + polyions
Hemoglobin	Haem-Fe^{3+}/Fe^{2+}	−0.320	0.8	Carbon electrode modified with *N*-butylpyridinium hexafluorophosphate ionic liquid
Photosystem I (from spinach)	Phylloquinone and [Fe–S] clusters, F_A/F_B	−0.540 and −0.190	7.2 and 65	Pyrolytic graphite electrode
Molybdoenzyme arsenite oxidase	Mo^{6+}/Mo^{5+}	−0.300	/	Pyrolytic graphite electrode
Cytochrome *c* oxidase–azurin and subunit II (Cu_A domain)	Haem-Fe^{3+}/Fe^{2+} and Cu^{2+}/Cu^{1+}	−0.300 and +0.150	/	Gold electrode modified with alkanethiole
Myoglobin	Haem-Fe^{3+}/Fe^{2+}	−0.240	90	Basal plane pyrolytic graphite electrode modified with titanate nanotubes
Myoglobin	Haem-Fe^{3+}/Fe^{2+}	−0.260	25	Basal plane pyrolytic graphite electrode modified with TiO_2
Hemoglobin		−0.070	5	Glassy carbon electrode modified with NiO
Sulfite dehydrogenase (from *Starkeya novella*)	Haem-Fe^{3+}/Fe^{2+} and $Mo^{6+/5+}$	0.180 and 0.210		Pyrolite graphite electrode
Respiratory nitrate reductase from *R. sphaeroides*	$Mo^{6+/5+}$ and [Fe–S] clusters	−0.160	/	Pyrolite graphite electrode
Flavodoxin D. (*from vulgaris* Hildenborough)	Quinone/semi-quinone and semiquinone/hydroquinone	−0.340 and −0.585	2	Mesoporus nanostructured SnO_2 electrode
Horseradish peroxidase	Haem-Fe^{3+}/Fe^{2+}	−0.340	/	Pyrolytic graphite electrode modified with polyethylene glycol
Cytochrome P450 BM3 (haem domain)	Haem-Fe^{3+}/Fe^{2+}	−0.330	10	Basal plane graphite electrode modified with sodium dodecyl sulfate
Cytochrome P450 BM3 (haem domain)	Flavin reductase domain and haem-Fe^{3+}/Fe^{2+}	−0.350 and −0.220	/	Pyrolytic graphite electrode modified with didodecyldimethylammonium
Cytochrome P450 BM3 (haem domain)	Haem-Fe^{3+}/Fe^{2+}	−0.340	/	Graphite electrode modified with pyrene-terminated tether
Hemoglobin	Haem-Fe^{3+}/Fe^{2+}	−0.350	/	Pyrolytic graphite electrode modified with multiwalled carbon nanotubes
[NiFe]-hydrogenases (from *Desulfovibrio vulgaris*)	Ni^{3+}/Ni^{2+} and [Fe–S] clusters	−0.290 and −0.340		Pyrolytic graphite electrode
Glucose oxidase		−0.430	2	Graphite electrode modified with carbon nanotubes
Hemoglobin	Haem-Fe^{3+}/Fe^{2+}	−0.050	1.5	Glassy carbon electrode modified with $CoCl_2$ nanoparticles
Hemoglobin	Haem-Fe^{3+}/Fe^{2+}	−0.040	/	Glassy carbon electrode modified with polytetrafluoroethylene
Hemoglobin	Haem-Fe^{3+}/Fe^{2+}	−0.340	2.5	Graphite electrode modified with carbon nanotubes
Cytochrome P450 2B4	Haem-Fe^{3+}/Fe^{2+}	−0.300	/	Graphite electrode modified with colloidal clay
Myoglobin	Haem-Fe^{3+}/Fe^{2+}	−0.310	/	Graphite electrode modified with zeolites
Hemoglobin	Haem-Fe^{3+}/Fe^{2+}	−0.310	/	Pyrolytic graphite electrode modified with Fe_3O_4 nanoparticles
Cytochrome c (from *Pseudomonas aeruginosa*)	Haem-Fe^{3+}/Fe^{2+}	+0.090	/	Pyrolytic graphite electrode
Cytochrome c (from *Hydrogenobacter thermophilus*)	Haem-Fe^{3+}/Fe^{2+}	+0.025	/	Pyrolytic graphite electrode
Cytochrome c (from *Nitrosomonas europaea*)	Haem-Fe^{3+}/Fe^{2+}	+0.050	/	Pyrolytic graphite electrode
Various Cu-containing enzymes				

Heme Oxygenase

Heme oxygenase catalyzes the degradation of heme to carbon monoxide, biliverdin, and free iron. In vivo, biliverdin is reduced to bilirubin by biliverdin reductase (figure 3).[4] Heme oxygenase exists in two isoforms, HO-1 and HO-2. A third isoform, HO-3, has been reported but not well understood.[5] The HO-1 enzyme, also known as heat shock protein HSP32, is inducible in the presence of stresses including oxidative, heavy metal, and inflammation. The HO-2 enzyme is constitutive and also serves as a heme-binding protein.[5,6] The highest concentration HO-2 is found in the mammalian brain and testes.[5]

