GENETICALLY MODIFIED MYOGLOBIN AS A MIMIC FOR HEME ENZYMES

by

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ABSTRACT

GENETICALLY MODIFIED MYOGLOBIN AS A MIMIC FOR HEME ENZYMES

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This study addresses the mechanistic relationships between formation of reactive oxygen species (ROS) and their catalytic oxidation functions in oxygenation and peroxynitration reactions in heme enzymes. Even though both cytochrome P450s (CYP P450s) and peroxidases have different catalytic activities, the involvement of common ROS (Compound 0 and Compound I) have been proposed. Therefore, to understand the generation and activation of peroxide to form ROS, genetically-engineered myoglobin (Mb) mutants were created by incorporating redox-sensitive 3-amino-L-tyrosine (NH₂Tyr) or L-3, 4-dihydroxyphenylalanine (DOPA) into its active site. Distal His 64 replaced with redox amino acids mutant Mb showed excellent turnover rates for thioanisole and benzaldehyde oxidation, compared to the wild-type protein. A 9-fold and 81-fold increase in activity, respectively, was observed in the presence of hydrogen peroxide (H₂O₂). The presence of a redox unnatural amino acid in the active site enhances the rate of compound I formation and stabilizes it to form one extra H-bond as compared to the wild type (WT) Mb. This increased oxidation activity in mutants offer insights into the role of the distal active site residues which are involved in acid-base catalysis and distal charge relay "pull" effect in peroxide activation and formation of ROS in heme proteins.
Furthermore, cyclic voltammetry (CV) and atomic force microscopy (AFM) were used to investigate the importance of active site orientation of an immobilized protein for direct electron transfer (DET) and electrocatalysis. While the bioconjugated wild-type myoglobin (WT Mb) was immobilized on the modified gold electrode surface in a random multilayered fashion, the Ser 3 replaced with NH$_2$Tyr in Mb mutant, was immobilized via a Diels-Alder reaction specific to the NH$_2$Tyr residue to form a homogeneous monolayer. Electrochemical calculations for the number of surface exposed redox-sensitive molecules on the electrode surface ($\Gamma$) and heterogeneous rate constant for DET were $1.29 \times 10^{-10}$ mol cm$^{-2}$; 2.3 sec$^{-1}$ for the WT Mb and $1.54 \times 10^{-10}$ mol cm$^{-2}$; 1.3 sec$^{-1}$ for the S3NH$_2$Tyr Mb mutant, respectively. Electro-catalytic conversion of thioanisole to sulfoxide products showed similar turnover frequencies (TOF) around $1.9 \times 10^3$ sec$^{-1}$ (with 87% conversion) for the WT Mb, and $1.5 \times 10^3$ sec$^{-1}$ for the mutant Ser 3-amino-L-tyrosine (S3NH$_2$Tyr) Mb (with 81% conversion). These results indicate that site-directed single monolayer immobilization affords almost the same number of surface exposed Mb active sites as the random multilayer immobilization strategy, though the latter contains a greater number of protein molecules on the electrode surface. The microarray concept development provides novelty to study protein-protein interactions, drug discovery, and biomedical and proteomic research.

Another aspect of this research was that the importance and significance of electron rich functional groups on the electronic nature of heme center had been extensively explored. It has been found that the axial His attached to the heme center plays a decisive role in dictating the electron cloud near the heme center. When the axial heme is replaced by a residue with an electron rich functional group like $\rho$NO$_2$ L-Phenyl alanine ($\rho$NO$_2$Phe), the electron density was higher near the heme center. The oxidation state of the metal center and the nature of the ligand play important role in the determination of back-bonding and direction of charge transfer.
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This study “Genetically modified myoglobin as a mimic for heme enzymes” is submitted by Subhash Chand to The University of Texas at Arlington for the degree of Doctor of Philosophy (Ph.D.), and has the following kind contributions. Chapter 4 was published as "S. Ray, S. Chand, Y. Zhang, S. Nussbaum, R. Krishnan, R. Perera, Immobilization of Myoglobin on the Gold Electrode to Promote Direct Electron Transfer, *Electrochimica. Acta*. 2013, 99, 85-93". In this study Dr. Ray has carried out electrochemistry studies. Figures (4.1, 4.5, 4.6, 4.7, and 4.8) and schemes (4.1 and 4.2) were kindly contributed by her. The microarrays studies in chapter 4 have been published as “B. Stamos, L. Loredo, S. Chand, T. Phan, Y. Zhang, S. Mohapatra, R. Perera, Biosynthetic Approach for Functional Protein Microarrays, *Anal. Biochem.* 2012, 424, 114-23".

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CHAPTER 1
INTRODUCTION

1.1 Heme proteins

Heme proteins are the big family of proteins and are widely distributed in nature. Some of these proteins act as oxygen storage (e.g., myoglobin) and some as an oxygen carrier (e.g., hemoglobin). While other heme proteins play important roles either in mediating electron transfers (e.g., Cyt b5, Cyt c), or in catalysis (e.g., cytochrome P450, peroxidase, and catalase) (1, 2). Along with heme, many enzymes contain other cofactors such as molybdopterin, copper, flavin, and iron-sulfur cluster (1, 2). In these proteins (e.g., flavocytochrome b2, flavocytochrome c3, p-cresolmethyl hydroxylase, sulfite oxidase, fructose dehydrogenase, alcohol dehydrogenase, and cytochrome c oxidase), charge transfer is the main function of heme domain. Iron porphyrin prosthetic groups are the most common among all heme proteins (1-3). Different varieties of porphyrins such as heme a, heme b, heme c, heme d, heme d1, heme P460, heme o, siroheme, and chloroheme are present in heme proteins (Figures 1.1 and 1.2, and Table 1.1). These porphyrins have a common basic skeleton, but due to substitution at various positions, they differ in their structural details. In heme proteins, nitrogen from the porphyrin ring occupy four coordination sites out of six of the heme iron, leaving only two positions available for further ligand interactions which strongly influence the reactivity and redox potential of the heme protein (1-3).

In many heme and globin enzymes, penta-coordinated heme iron is found (1, 3). In globins, the proximal histidine (His) residue act as fifth ligand while axial heme position remains open for O2 coordination, while the sixth coordination position is used for substrate binding in some heme enzymes (1, 3). The redox potential of the heme center depends on the variety of proximal ligands while distal ligands are responsible for the catalytic reaction in general (2, 3).
The spin state of the heme center is governed by the nature of axial ligands (1, 2). If a water molecule is present as the sixth ligand, the center is in a high spin state, while presence of carbon monoxide (CO), nitric oxide (NO), O₂ azide (N₃⁻), and cyanide (CN⁻) as the sixth ligand makes it in a low spin state (2, 3).

Heme type b is the simplest form of all known hemes and contains methyl group at 1, 3, 5, and 8 positions. At the same time 6 and 7 positions have two propionate groups and also 2 and 4 positions have vinyl groups (1). Energetically favored orientation of heme is the coplanar structure, where vinyl group and the aromatic heme plane have the capability to interact with the amino acids scaffold in the proteins (1, 2).