The decomposition catalyzed by HO proceeds through three successive oxygenation reaction, sequentially forming α-meso-hydroxyheme, verdoheme (dark green) and biliverdin (green). Biliverdin is reduced to bilirubin which is yellow in color. The HO catalyzed heme degradation is responsible for the color change from purple to yellow observed in a bruise. The reaction requires seven electrons, which is provided by NADPH-cytochrome P450 reductase in mammals.[6,7] HO catalysis is unique in that the heme prosthetic group serves as the substrate and the cofactor.[2]

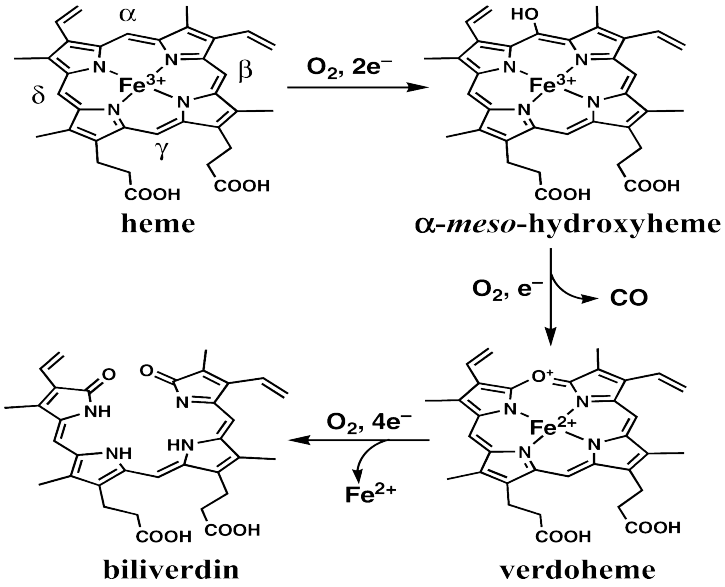

Figure 3. The sequential oxygenation of heme catalyzed by the enzyme HO.

Heme Oxygenase Mechanism

Transfer of electron from NADPH-cytochrome P450 reductase, which reduces the ferric heme-iron to the ferrous state, initiates the catalytic turnover of heme oxygenase.[4] This is followed by the binding of O_2 and transfer of another electron from reductase and a proton from the distal pocket water which leads to the formation of a hydroperoxide intermediate (Fe—OOH). [6,7] The terminal oxygen of the hydroperoxide intermediate attacks the α carbon of the porphyrin ring to form α-meso-hydroxyheme. The reaction of HO-1: heme complex with H_2O_2 supports the involvement of the ferric peroxo intermediate and supports the α-meso-hydroxyheme formation. [6,7]

In the second step, the α-meso-hydroxyheme is converted to Fe(II) verdoheme and CO in the presence of molecular oxygen and one electron (Figure 2).

Although there are multiple proposed pathways proposed to yield verdoheme, the molecular oxygen is thought to react on the porphyrin ring and form α-, β-, γ-, δ-isomers of verdoheme. [4, 6]

The third step of the HO mechanism is not well understood. The conversion of verdoheme to biliverdin requires one O_2 molecule and four electrons (Figure 2). According to *Matsui et al.*, Fe (II)-verdoheme is first converted to Fe (III)-biliverdin, and subsequent one electron reduction of iron liberates free Fe (II) (Figure 3).[5]

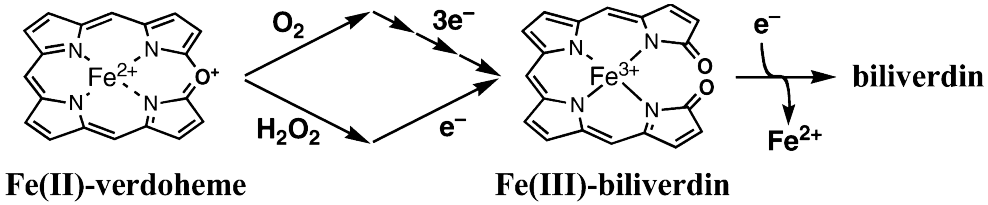

Figure 4. Dual pathway of verdoheme ring-opening supported by O_2 and H_2O_2.

The HO enzyme is known to incorporate the oxygen from O_2 rather than the oxygen from water, which suggests that the reaction is not a true hydrolysis reaction. [4, 6]

Protein Film Voltammetry of Heme Oxygenase

Human heme oxygenase-1 (hHO-1) is a 33-kDa membrane bound protein with 288 amino acid residues (Figure 4).[8] There are difficulties associated with the expression and purification of hHO-1.

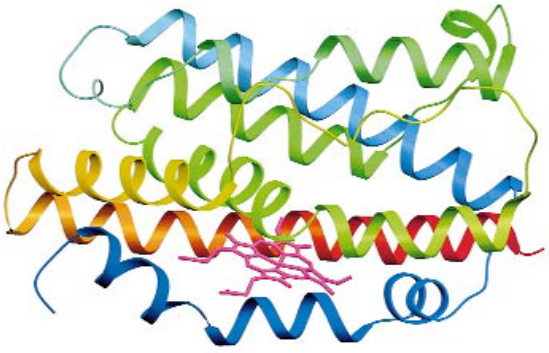

Figure 5. Crystal structure of heme oxygenase

Hence, a truncated form of hHO-1 without the 23 amino acid binding domain, which is important for the insertion of HO into the endoplasmic reticulum, is used in this experiment, which is easily purified and fully active. The truncated form of HO is provides a greater yield of the enzyme which is soluble and fully active.[9] *Ortiz de Montellano et al.* and colleagues determined that the surface of hHO-1 around the exposed heme is electropositive which provides the ideal site for the binding of P450 reductase which is negatively charged.[8] Alanine mutagenesis performed on the residues surrounding the exposed heme suggests that the mutation to a positively charged amino acids including Lys^{18}, Lys^{22}, Lys^{39}, Arg^{183}, Arg^{185}, Arg^{198} and negatively charged Glu^{19}, Glu^{127}, and Glu^{190} caused a decrease in the binding affinity of P450 reductase.

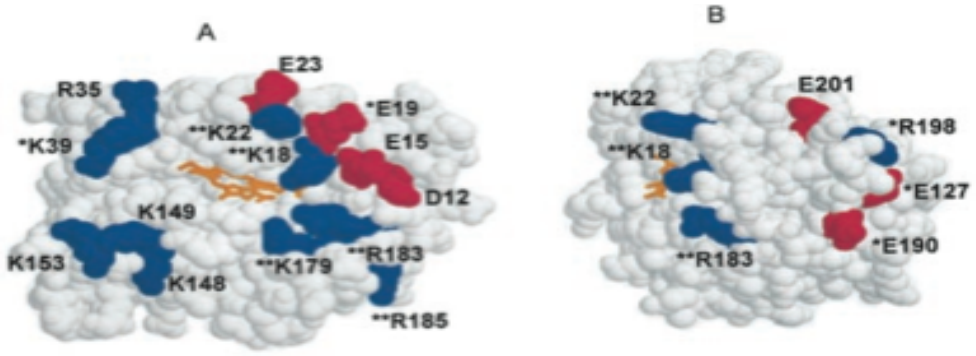

Figure 6. Space-filling structure of hHO-1. *(A)* front surface where heme is partially exposed; (*B),* the side view of $hHO\text{-}1_{233}$ obtained by a 90 ° rotation of the view in (*A).* The heme is shown in *orange,* positively charged residues in *blue,* and negatively charged residues in *red.* The indicated residues were mutated to an alanine. An *asterisk* indicates a mutant with a significantly decreased binding affinity for P450 reductase, and a *double asterisk* indicates mutant with a low binding affinity for both P450 reductase and biliverdin reductase.

Electrochemistry studies of heme oxygenase can provide insight into the mechanism of heme oxygenase. Voltammetry studies of heme oxygenase were performed on pyrolytic graphite electrode (PGE) trapped in nafion films. The activity of heme oxygenase was tested by SDS PAGE, UV-VIS spectroscopy and kinetic studies.

CHAPTER TWO

Materials and Methods

Experimental Procedure

Human heme oxygenase -1 was expressed in *E. coli* and purified as adapted from *Wilks et al.*[11]

Transformation

The HO was expressed using Δ^{233}-HO-1 gene in competent DH5α *E. coli* cells. After thawing the cells on ice, 1 µl of plasmid was added to 10 µl of competent cells in an Eppendorf tube and mixed using a vortex for 2 seconds. The cells were heat shocked for 45 seconds at 42 °C. A volume of 50 µl of LB media was added to the cells and incubated in the shaker at 215 rpm at 37 °C. The cells were transferred to an agar plate containing ampicillin. The plate was incubated overnight at 37 °C.

Expression

5 mL of LB media containing 6 µl of 100 µg/mL of ampicillin was placed in a culture tube. Single colonies were picked from the agar plate and overnight cultures were grown for 8 hours at 215 rpm in the incubator-shaker. 500 µl of the overnight culture was transferred to 2.8 L Fernbach flasks containing 1 L of LB media and 1 mL of 100 µg/mL ampicillin. The flasks were placed in the incubator-shaker at 37 °C and a shaking speed of 150 rpm. The cells were grown to an optical density at 600 nm (approximately 3-5 hours) of .3-.5 absorbance and induced with 1 mL of 1mM IPTG (isopropyl-P-D-thiogalactoside). The shaking speed was decreased to 115 rpm

and the cells were allowed to over express for 12-18 hours. A Beckman Avanti J-25 centrifuge was used to harvest the cells by spinning at 5000 rpm for 20 minutes.