![Heme a and Heme b](image)

*Figure1.1 Structures of heme a and heme b.*
Figure 1.2 Structures of naturally occurring iron porphyrines.
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<td></td>
<td>Fe(III) (S = 1/2)</td>
</tr>
<tr>
<td>Mb Hemoglobins</td>
<td>O₂ storage</td>
<td>b</td>
<td>His</td>
<td>Deoxy Fe (II) (S = 2)</td>
</tr>
<tr>
<td></td>
<td>O₂ transport</td>
<td>b</td>
<td></td>
<td>Oxy Fe(II) (S = 0)</td>
</tr>
<tr>
<td>Peroxidases</td>
<td>A-H₂+H₂O₂ → A+2H₂O</td>
<td>b</td>
<td>His</td>
<td>Fe(III) (S = 5/2)</td>
</tr>
<tr>
<td>Catalases</td>
<td>H₂O₂+ H₂O₂ → O₂ + 2H₂O</td>
<td>b</td>
<td>Tyr</td>
<td>Fe(III) (S = 5/2)</td>
</tr>
<tr>
<td>P450 proteins</td>
<td>RH +2e⁻ + 2H⁺ + O₂ → ROH + H₂O</td>
<td>b</td>
<td>Cys</td>
<td>Fe(III) (S = 5/2)</td>
</tr>
<tr>
<td>Cytochrome bo quinol oxidases</td>
<td>O₂ + 4 Fe(II)cyt c + 4e⁻ + 4H⁺ → 4 Fe(III)cyt c + 2H₂O</td>
<td>o</td>
<td>His</td>
<td>Fe(III) (S = 5/2)</td>
</tr>
<tr>
<td>Hydroxylamine oxidoreductase</td>
<td>H₂O + NH₂OH → NO₂⁻ + 4e⁻ + 5H⁺</td>
<td>P 460</td>
<td>His</td>
<td>Fe(III) (S = 5/2) Or (S = 3/2)</td>
</tr>
<tr>
<td>Nitric reductases (cyt d₁)</td>
<td>2H⁺ + NO₂⁻ + e⁻ → NO + 2H₂O</td>
<td>d₁</td>
<td>His</td>
<td>Fe(III) (S = 1/2)</td>
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<tr>
<td></td>
<td>4H⁺ + O₂⁻ + 4e⁻ → 2H₂O</td>
<td></td>
<td>His+ Tyr</td>
<td></td>
</tr>
<tr>
<td>Sulfate Reductase</td>
<td>8H⁺ + NO₂⁻ + 6e⁻ → NH₄⁺ + 2H₂O</td>
<td>Siroheme</td>
<td>Cys</td>
<td>Fe(II) (S = 1 or 2)</td>
</tr>
</tbody>
</table>
1.1.1 Myoglobin

Myoglobin (Mb) is a relatively small heme protein (17 kDa) that is less affected by environmental conditions when compared to most of the other heme proteins. Mb is a single-chain globular protein of 153 amino acids, as found in the sperm whale, and serves as oxygen storage (4, 5). It consists of eight separate right-handed α helices in its apoprotein (Figure 1.3). Oxygen can bind at the distal site of proteins, directly to the iron atom of the heme. The axial His (His 93) links protein to the heme center through a bond between the iron atom and the His (2, 4). As shown in figure 1.2, a wide variety of amino acid residues are present near the binding site of Mb. These residues play a critical role in determining the electronic nature of the heme center and catalytic nature of the heme proteins (4-6). Studies shows that in Mb, the positions such as 64, 68, and 93 are the most crucial residues (4). Due to its ability to prepare mixed ligand complexes with different valences (ferric, ferrous, and ferryl species), Mb is often used as a model to mimic other heme proteins such as cytochromes, peroxidases, catalases, chloroperoxidases, and many more (5-10).

The role of the proximal ligand in controlling the reactivity and stabilizing the heme has been a matter of interest for many years. In Mb, the role of proximal and distal His has been assessed by replacing it with different residues (11-15).

Mb is an oxygen storage heme protein; however, in presence of H₂O₂, it catalyzes a variety of oxidative reactions such as sulfoxidation and epoxidation. Since Mb does not form a stable Mb compound I in presence of H₂O₂, the rate of epoxidation and sulfoxidation are significantly slow in Mb as compared to those of peroxidases (16-19). Watanabe and coworkers have created mutations at 64, 43, and 29 positions of Mb, replacing respective amino acids with Asp, His, and Trp, as well as with Leu and His in double mutants. They have been able to introduce catalase monooxygenase and peroxidase activity in Mb by generating compound I in different Mb mutants (16-27).
Figure 1.3 Overall structure of sperm whale Mb. (A) WT Mb showing heme center and His at 64 and 93 positions and (B) schematic representation of binding site of WT Mb.
Dawson and coworkers have created an H93G Mb cavity mutant and explored it as an excellent structural model for His-ligated and other heme containing proteins (15, 28-32). They have successfully compared and used Mb mutants as a model for the study of cytochrome f, nitrite reductases, peroxidases, cytochrome P450, and many other heme proteins (15, 29, 33-43). They have also extensively studied the ligand binding properties of heme proteins (6, 35, 43).

1.2 Incorporation of unnatural (noncanonical) amino acids into proteins

There are two strategies for the incorporation of noncanonical amino acids/unnatural amino acids (UAA) into proteins: (1) site-specific and (2) residue-specific (44, 45). The site-specific approach involves replacement of a single amino acid residue (Figure 1.4) while the residue-specific method allows replacement of all or a fraction of one of the natural amino acids by UAA (44-52). In the site-specific method, to deliver the analog in response to a stop or four-bases codon, a heterologous tRNA/ami noacyl-tRNA synthetase pair is used (53). Amber codon suppression in mammalian cells has been used to incorporate unnatural amino acids by using the E. coli tRNA/ami noacyl-tRNA synthetase pair; none of the suppressor tRNA was charged by any of the mammalian aminoacyl-tRNA synthetases (54). For the site-specific incorporation of UAA, amber codon suppression is the most frequently used method (Figure 1.3) and to date more than 50 UAA have been introduced in recombinant proteins using these methods (Figure 1.4) (44, 52). For the incorporation of structurally, chemically and spectroscopically amino acids, Schultz and coworkers have produced orthogonal suppressor tRNA/ami noacyl-tRNA synthetase pairs and used it to incorporate many UAA in recombinant proteins (55-60). By using both four base codon and nonsense codon suppression, Dougherty and coworkers have successfully incorporated two UAA into an ion channel protein (61-63). Four bases codons, commonly known as frameshift suppression, have also been used for the site-specific insertion of UAA into fluorophore proteins and in many more recombinant proteins (50, 63-65).
Figure 1.4 A general method for genetically encoded unnatural amino acids in live cells.
Figure 1.5 The list of unnatural amino acids that have been introduced to the genetic codes of E. coli, yeast, and mammalian cells.
Furthermore, for the site-specific incorporation of UAA, analogous five bases codon and reassignment of sense codons can also be used, but fidelity of the method is much lower than that of anti-sense or frameshift suppression methods (66-68). It is possible in the genetic code that one amino acid can be coded by more than one codon, as there are 61 codons for 20 natural amino acids; this makes the genetic code highly degenerate. In *E.coli*, many amino acids are coded by more than one codon. Phe is coded by UUU and UUC. Only one tRNA has been assigned for both codons. The tRNA uses base pairing and wobble interaction to decode UUC and UUU, respectively. tRNA synthetases from different organisms have been used for the reassignment of UUU into host *E.coli*. The discriminating capability of synthetases between unnatural and natural amino acids plays a crucial role in the incorporation of UAA and yield of recombinant proteins.

Similar to post-translational modification, chemical methods for protein modification are equally important and they have been used to enhance the performance of therapeutic proteins and labeling with fluorescent dyes (69, 70). Highly reactive Cys and Lys residues have been most commonly used in the process of protein modifications (70).