Purification

The cells were purified by re-suspending the pellets in a pH 8.0 lysis buffer containing 50 mM TRIS and 2mM EDTA buffer. The appropriate amount of 1 M stock solution of PMSF was added to the cell to achieve a final concentration of 1 mM PMSF in solution. The cells were lysed using a French press. To the crude lysate of Δ^{233}-HO-1, 35 uL/mL of 10% polyethylenimine was added and the mixture was placed on ice for 25 minutes. The lysate was brought to 40% ammonium sulfate saturation with gentle stirring for 30 minutes. The sample was centrifuged at 15,000 rpm for 24 minutes and the pelleted material was discarded. The supernatant was then brought to 70% ammonium sulfate saturation with gentle stirring for 30 minutes followed by centrifugation at 10,000 rpm for 15 minutes. The liquid was discarded and the green-pelleted material was resuspended and dialyzed. 3 x 4 hours each in the 50 mM TRIS 2 mM EDTA buffer. The precipitate formed during dialysis was removed using a Nalgene vacuum filtration system containing a 0.2 μm PES membrane. The protein was filtered using a Sephadex G-75 gel filtration column using 20 mM pH 7.4 phosphate buffer. The collected sample was analyzed using SDS-PAGE and UV-VIS spectroscopy. UV –Vis: Fe - λ_{max}=280 nm.

HO Reconstitution

A 2.5 excess of hemin was dissolved in minimal volume of 0.1 M NaOH and neutralized by an equal amount of 1 M phosphate buffer. These solutions were added to the purified protein in 200 uL increments and the reconstitutions was monitored by the growth of the soret band at 404 nm for hemin via UV-Vis spectroscopy. The addition of hemin was stopped after the soret band reached its maximum. Fe (III)-HO was placed on a shaker for 2 hrs. The protein was concentrated and filtered using G-25 Sephadex column with pH 7.4 50 mM Sodium Phosphate buffer to remove unbound hemin.

Reaction of Fe(III)HO with sodium ascorbate and H_2O_2

For the reactions with H_2O_2, 20 mM (4 µl) H_2O_2 was added to the 100 uM of Fe(III)HO in pH 7.4 50 mM carbonate buffer. For the reactions with sodium ascorbate, 10 mM sodium ascorbate (46 µl) was added to the 100 uM Fe(III)HO in pH 7.4 50 mM carbonate buffer. The spectra for each reaction were taken at 10-s intervals for 20 min immediately after the addition of sodium ascorbate and H_2O_2. For H_2O_2, the absorbance at 520, 556, and 680 nm were followed. For sodium ascorbate, the absorbance at 544, 574, and 680 nm were followed.

Protein Film Preparation

Pyrolytic graphite (PG) working electrode was cleaned by 20-min sonication in methanol followed by 20-min sonication in water. 100 uL of nafion was added to 9.9 mL of methanol to form a 1% nafion solution. 5 uL of Fe(III)HO was cast onto a

circular pyrolytic graphite working electrode and allowed to dry overnight. Then 5 uL of 1% nafion solution was cast onto the electrode and allowed to dry for 6 hours. The PG electrode was rinsed with water to wash excess film. The resulting stable film also gave reproducible voltammetric currents for weeks.

Electrochemistry

A three-electrode setup was used to perform cyclic voltammetry which included a pyrolytic graphite working electrode, a platinum wire as the counter electrode, and a saturated calomel electrode (SCE) as the reference electrode. All electrochemistry was performed in a pH 7.1 20 mM sodium phosphate buffer (NaPi). The buffer was purged with nitrogen for 15 minutes before cyclic voltammetry was performed. Various different potential ranges were used. Three different scan rates were performed: 10 mV/s, 50 mV/s, and 100 mV/s.

CHAPTER THREE

Results and Discussion

Expression and Purification of truncated hHO-1

Truncated form of hHO-1 was used to achieve a high yield. Expression of the truncated hHO-1 turned the media green as a result of biliverdin accumulation. This suggests that the E. coli cells are capable of reductase activity and the hHO-1 undergoes a catalytic turnover. The truncated form of hHO-1 is constructed without the 23 amino acid membrane binding domain, which forms the C-terminal lipophilic peptide necessary to the anchor the HO-1 enzyme to the membrane. Prior to the construct of a truncated hHO-1 enzyme by *Wilks and Ortiz de Motellano et al*., it was difficult to purify and study the structure and mechanism of heme oxygenase. The purified truncated HO protein is easily purified, soluble and catalytically active and was characterized via UV-VIS spectroscopy and SDS-PAGE. The purification process resulted in apo-HO which has an absorbance of 280 nm mainly due to the aromatic rings of tryptophan and tyrosine (Figure 6A). The truncated hHO-1 protein was further characterized by a 27 kDa band on SDS-PAGE (Figure 6B).

(A) (B)

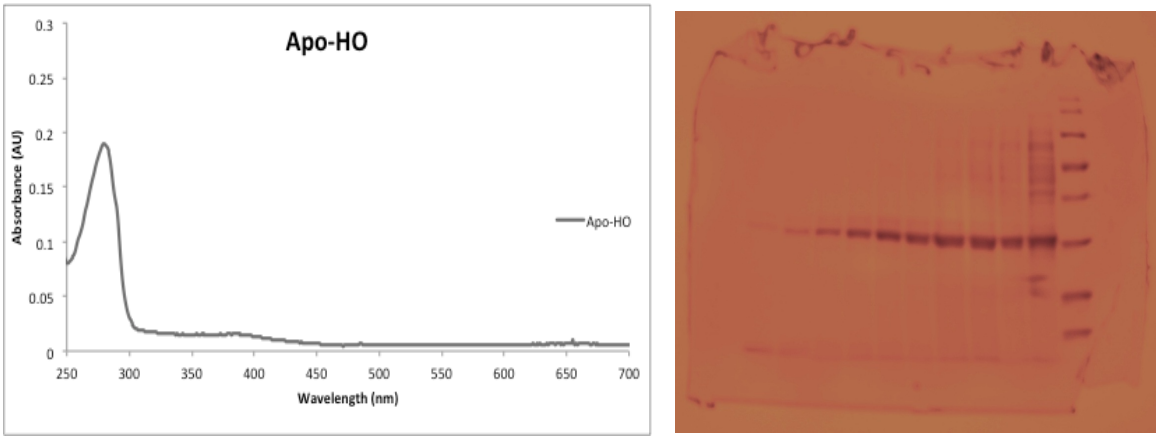

Figure 7. (A) Absorption spectrum of purified apo-HO. (B) SDS-PAGE of purified apo-HO: lane 1, molecular mass markers; subsequent lanes, purified apo-HO.

The purified truncated hHO-1 was reconstituted with 2.5x excess of hemin. The hemin was dissolved in 0.1 M NaOH and added in 200 uL increments to the purified hHO-1. Growth of the Soret band at 404 nm was observed as Fe(III)-HO complex was formed (Figure 7).

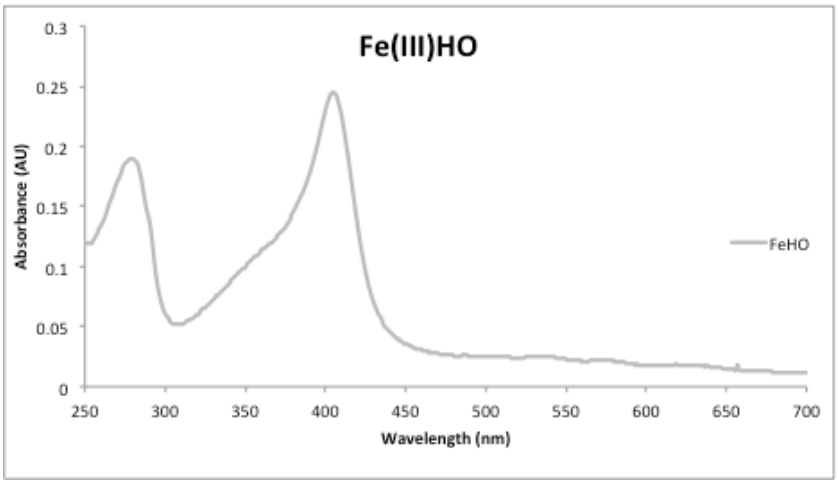

Figure 8. Absorption spectrum of complex of heme with purified HO after passage through a G-25 sephadex column to remove excess heme.

In the native configuration of heme oxygenase, iron is bound to the heme group present. To test the activity of the enzyme, Fe(III)HO was reacted with sodium ascorbate and H_2O_2 under aerobic conditions.

Reactivity of Fe(III)HO with sodium ascorbate and H_2O_2

Wang et al. provides evidence that Fe(III)-verdoheme and Fe(III)-biliverdin is produced with Fe(III)HO in the presence of H_2O_2 and sodium ascorbate, respectively. [13] To test the reactivity of the reconstituted Fe(III)HO, reactions with sodium ascorbate and H_2O_2 were performed and the results were compared to literature.