1.3 Direct electron transfer and bioelectrocatalysis

Electrochemical methods are very useful in determining the redox properties of proteins and kinetics of electron transfer (71). Electrochemistry can be used as a powerful tool in the biocatalytic study of a protein and in biosensors and biofuel cell production (72). In general, to facilitate the electron communication in between electrode and redox-sensitive protein, small soluble mediators were used (72, 73). Due to the possibility of their unspecific side reaction, these mediators lead to erroneous results; to overcome this problem, direct electron transfer (DET) methods were developed. For the electron communication, the DET methods do not require a mediator between the enzymes and the electrodes. These methods at times have
their own limitation such as denaturation of protein upon adsorption on electrodes, which can be solved by using different varieties of linkers and insulators.

The acceleration of the electrochemical reactions by a biological enzyme comes under the realm of bioelectrocatalysis, and electrons can be transferred between an enzyme active site and the electrode without any mediator. Electrodes for fuel cells and for the biosensors are the two most common applications of bioelectrocatalysis. It was essential to achieve DET in redox enzymes on solid electrode for its further development. In addition, to achieve the most effective electron transfer, the importance of protein orientation on the solid electrode surface has been investigated (71-73).

In principles, bioelectrocatalysis involves acceleration of the electrochemical reactions by biological catalysis (72). Figure 1.6 indicates the involvement of the enzymes in direct bioelectrocatalysis. The communication of electrons with the electrode was provided by enzyme catalyzing redox reactions. Oxidation and reduction reactions are the two halves of redox reactions and one of these half reactions can be substituted in bioelectrocatalysis (72).

Heme is among those prosthetic groups which can communicate directly with the electrode. Heme can be used both in formation of the enzyme active center as well as for the communication with the redox mediator, thereby leading to communication with the electrode. This makes heme one of the most important constituents of direct bioelectrocatalysis systems and hence heme proteins are frequently used in bioelectrocatalysis (74, 75).
Figure 1.6 Schematic representations of bioelectrocatalysis involving direct electron transfer in an enzyme. One of the two coupled half-reactions is substituted by the electrochemical reaction.

1.4 Dissertation overview

Creation of genetically engineered Mb mutants by incorporating a non-cannonical redox-active NH$_2$Tyr into its active site for the mechanistic insight and oxidative activity has been extensively explored in chapter 2. This study proposed the importance of the distal binding site residues in acid-base catalysis and formation of reactive oxygen species (ROS) in heme proteins.

In chapter 3, I reported the incorporation of another unnatural amino acid DOPA to a specific position in the binding site of Mb. The mutant Mb protein could carry out the oxidation of thioanisole and benzaldehyde and support the idea that the distal His is important for the formation of compound I to exhibit peroxynogenase activity in Mb.

In chapter 4, I reported monolayer and multilayer immobilization of Mb using two unique immobilization techniques, onto a chemically-modified gold electrode surface. Further, using
DET method bioelectrocatalysis of thioanisole oxidation was carried out successfully with both the protein immobilized electrodes. The results described in chapter 4 suggest the importance of proper orientation of recombinant heme proteins containing UAA in bioelectrocatalysis and effective electron transfer as comparison to the random multilayered WT Mb case. I have also analyzed the protein-protein interactions of five different proteins which have been covalently attached onto a solid support. Functional protein microarray concept development was confirmed by catalytic activity assay using spectroscopic characterization, which will be of huge importance in the field of drug discovery, and biomedical and proteomic research.

In chapter 5, I have summarized the importance and significance of electron rich residues on the electronic nature of heme center. I have observed that the axial His attached to the heme center plays a more crucial role in dictating the electron cloud near the heme center and hence in formation of reactive species. When axial heme residue was replaced by electron rich residue like pNO2Phe, it contributed to higher energy and red shift of the spectra due to the higher electron density near the heme center. This study also explored the oxidation state of the metal center, the nature of the ligand, the type of residues near the heme center that play an important role in determining of backbonding, the direction of charge transfer, and the possibility of hydrogen bonding. I also underlined that the size of UAA residue plays a crucial role in the stability of ligand adducts.

Overall in this study I have used Mb as a model for the heme protein to study their monooxygenase and peroxidase activity. No prior studies were reported using mutant protein that utilizes its UAA acid residue to immobilize site specifically onto the electrode surface. I also explored the DET and bioelectrocatalysis and importance of the proper orientation of protein using Mb mutants as a model for heme proteins. This study also offers a similar orientation concept while dealing with the protein microarray problems. The Mb mutants have also been used to explore the role and effect of different varieties of residues near the heme center and their effect in ligand binding spectra.
CHAPTER 2
MECHANISTIC INSIGHTS AND IMPROVED OXIDATION ACTIVITY USING GENETICALLY ENGINEERED MYOGLOBIN

2.1 Introduction

Peroxidase enzymes couple substrate oxidation to $\text{H}_2\text{O}_2$ reduction (as generically represented by Reaction 1). They are widely distributed throughout the plant and animal kingdoms and are frequently isolated from bacteria, mold, and microorganisms (76-78). Their main biological function is to act as antioxidants by protecting cells, tissues, and organs against the oxidation of a variety of organic and inorganic compounds by $\text{H}_2\text{O}_2$, organic hydroperoxides, peracids, or inorganic oxysalts, such as periodate or chlorate (79-85).

$$\text{AH}_2 + \text{H}_2\text{O}_2 \rightarrow \text{A} + 2\text{H}_2\text{O} \quad (1)$$

Most heme-containing peroxidases (e.g., horseradish peroxidase, HRP) have a proximal His and a polar distal pocket, with a non-ligated His as an $\text{H}^+$ acceptor/donor and a cationic arginine to stabilize developing negative charges during O-O bond cleavage. Peroxidases transform the oxidizing power of $\text{H}_2\text{O}_2$ into high-valent, protein-bound oxidants. The general peroxidase pathway (Figure 2.1) involves addition of $\text{H}_2\text{O}_2$ or other oxygen atom donors ([O]) to the ferric resting state (1) to form Compound I (5), a ferryl (Fe$^{IV}$) radical species where the π-cation radical is usually centered on the porphyrin (86). Compound I typically oxidizes substrates by one electron to yield an organic radical and Compound II (6), a ferryl heme complex (79, 87). Compound II then oxidizes another substrate molecule and returns to the ferric state (1). A ferryl state-like peroxidase, Compound I, is thought to be the active species in the catalytic cycle (88, 89). Involvement of a hydroperoxo-ferric intermediate (Compound 0) has also been proposed (6, 82). Alternatively, addition of $\text{H}_2\text{O}_2$ to ferric-heme protein can generate Compound 0 (4) via a peroxide "shunt" pathway. Heme-containing peroxidases such as HRP
form Compound I from Compound 0 using distal catalytic arginine amino acids to “pull” the O-O bond apart heterolytically.

Figure 2.1 Mechanistic pathway for generation of the highly reactive Compound I (5) from the ferric resting form (1) in heme-proteins.

Lacking this distal machinery, WT Mb undergoes nearly equal amounts of homo- and heterolytic cleavage upon H$_2$O$_2$ addition. Compound II, the stable product of homolytic cleavage of the O-O bond of (6), is the prime product of Mb but has no catalytic activity. Previous research shows that site-directed mutagenesis is a versatile technique to change the functional properties of Mb. Thus the distal His (H64) has been replaced by amino acids such as Ala, Ser, Leu and Asp, and the corresponding effect on the activity of Mb has been probed (90). Recent
reports evaluated the His93Gly (H93G) “cavity” mutant of Mb as a versatile scaffold for modeling heme states (29, 35, 36). The difference in accessibility of the two sides of the heme have frequently made it possible to prepare mixed ligand adducts in ferrous, ferric, as well as ferryl, oxidation states with this mutant. In addition, the protection provided to the heme by the protein environment allows for the preparation of stable oxyferrous and oxo-iron (IV) complexes at near-ambient temperatures with sperm whale Mb. Similar studies have been carried out to obtain spectroscopic characterization of homogeneous oxyferrous complexes of the cytochrome P450 BM3 (CYP102) holoenzyme and heme domain (BMP) at –55 °C in presence of 70/30 (v/v) glycerol/buffer cryosolvent (91).