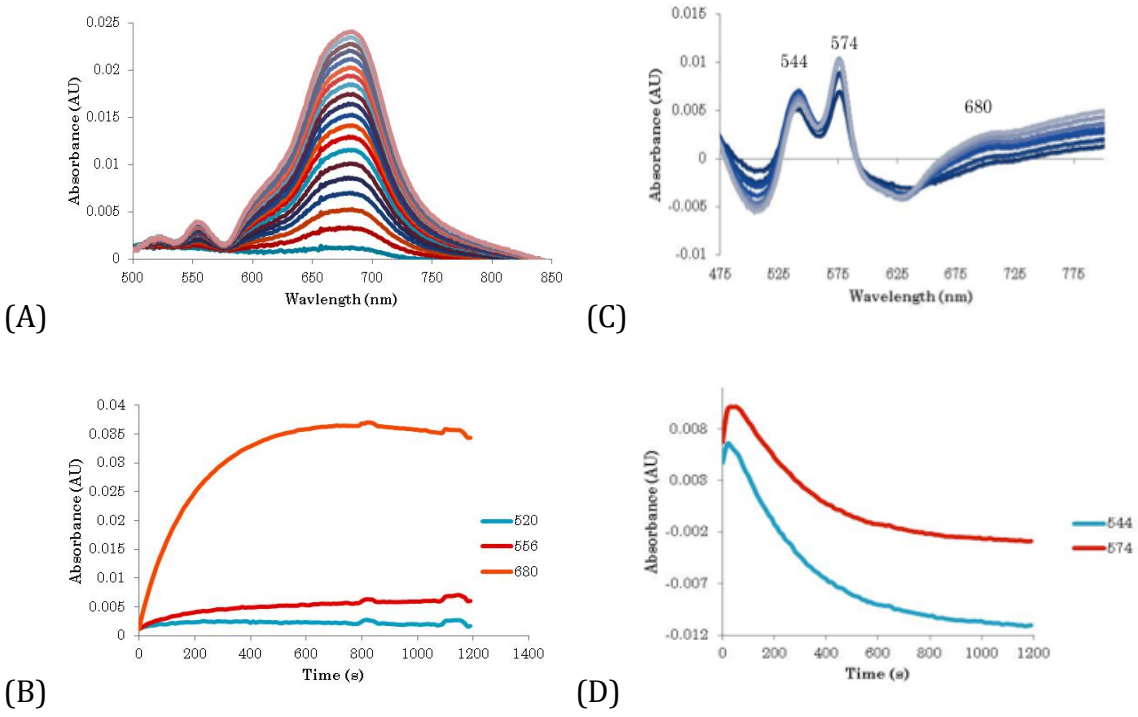

Figure 9: Fe(III)HO reaction with H_2O_2 and sodium ascorbate. (A) UV-VIS spectra of reaction with H_2O_2 at 10-s interval. (B) Time plot following absorbance at 520 nm, 556 nm, and 680 nm of Fe(III)HO reaction with H_2O_2. (C) UV-VIS spectra of Fe(III)HO reaction with sodium ascorbate. (D) Time plot following absorbance at 544 nm and 574 nm of reaction with sodium ascorbate.

Figure 9 shows the formation of Fe(III)verdoheme in the presence of H_2O_2 and Fe(III)biliverdin in the presence of sodium ascorbate. The spectra were taken 10-s intervals for 20 min immediately after the addition of H_2O_2 and sodium ascorbate. In figure 9A and 9B the accumulation of Fe(III)-verdoheme is evident in the presence of 20 μM H_2O_2 as indicated by the increase in absorbance of 520 nm, 556 nm, and 680 nm which are characteristic absorbance values of verdoheme in wild-type reactions. Hydrogen peroxide serves a surrogate to O_2 and provides electrons for the turnover of HO.[7] The reaction of Fe(III)HO in the presence of H_2O_2 produces Fe(III)verdoheme because a reductant is not present to covert verdoheme into biliverdin.

In figure 9C and 9D, the UV-VIS spectra and time plot of the reaction of Fe(III)HO with sodium ascorbate is shown. Wang et al. suggests the accumulation of Fe(III)biliverdin in the presence of sodium ascorbate which has a spectrum with two distinct absorbance at 544 nm and 574 nm.[13] Unlike the sharp peak at 680 nm for verdoheme, biliverdin has a broad absorbance at 680 nm as evident in figure 9C. However, figure 9D shows an initial increase in the 544 nm and 574 nm bands but steadily decreases. This suggests that the Fe(III)biliverdin complex is initially formed but the Fe(III) is ultimately reduced to Fe(II) then released which is indicated by the decay in the time plot. In the presence of sodium ascorbate, Fe(III)HO produces biliverdin because sodium ascorbate serves as the reductant and causes a turnover of the enzyme.

Electrochemistry of Heme Oxygenase

Fe(III)HO was immobilized on a pyrolytic graphite (PG) electrode using Nafion and Electrochemical properties of Fe(III)-HO were investigated using cyclic voltammetry (CV). A typical cyclic voltammogram for Fe(III)HO/Nafion film on PG electrode in pH 7.1 20 mM NaPi buffer in anaerobic condition is shown in Figure 8. The potential for the CVs were held constant between -0.8 V to +0.8 V but the scan rates were altered. After the solution was purged with N_2 for 15 minutes, the CVs for Fe(III)HO/Nafion film at a scan rate of 0.01 V/s and 0.1 V/s show cathodic-anodic peak pair (figure 10).