Taking the above facts into account, I can effectively model the heme protein active site using a natural probe. Here in this study, Mb mimic of peroxidase enzymes using unnatural amino acids was used to characterize the active species in the catalytic cycle, investigate the catalysis mechanism, and to model the heme protein active sites (44, 92). To this end, Mb, an oxygen storage and carrier protein, was converted into a catalytically active peroxygenase using a genetically incorporated redox-active NH₂Tyr into its active site (Figure 2.2) (44, 93-95). The mutant H64NH₂Tyr Mb was then employed to investigate the structure-function relationship in heme proteins (based on acid-base catalysis and distal charge relay effect).

A Mb mutant in which His 64 was replaced with NH₂Tyr (H64NH₂Tyr) showed high turnover rates for thioanisole and benzaldehyde oxidation (9-fold and 81-fold), when compared to WT, in the presence of H₂O₂. Our results indicate a possible situation where acid-base catalysis of NH₂Tyr coupled with distal “pull” effect via the hydrogen bonding network, play an important mechanistic role to mimic peroxidase. Furthermore, peroxygenase activity of this mutant exhibited remarkable stability against heme bleaching with the generation of active catalytic species.
Figure 2.2 Schematic representation of the oxyferrous complex of Mb. (A) binding site of WT Mb. (B) active site of H64NH₂Tyr, (PDB number 1MBO).

2.2 Experimental procedure

2.2.1 Chemicals

The gases (O₂ and N₂) were purchased from Air Liquide USA while NH₂Tyr, sodium dithionite (Na₂S₂O₄), thioanisole, benzaldehyde, 30% H₂O₂, and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) were purchased from Sigma-Aldrich USA, and used without any further purification. All chemicals were at least analytical grade.

2.2.2 Preparation of WT Mb and H64NH₂Tyr Mb mutant constructs

The WT Mb expression constructs, H64NH₂Tyr Mb gene expression vector and aminoacyl tRNA synthetase (aatRNA S) plasmids were gifts from Dr. Peter Schultz (The Scripps Research Institute, La Jolla, CA USA). The H64NH₂Tyr Mb expression vector and tRNA synthetase plasmid were co-transformed in DH10B E. coli bacteria cells. The double antibiotic resistant colonies were picked, grown, and the cell stocks were stored at –80 °C prior to protein expression and purification.
2.2.3 WT Mb and H64NH₂Tyr Mb protein purification

The H64NH₂Tyr Mb was expressed in *E. coli* grown in glycerol minimal media (GMML) containing 25% Luria Broth (LB) media and suitable antibiotics (ampicillin and tetracycline). Both the WT Mb and mutated (H64NH₂Tyr) Mb proteins were induced by adding 0.02% arabinose to bacterial cultures when optical density at 600 nm (O.D₆₀₀) was 0.5. Cells were harvested by centrifugation at 13000 g and lysed in lysis buffer (Tris HCl buffer at pH 8.0) by addition of RNase, Dnase, and lysozyme to the cell contents, followed by three freeze (liquid N₂) thaw (37 °C) cycles and 10 rounds of 40 sec sonications on ice. The crude lysate was mixed thoroughly with Ni-NTA resin and the protein-bound nickel-nitrilotriacetic acid (Ni-NTA) was collected in a column. Purified proteins were eluted from the column using an elute buffer (300 mM NaCl, 250 mM imidazole, 50 mM phosphate buffer, pH 7.0). The purified WT Mb and mutant proteins were stored at −80 °C until further use.

2.2.4 WT Mb and H64NH₂Tyr Mb protein analysis

The sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis were shown in figure 2.3. The mass of mutant H64NH₂Tyr Mb protein was analyzed by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) and was observed to be 18397 Da, (Figure 2.4) which matched with the theoretically calculated mass.
Figure 2.3 Coomassie-stained SDS-PAGE analysis of expression of WT Mb and H64NH₂Tyr Mb. (A) Lane 1, Molecular weight standards as indicated in kiloDalton (kDa); Lane 2, expressed WT Mb; Lane 3, expressed H64NH₂Tyr Mb. (B) Lane 1, Molecular weight standards as indicated in kDa; Lane 2, expressed H64NH₂Tyr mutant Mb in the absence (−) of 1 mM NH₂Tyr; Lane 3, expressed H64NH₂Tyr Mb in the presence (+) of NH₂Tyr. No Mb protein was found by SDS-PAGE analysis in the absence of NH₂Tyr.
2.2.5 Spectroscopy

UV-visible electronic absorption spectra of WT Mb and mutant Mb proteins were acquired on a Varian Cary 50 Bio UV-visible spectrophotometer. The concentration of WT Mb was determined using pyridine hemochromogen method (96).

2.2.6 Preparation of oxyferrous complexes

The oxyferrous complexes were prepared in a chest freezer at −35 to −45 °C. The protein sample was taken in 100 mM potassium phosphate buffer (pH 7.0) which contained 65% glycerol (v/v). The ferric protein was first degassed with N₂ gas for 2 hr. Then it was reduced to a deoxyferrous species by addition of Na₂S₂O₄ (20 mg/mL stock) under N₂.
atmosphere in a sealed cuvette at 4 °C. Pre-cooled O₂ gas was bubbled into the cuvette for 60 s and the UV-visible spectra were recorded (Figure 2.8) as indicated in the previous study (32).

2.2.7 Effect of H₂O₂

The effect of H₂O₂ on ferric proteins was observed in the presence of 5 mM H₂O₂. The protein concentration was 5 μM and heme bleaching of proteins was observed at 20 °C (Figures 2.6 and 2.7).

2.2.8 Catalytic sulfoxidation of thioanisole and oxidation of benzaldehyde

In the total volume of 500 μL, 100 mM potassium phosphate buffer pH 7.0, the 5 μM protein (WT Mb or H64NH₂Tyr mutant Mb) was added. To that 1 mM substrate, either thioanisole or benzaldehyde was added. To initiate the reaction, 5 mM H₂O₂ was added to a final volume of 500 µL. The reaction mixture was incubated for 1 hr After incubation the organic products were extracted in dichloromethane. The solvent was reduced and the organic products were analyzed by gas chromatography-mass spectrometry (GC-MS) (Figure 2.5).