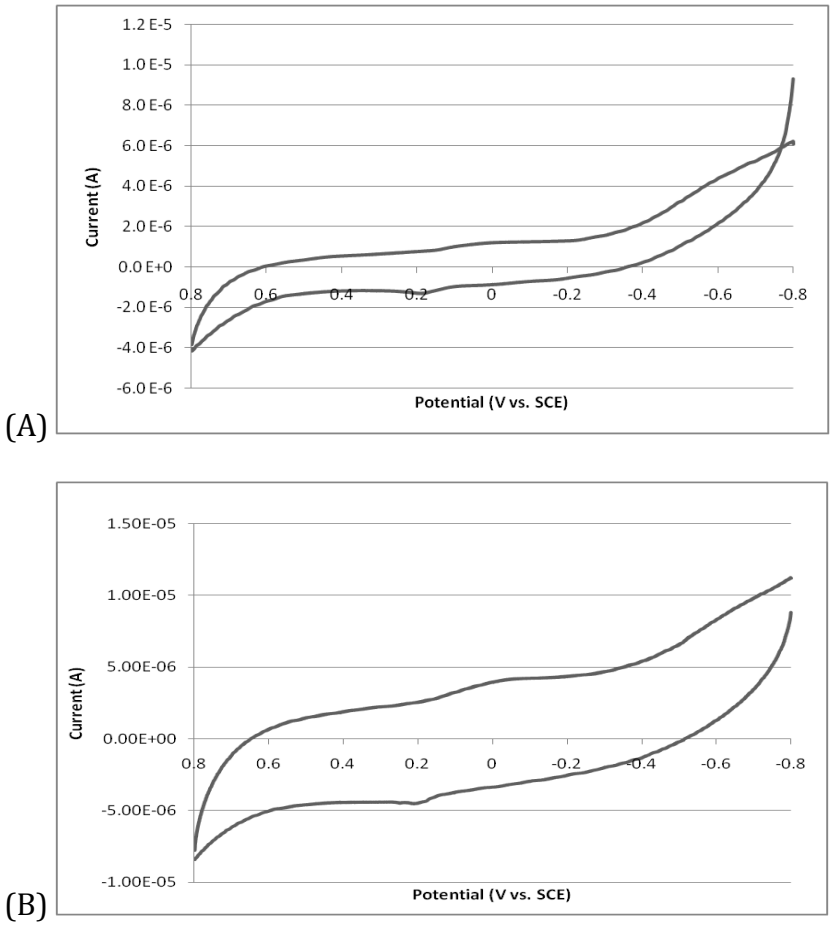

Figure 10. CV of 0.5 mM Fe(III)HO with nafion film. Potential scan range is from -0.8 V to +0.8 V. (A) Scan rate of 0.01 V/s (B) Scan rate of 0.1 V/s.

The cathodic-anodic couples at -0.05 V vs SCE are assigned to the reduction of Fe(III)to Fe(II) which occurs as HO is converted from heme to α meso-hydroxyheme (figure 9). The catalytic conversion of heme to meso-hydroxyheme by HO requires two electrons and one molecule of O_2. The first step in O_2 activation is the reduction of Fe(III) to Fe(II) and the reduction peak at approximately -0.05 V vs SCE is assigned to this reduction. The peak at around 0.2 V vs SCE is assigned to subsequent oxidation of Fe(II) to Fe(III) to form meso-hydroxyheme. [6]

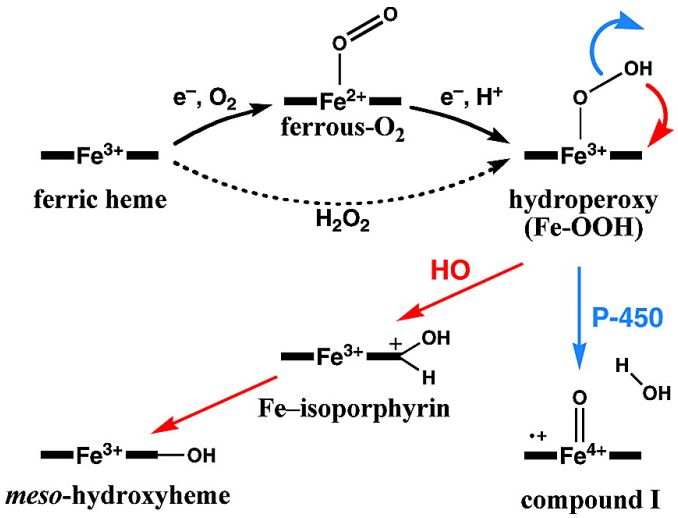

Figure 11. The electron transfer required for the conversion of ferric heme to meso-hydroxyheme.