2.2.9 Electrochemical instrumentation

Differential pulse voltammetry (DPV) was carried out using a single-compartment, three-electrode cell at 22 °C on a CHI720C electrochemical analyzer (CH Instruments, Austin, TX). The working electrode was polycrystalline gold, the counter electrode was a platinum wire, and the reference electrode was Ag/AgCl/3.5 M KCl (BAS, West Lafayette, IN). All potential values below are reported with respect to the Ag/AgCl reference electrode. The working electrode surface was first polished on microcloth with alumina slurry suspension (0.05 μm), then sonicated in ethanol, and finally rinsed thoroughly with Milli-Q water. The electrolyte solution was 0.1 M K₂HPO₄/KH₂PO₄ buffer at a pH of 7.0. The solutions were deoxygenated by bubbling nitrogen prior to each experiment. The concentration of H₂O₂ used was 5 mM. The
DPV measurements were recorded with pulse amplitude of 30 mV, step size set at 4 mV, pulse width at 0.05 sec, and pulse period at 0.20 sec (Figure 2.9).
Figure 2.5 GC-MS analysis of oxidation products of thioanisole and benzaldehyde. Conversion of thioanisole (a) to its sulfoxidation product (b) by 5 mM H$_2$O$_2$. (A), GC data of WT Mb; (B), GC data of H64NH$_2$Tyr mutant Mb, (C), mass spectrum of thioanisole sulfoxide product, (D) mass spectrum of benzoic acid product, (E) and (F) show GC and MS data of oxidation of benzaldehyde (a) to benzoic acid (b) by 5 µM Mb and H$_2$O$_2$ for WT Mb and H64 H64NH$_2$Tyr respectively. The reaction was carried out by adding 5 mM H$_2$O$_2$ to 5 µM WT or mutant Mb in 0.5 mL of 100 mM potassium phosphate buffer (pH 7.0) with 1 mM thioanisole or benzaldehyde at 25 °C.
2.3 Results and Discussion

2.3.1 Genetic design rationale and characterization

In the heme-containing peroxidases, the generation of active Compound I species depends on precise delivery of two protons at the distal oxygen of the peroxo-ferric intermediate (4). Another essential feature is the presence of the distal His and the Arg in the active site pocket of the peroxidases (8, 21, 97). In HRP, the distal Arg influences the distal His to react with H$_2$O$_2$ and form the active Compound I through an acid-base mechanism. But in WT Mb, the distal His is not affected by any other distal amino acid and cannot induce the formation of Compound I efficiently. Therefore, I decided to incorporate a redox-sensitive NH$_2$Tyr amino acid in place of the distal His. In the presence of an oxidant (e.g., H$_2$O$_2$), the redox-active NH$_2$Tyr amino acid should be able to facilitate an oxidation reaction which releases two electrons and two protons (Figure 2.2) as well as to induce the formation of Compound I. Therefore, I expected the H64NH$_2$Tyr mutant Mb to behave as a considerably more potent peroxynasen catalyst due to the increased oxygenase activity around the heme environment.

To facilitate the de novo design of Mb with unique peroxidase functionality, I have genetically encoded NH$_2$Tyr into Mb, replacing His 64 with the amber nonsense codon TAG into a pBAD expression vector, using a previously reported technique. The method includes the genetic incorporation of non-canonical amino acids into proteins using orthogonal tRNA$_{CUA}$/aminoacyl-tRNA synthetase pairs in bacteria. The expressed H64NH$_2$Tyr Mb showed were analyzed by SDS- PAGE (Figure 2.3). Mass spectral analysis (MALDI-TOF) showed a parent ion mass of 18397 Da as expected for the H64NH$_2$Tyr Mb mutant (Figure 2.4).

The electronic absorption spectra of purified H64NH$_2$Tyr mutant displayed a sharp Soret absorption peak at 410 nm (visible peaks around 539 nm, 579 nm and 624 nm), consistent with the aquo-ferric complex. Surprisingly, addition of 5 mM H$_2$O$_2$ did not show significant Soret shift (A$_{max}$ = 410 nm) or heme-bleaching for the NH$_2$Tyr mutant Mb (Figure 2.6).
Figure 2.6 Electronic absorption spectra of the WT and mutant Mb. H64NH2Tyr Mb in the presence of 5 mM H2O2 (black solid line), in absence of H2O2 (blue dashed dot dot line) and WT Mb in the presence of 5 mM H2O2 (green dot line) and in absence of H2O2 (red dashed line). $\varepsilon =$ Molar extinction coefficient.

However, there were isosbestic points in the spectra between the peaks in the presence and absence of H2O2 with the mutant to indicate that a novel catalytic species had been generated (Figure 2.6). As expected, the WT protein formed Compound II (the Soret around 418 nm) with 5 mM H2O2 with significant heme loss within the first 15 min of the reaction (Figure 2.7) (98).
Figure 2.7 Effect of H$_2$O$_2$ on WT and mutant Mb. Absorbance changes monitored over a 30 min period at $\lambda_{\text{max}} = 418$ nm for WT Mb (blue dashed dot dot line) and $\lambda_{\text{max}} = 410$ nm for H64NH$_2$Tyr mutant Mb (black solid line) in the presence of 5 mM H$_2$O$_2$ and control (red dashed line). The spectra were measured in 100 mM potassium phosphate buffer, pH 7.0 at 20 °C with 10 μM protein.

In addition, the oxyferrous and deoxyferrous complexes of the H64NH$_2$Tyr mutant and the WT Mb were prepared at −35 to −45 °C. The UV-visible spectra were recorded (Figure 2.8), which exhibited that the inherent characteristics of the native heme were intact in both the WT and the H64NH$_2$Tyr mutant Mb.
Figure 2.8 Deoxyferrous and oxyferrous spectra of WT and mutant Mb. The spectra of WT Mb deoxyferrous (blue dashed line), H64NH₂Tyr Mb deoxyferrous (red dashed line), WT Mb oxyferrous (green dotted line) and H64NH₂Tyr Mb oxyferrous (black solid line) species. The spectra were taken at −35 to −45 °C and the samples were examined in 60% glycerol, 100mM phosphate buffer at pH 7.0.

2.3.2 Catalytic activity

A number of substrates including thioanisole and benzaldehyde were successfully oxidized to their products by the mutant. Sulfoxidation of thioanisole (Reaction 2) and oxidation of benzaldehyde to benzoic acid (Reaction 3) by 5mM H₂O₂ for H64NH₂Tyr mutant Mb showed 2.4 min⁻¹ (9-fold higher activity compared to WT Mb) and 4.05 min⁻¹ (81-fold higher activity than WT Mb) turnover rates, respectively (Table 2.1). However, under the same conditions WT Mb shows only 0.25 min⁻¹ for sulfoxidation and 0.05 min⁻¹ for the oxidation of benzaldehyde.
Table 2.1 Rate of sulfoxidation of thioanisole (Reaction 2) and benzaldehyde oxidation (Reaction 3) by WT Mb and mutant H64NH₂Tyr Mb. Kinetic values are based on the average of at least 2 determinations and the unit for rate is turnover per min.

<table>
<thead>
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<th>Protein</th>
<th>$K_m$ (µM)</th>
<th>$V_{\text{max}}$ (µM min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT Mb</td>
<td>19.3</td>
<td>25.6[a]</td>
</tr>
<tr>
<td>H64NH₂Tyr Mb</td>
<td>50.25</td>
<td>69.93</td>
</tr>
</tbody>
</table>

[a] This value is in agreement with data previously reported (26)

2.3.3 ABTS peroxidase assay

2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) or ABTS has often been used to estimate the reaction kinetics of enzymes like peroxidases. In the presence of H₂O₂ and peroxidase enzyme, it is converted to its radical cation. This blue colored radical cation is utilized to indirectly identify the formation of the ferryl radical species in peroxidases (26, 99). In order to detect the development of the reactive species in the WT and mutant H64NH₂Tyr Mb, the ABTS peroxidases assay was performed. The results are contained in Table 2.2. The
reaction mixture contained 1 µM protein, 5 mM H$_2$O$_2$ and ABTS. The concentration of ABTS was varied from 0.02 mM to 2 mM (97, 100, 101).

Table 2.2 Peroxidase assay of WT and mutant Mb with ABTS in presence of H$_2$O$_2$. ABTS oxidation reaction assays of Mb and its mutant in the presence of 5 mM H$_2$O$_2$. Kinetic values are based on the average of at least 2 determinations and the unit for rate is turnover per min. Reaction has been done at pH 7.0 and at 20 °C.

<table>
<thead>
<tr>
<th>Protein</th>
<th>$K_m$ (µM)</th>
<th>$V_{max}$ (µM min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT Mb</td>
<td>19.3 [a]</td>
<td>25.6 [a]</td>
</tr>
<tr>
<td>H64NH$_2$Tyr Mb</td>
<td>50.25</td>
<td>69.93</td>
</tr>
</tbody>
</table>

[a] This value is in consistent with data previously reported (99).

The ABTS radical cation formation was monitored at 730 nm. The $K_m$ and $V_{max}$ values were calculated from Lineweaver-Burk plots. The detection of the ABTS radical cation, in turn, corroborated the peroxidase activity of the WT and mutant H64NH$_2$Tyr Mb (101).

2.3.4 Electrochemical characterization

To understand the effect of the distal proton and electron delivery network via NH$_2$Tyr residue in Mb, we measured the reduction potentials ($E^\circ$) for Mb and its mutant in the presence of H$_2$O$_2$ at pH 7 as well as at pH 10 (Table 2.3) using differential pulse voltammetry (DPV). The $E^\circ$ values confirmed that the primary amine and hydroxyl groups in NH$_2$Tyr amino acid influence the electronic nature of the iron at the heme active site. In the presence of H$_2$O$_2$ at pH 7.0, the mutant (−360 mV) exhibited a 40 mV shift from the WT Mb (−320 mV), which is more than the difference observed (27 mV) at pH 10.0 (Figure 2.9 and Table 2.3). Since a single hydrogen bond results in a 25-60 mV change in the $E^\circ$ values (102, 103), the observed potential
difference between the mutant and the WT Mb is consistent with the presence of an additional hydrogen bond for H64NH$_2$Tyr mutant than the His-64 in WT Mb.
Figure 2.9 DPV of WT and mutant Mb in presence and absence of H₂O₂. The DPV response of 5 µM WT and mutant H64N²TYr Mb in nitrogen saturated 100 mM phosphate buffer and 10 mM KCl solutions (A and C: at pH 7.0; B and D: at pH 10.0) in absence (A and B) and in presence (C and D) of H₂O₂ at room temperature conditions. Pulse amplitude = 30 mV, step size = 4 mV, pulse duration = 0.05 sec and pulse period = 0.20 sec.
Table 2.3 Reduction potential of WT and mutant Mb. Reduction potentials were measured at pH 7.0 and 10.0 in the absence and presence of 5 mM H$_2$O$_2$. All reduction potentials are reported with respect to Ag/AgCl (3.5 M KCl) reference electrode.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Without H$_2$O$_2$</th>
<th>With 5 mM H$_2$O$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH 7.0</td>
<td>pH 10.0</td>
</tr>
<tr>
<td>WT Mb</td>
<td>–0.232</td>
<td>–0.256</td>
</tr>
<tr>
<td>H64NH$_2$Tyr Mb</td>
<td>–0.244</td>
<td>–0.256</td>
</tr>
</tbody>
</table>

A possible reason for the 27 mV shift with H$_2$O$_2$ at pH 10 (than the 40 mV at pH 7) is that the redox-sensitive amino acid (NH$_2$Tyr) can be more easily oxidized ($pK_a$ is ~10 for hydroxyl group in NH$_2$Tyr) to $\alpha$-imino-quinone by releasing two electrons and two protons, which would preferentially promote the formation of the heme high valent (ferryl) species in the Mb mutant.

2.3.5 Ligand binding study of WT and H64NH$_2$Tyr Mb mutant adducts

The replacement of distal His with redox residue like NH$_2$Tyr gave us an important insight of the nature of electronic absorption spectra. From Figure 3.6, I observed that after addition of neutral ligands such as CO, NO and O$_2$ to WT and mutant Mb, the Soret became sharper at respective places. The CO adduct is linear and both of its $\pi^*$ orbitals are empty, which allows d$\pi$-$\pi^*$ orbitals to overlap in a perpendicular direction resulting in greater overlapping. It provides the perfect condition for $\pi$ backbonding, while under similar conditions NO and O$_2$ adducts have less $\pi$ backbonding because they have one or two electrons in their orbitals (104). Since mutant Mb form an extra hydrogen bond with the ligands, the adduct with
mutant Mb were stronger. The bigger size residue like NH₂Tyr, if present near the active site, also prohibits the release of ligands from the heme center and hence increases the stability. CN⁻ and N₅⁻, which are rich in electrons, also show the similar effect with lesser intensity as compared to neutral ligands (Figure 2.10).
Figure 2.10 Characterization of WT and H64NH₂ Mb ligand complexes. Electronic absorption spectra of (A) CO (B) NO, (C) cyanoferric and (D) ferric N₅⁻ complexes of WT Mb (red solid line) and H64NH₂Tyr (blue dashed line) Mb. The spectra were taken at 4 °C and the samples were examined in 100 mM phosphate buffer at pH 7.0.
2.3.6 Proposed mechanical feature

The above spectroscopic and electrochemical characterizations demonstrate the role of the redox-sensitive NH$_2$Tyr in the introduction of peroxidase activity in Mb (Figure 2.11). When H$_2$O$_2$ is present in the active site of the mutant Mb, the amine functional group of the NH$_2$Tyr can abstract a proton from the H$_2$O$_2$, thereby forming the Fe-O-OH moiety (Compound 0). This in turn loses one molecule of H$_2$O to form the active ferryl radical cation (Compound I). The presence of the hydroxyl and the amine groups on the NH$_2$Tyr is absolutely essential for this step as it involves an acid-base catalysis mechanism. The distal "pull" effect via the hydrogen bonding network facilitates heterolytic cleavage of the hydroperoxide-ferric O-O bond in Compound 0. The substrate can now approach the ferryl (Fe$^{IV}$) radical species become oxidized, thereby regenerating the resting ferric state in the mutant Mb.

This indicates that the redox-properties of NH$_2$Tyr can be exploited to generate the elusive ferryl radical cation (Compound I) in the mutant Mb. Hence, H64NH$_2$Tyr Mb peroxygenase activities as well as isolation of catalytically active species in this mutant will significantly increase our understanding of how nature utilizes the heme iron cofactor and the protein scaffold around the active site in essential biological transformations involving peroxides and O$_2$. 
Figure 2.11 Mechanism of oxidation of NH$_2$Tyr which releases 2 electrons and two protons to produce quinone product in the presence of an oxidant [O], such as H$_2$O$_2$.

2.4 Conclusions

In this chapter, I report the creation of a genetically engineered Mb mutant by incorporating a non-canonical redox-active NH$_2$Tyr into its active site. The replacement of His in the distal His 64 with NH$_2$Tyr mutant Mb provided an insight into the role of the distal binding site residues in acid-base catalysis and distal charge relay "pull" effect in peroxide activation and formation of ROS in heme proteins. The H64NH$_2$Tyr mutant Mb showed high turnover rates for thioanisole oxidation (9 times) and benzaldehyde oxidation (81 times) when compared with the WT Mb, in the presence of H$_2$O$_2$. The ligand binding studies also explored the importance of redox residues (e.g., NH$_2$Tyr) near the heme center.
CHAPTER 3
INTRODUCTION OF PEROXIDASE ACTIVITY IN MYOGLOBIN BY INCORPORATING UNNATURAL AMINO ACIDS AT THE DISTAL HISTIDINE POSITION

3.1 Introduction

Among the various heme containing metalloproteins, Mb is found in the muscle tissue of most vertebrates and all mammals (2). It is an oxygen binding single chain globular protein with 153 amino acids (sperm whale Mb) (1, 2, 5). The binding site of Mb contains iron-heme as the prosthetic group (105). Since Mb has been extensively characterized, it is frequently used as a model to study the structure and function of the heme proteins. X-ray crystallography, NMR, and EPR, have been successfully used to study the structure and function of the Mb protein (106-108). The Mb gene of many organisms, including human and sperm whale, have been cloned, and heterologous expression of recombinant protein in E. coli has been carried out effectively (10, 109-112).