Rusling and coworker's extensive work on Mb/DDAB films report similar redox couple at -0.1 V vs SCE due to the conversion of Fe(III) to Fe(II) in Mb.[10] Although the oxidation peak is slightly shifted to 0.2 V, the reduction of Fe(III) to Fe(II) is a reversible process.

In cyclic voltammetry of an enzyme, two different types of waves are possible: a "nonturnover" wave and a catalytic wave.[11] As seen with the reduction of Fe(III) to Fe(II), a nonturnover signal is due to the transfer of electrons from the active site of the protein to the electrode surface. The catalytic wave reports the activity of the enzyme and presents a large amplification of the current which is directly related to the enzymes turnover rate.[11, 12]

Figure 11 shows the cyclic voltammogram of heme oxygenase under aerobic conditions. Heme oxygenase catalyzes the degradation of heme into biliverdin, ferrous iron and CO in the presence of three molecules of oxygen and seven electrons. Figure 11 shows an increase in current at -0.4 V vs. SCE when oxygen is introduced in the system. The increase in current suggests that oxygen is being reduced by HO which might indicate a possible turnover of the enzyme. Although the voltammogram indicates a slight change in current, the increase is not significant to represent a catalytic wave.

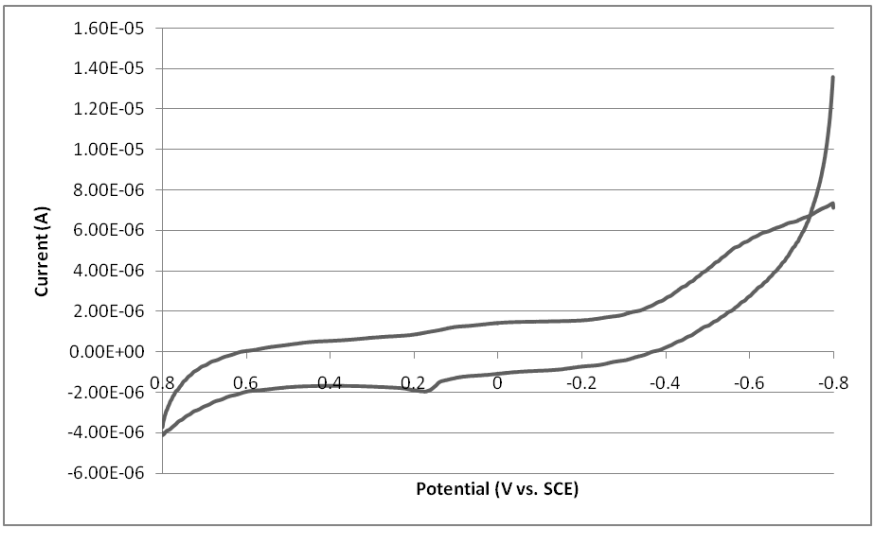

Figure 12: Cyclic voltammogram of Fe(III)HO under aerobic conditions

REFERENCES

1. Gulaboski, R.; Mirceski, V.; Bogeski, I.; Hoth, M. *J. Solid State Electrochem*. 2012,16, 2315−2328.

2. Shen, L.; Hu, N. *Biochim. Biophys. Acta* 2004, 1608, 23-33.

3. Lin, R.; Immoos, C.; Farmer, P. J. *J. Biol. Inorg. Chem*. 2000, 5, 738-747.

4. Ortiz de Montellano, P.; Auclair, K. *The Porphyrin Handbook*. 2003, 11, 1-27.

5. G. Kikuchi, T. Yoshida, M. Noguchi. *Biochem. Biophys. Res. Commun*. 2005,338, 558–567.

6. Wilks, A.; Black, S. M.; Miller, W. L.; Ortiz de Montellano, P. R. *Biochemistry*. 1995, 34, 4421.

7. Matsui, T.; Unno, M.; Ikeda-Saito, M. *Acc. Chem. Res.* 2009, 43, 240–247.

8. Wang, J. and de Montellano, P.R. *J. Biol. Chem*. 2003, 278, 20069–20076.

9. Wang,J., Niemevz, F.,Lad,L., Huang,L., Alvarez,D.E., Buldain,G., Poulos, T. L., and Ortiz de Montellano, P. R. (2004) *J. Biol. Chem.* **279,** 42593– 42604.

10. J. F. Rusling and A.-E. F. Nassar, *J. Am. Chem. Soc.*, **1993**, *115*, 11891-11897.

11. Vincent, K. A.; Armstrong, F. A. *Inorg. Chem*. 2005, 44, 798.

12. Leger, C.; Elliott, S. J.; Hoke, K. R.; Jeuken, L. J. C.; Jones, A. K.; Armstrong, F. A. *Biochemistry* **2003,** *42,* 8653.

13. J. Wang, L. Lad, T. L. Poulos and P. R. Ortiz de Montellano, *J. Biol. Chem*., 2005, 280, 2797–2806.