While studying other heme-proteins, like horseradish peroxidase (HRP) and cytochrome c peroxidase (CcP), it has been revealed that the presence of distal His is very critical for the formation of compound I and peroxidase activity (Figure 3.1) (106-108, 113-115). The successful oxidation of thioanisole and benzaldehyde by HRP and CcP has demonstrated their peroxidase property. In contrast, the rate of formation of compound I in Mb is quite slow and the myoglobin compound I (Mb Cpd I) is also very unstable, hence the WT Mb cannot catalyze sulfoxidation of thioanisole or benzaldehyde oxidation significantly. To carry out these reactions, it is essential to increase the rate of compound I formation and provide a suitable environment at the active site to prohibit its decay to compound II. The compound II is a
catalytically inactive and stable iron-oxygen species, which effectively stores the oxygen in the tissues.

Figure 3.1 Schematic representation of the formation of compound I and compound II in Mb and HRP. (A) Formation of compound II by homolytic cleavage of O-O bond in Mb; (B) Compound I formation in HRP by heterolytic cleavage.

In order to increase the rate of formation of compound I and to stabilize it, we have incorporated an unnatural amino acid, DOPA, replacing the distal His in the binding site of Mb. This H64DOPA Mb mutant was constructed on the basis of accommodating a redox-sensitive amino acid in the binding site of Mb. Oxidation of thioanisole and benzaldehyde were carried
out in the presence of WT and mutant Mb to monitor their peroxidase property. Herein, we report that the H64DOPA Mb mutant exhibited significantly high peroxidase activity as compared to the WT Mb.

Furthermore, I have studied the electron rich and neutral ligand adducts of mutant and WT Mb to understand the effect of redox amino acids such as DOPA, which is capable of giving electrons to heme center and to ligand when present near the active site. It also was interesting to explore the role played by hydrogen bonding favoring residues on the stability of ligand adducts.

3.2 Experimental procedures

3.2.1 Chemicals

The gasses (O₂, NO, CO and N₂) were purchased from Air Liquide while DOPA, Na₂S₂O₄, sodium azide, potassium cyanide, thioanisole, benzaldehyde, and 30% H₂O₂ were purchased from Sigma-Aldrich USA, and have been used without any further purification. All chemicals were of analytical grade.

3.2.2 Preparation of WT Mb and H64DOPA Mb mutant constructs

The WT Mb and H64DOPA Mb expression constructs were made by the following previous method (30). The H64DOPA Mb expression vector and tRNA synthetase plasmid have been co-transformed in DH10B E.coli. The double antibiotic resistant colonies were picked, grown, and the cell stocks were stored at –80 °C prior to protein purification.

3.2.3 WT Mb and H64DOPA Mb protein purification and analysis

The H64DOPA Mb was expressed in E. coli that was grown in GMML containing 25% LB media and suitable antibiotics (ampicillin and tetracycline). Both the WT and mutated
(H64DOPA) Mb proteins were expressed, purified and analyzed as described in chapter 2 (Figure 3.2).

Figure 3.2 *Analysis of WT and H64DOPA mutant Mb by SDS-PAGE and MALDI-TOF*. (A) Lane 1, expressed WT Mb; Lane 2, expressed H64DOPA Mb; Lane 3, Molecular weight standards as indicated in kDa (B) MALDI-TOF of the H64DOPA mutant Mb protein. A mass of 18397 Da matches the calculated mass for the Mb mutant. No WT protein (predicted mass, 18356 Da) was observed.
3.2.4 Spectroscopy

UV-visible electronic absorption spectra of the WT Mb and mutant Mb proteins were taken using Varian Cary 50 Bio UV-visible spectrophotometer. The concentration of WT and mutant Mb was determined by heme chromatogen method (96).

3.2.5 Effect of H\textsubscript{2}O\textsubscript{2}

The effect of H\textsubscript{2}O\textsubscript{2} on ferric proteins has been observed in the presence of 5 mM H\textsubscript{2}O\textsubscript{2}. The protein concentration was 5 μM and heme bleaching of proteins was observed at 20 °C.

3.2.6 Preparation of oxyferrous complex

The oxyferrous complexes were prepared in a chest freezer at −45 to −55 °C. The protein sample was taken in 100 mM potassium phosphate buffer (pH 7.0) which containing 65% glycerol (v/v). The ferric protein was first degassed with N\textsubscript{2} gas for 2 hr Then, it was reduced to deoxyferrous by addition of Na\textsubscript{2}S\textsubscript{2}O\textsubscript{4} (20 mg/mL stock) under N\textsubscript{2} atmosphere in a sealed cuvette at 4 °C. Pre-cooled O\textsubscript{2} gas was bubbled into the cuvette for 60 sec and the UV-visible spectra were recorded.

3.2.7 Preparation of CO, NO, CN\textsuperscript{−} and N\textsubscript{3}\textsuperscript{−} adduct samples

The ferrous-CO adducts were generated by gentle bubbling of CO in deoxyferrous enzymes for 30 sec at 4 °C in 0.1 M potassium phosphate buffer pH 7.0. To prepare the ferrous-NO adducts, minimal amount of buffer saturated with NO gas was added to the deoxyferrous enzymes under N\textsubscript{2} at 4 °C in 0.1 M potassium phosphate buffer pH 7.0. The ferric N\textsubscript{3}\textsuperscript{−} and cyanoferric adducts were formed by addition of minimal amounts of NaN\textsubscript{3} and KCN from their respective stocks (40 mM NaN\textsubscript{3} and 1 M KCN) to the degassed ferric proteins. The ferric proteins were in 100 mM potassium phosphate buffer pH 7.0 and the temperature was 4
°C. The deoxyferric enzymes were prepared by bubbling of N₂ gas in the ferric enzymes for at least 30 min.

### 3.2.8 Catalytic sulfoxidation of thioanisole and oxidation of benzaldehyde

The reactions were performed and the products were analyzed as described in chapter 2 and the data were shown in figure 3.8 and table 3.2.

### 3.2.9 Electrochemical instrumentation

DPV was carried out following the method as described in chapter 2 and the data were shown in figure 3.7.

### 3.3 Results and discussion

The inherent property of Mb is to store and transport oxygen. The WT Mb forms a stable and catalytically inert oxy-complex. It has been reported that the His 64 stabilizes the sixth ligand water molecule through hydrogen bonding (110, 116, 117).

#### 3.3.1 UV-visible spectroscopic characterization of WT and H64DOPA mutant Mb

The mutant protein and WT Mb exhibited similar electronic spectra as observed for the ferric heme species. The maximum absorbance in Soret band was found to be 409 and 411 nm for the WT and mutant Mb, respectively (Figure 3.3). In the case of WT Mb, small peaks were observed at 632 nm, 580 nm, 544 nm, and 507 nm, while for H64DOPA Mb, only two peaks were observed at 632 nm and 530 nm in addition to the 411 nm peak. His 64 has been replaced by DOPA and the unnatural amino acid was expected to stabilize it further by forming an extra hydrogen bond. Therefore, I have genetically encoded DOPA into Mb, replacing His 64 with the amber nonsense codon TAG into a pBAD expression vector, using a previously reported technique. The method includes the genetic incorporation of non-canonical amino acids into
proteins using orthogonal tRNA\textsubscript{CUA}/aminoacyl-tRNA synthetase pairs in bacteria. Mass spectral analysis (MALDI-TOF) showed a parent ion mass of 18597 Da as expected for the H64DOPA Mb mutant (Figure 3.2 and Figure 3.3).

Furthermore, the H64DOPA Mb mutant showed remarkable tolerance to H\textsubscript{2}O\textsubscript{2}. Even at 25 mM concentration of H\textsubscript{2}O\textsubscript{2}, heme bleaching was not observed to a significant extent (Figure 3.4). The UV-visible absorption spectrum of H64DOPA Mb mutant in presence of H\textsubscript{2}O\textsubscript{2} also exhibited a Soret band at 411 nm similar to that of ferric resting species. However, the isosbestic points present in the spectra indicate that an active catalytic species had been formed (Figure 3.3). Under similar conditions, when H\textsubscript{2}O\textsubscript{2} was added to the WT Mb, hemin was bleached out and it formed an inactive compound II species as expected. This fact was verified by the presence of a Soret at 418 nm (Figure 3.4) in the absorption spectrum of the WT Mb.

![Figure 3.3](image)

**Figure 3.3** High-valent heme complexes of WT and H64DOPA Mb. Electronic absorption spectra of the H64DOPA Mb in presence (black solid line), in absence (red dashed line) and WT Mb in presence (green dotted line) and in absence (blue dashed dot line) of 5 mM H\textsubscript{2}O\textsubscript{2}.
Figure 3.4 Stability of WT and H64DOPA Mb in presence of \( \text{H}_2\text{O}_2 \). Absorbance changes monitored over a 30 min period at \( \lambda_{\text{max}} = 418 \text{ nm} \) for WT Mb (blue dashed), \( \lambda_{\text{max}} = 410 \text{ nm} \) for H64DOPA mutant Mb (black dash dot line) in the presence of 5 mM \( \text{H}_2\text{O}_2 \), and control protein without \( \text{H}_2\text{O}_2 \) (red solid line). The spectra were measured in 100 mM potassium phosphate buffer, pH 7.0 at 20 °C with 10 μM protein.
3.3.2 Deoxyferrous and oxyferrous species of WT Mb and H64DOPA Mb

The substrate free ferric (low spin) of WT Mb and H64DOPA Mb have been reduced to ferrous (high spin) with the Na$_2$S$_2$O$_4$ in 65% (v/v) glycerol under N$_2$ at 4 °C. The deoxyferrous protein shows a shift in the Soret from 409 to 430 nm and from 411 to 432 nm in WT Mb and mutant Mb respectively (Figure 3.5). In the visible region, a peak at 558 nm was observed in the deoxyferrous WT Mb, while in mutant H64DOPA Mb, peaks were observed at 560 nm and 515 nm (118).

![Figure 3.5 Deoxyferrous and oxyferrous spectra of WT and H64DOPA mutant Mb.](image)

In the oxyferrous WT Mb and mutant H64DOPA Mb, Soret bands at 416 nm and 418 nm were observed, respectively. In the visible region, peaks at 581 nm and 540 nm for the WT Mb and at 580 nm and 536 nm for the mutant H64DOPA have been recorded. Since DOPA can
give electrons to the heme and can also participate in hydrogen bonding, the Soret ($\lambda_{\text{max}} = 418$) has been observed to be sharper in the mutant protein as compared to WT mb. (Figure 3.5)

3.3.3 CO, NO, CN$^-$ and N$_3^-$ adducts of WT and H64DOPA Mb

Figure 3.6 clearly indicates that when CO is bound to the enzyme, the Soret ($\lambda_{\text{max}} = 422$) became sharper for the ferrous enzyme bonded with CO. As Fe-CO is a linear adduct and both the $\pi^*$ orbitals are empty, it allows d$\pi$-$\pi^*$ orbitals to overlap in a perpendicular direction, making the sharper Soret (104). Under similar conditions addition of NO and O$_2$, (because of having one or two electrons in their orbitals) reduce the chances of backbonding. These adducts were stronger in the case of mutant protein because of the formation of extra hydrogen bonding. The electron rich ligands such as CN$^-$ and N$_3^-$ have similar effects with less intensity as $\pi$ backbonding was observed less in these cases (32, 119).
Figure 3.6 Study of WT and H64DOPA Mb ligand complexes. The electronic absorption spectra of (A) CO (B) NO, (C) cyanoferric and (D) $N_3^-$ complexes of WT Mb and H64DOPA Mb. The spectra were taken at 4 °C and the samples were examined in 100mM phosphate buffer at pH 7.0.
3.3.4. Redox potentials of WT and mutant Mb

Both the WT and the mutant H64DOPA Mb were further analyzed to obtain their redox potential. The electrochemistry of the protein solutions were probed by differential pulse voltammetry. The redox potential of WT and mutant H64DOPA Mb protein in the absence of H$_2$O$_2$ WT were found to be the same, i.e., –232 mV, while in the presence of H$_2$O$_2$ it was observed to be –260 mV and –300 mV, respectively (Figure 3.7 and Table 3.1). This observation was expected as H$_2$O$_2$ was bonded to the heme center (120). The binding of H$_2$O$_2$ to the heme center replaced the H$_2$O molecule, which was the sixth ligand, and a high spin ferric state was formed. The difference in the reduction potential also removed the energy barrier of electron transfer to the heme center (79, 120). Upon addition of H$_2$O$_2$ in the mutant H64DOPA Mb protein, the reduction potential had decreased, which clearly indicates that an extra hydrogen bond is formed at the active site of the mutant protein when compared to the WT Mb binding site (121).

Table 3.1 Reduction potential of WT Mb and H64DOPA Mb mutant at pH 7.0 and pH 10.0 in presence and absence of H$_2$O$_2$ as obtained from DPV. All protein concentrations were 5 µM and the potentials were recorded vs. Ag/AgCl (3.5 M KCl).

<table>
<thead>
<tr>
<th>Protein</th>
<th>Reduction Potential, $E$ (V)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without H$_2$O$_2$</td>
</tr>
<tr>
<td></td>
<td>pH 7</td>
</tr>
<tr>
<td>WT Mb</td>
<td>–0.232</td>
</tr>
<tr>
<td>H64DOPA Mb</td>
<td>–0.232</td>
</tr>
</tbody>
</table>
Figure 3.7  Reduction potential of WT and H64DOPA Mb in presence and absence of H₂O₂ at pH 7.0 and at pH 10.0. The DPV response of 5 µM WT and mutant H64DOPA Mb in nitrogen saturated 100 mM phosphate buffer and 10 mM KCl solutions (A and C: at pH 7.0; B
and D: at pH 10.0) in presence (A and B) and absence (C and D) of H\textsubscript{2}O\textsubscript{2} at room temperature conditions. Pulse amplitude = 30 mV, step size = 4 mV, pulse duration = 0.05 sec and pulse period = 0.20 sec.
3.3.5 Thioanisole sulfoxidation and benzaldehyde oxidation

As reported earlier (98, 122), WT Mb reacts with H$_2$O$_2$ and forms ferryl heme (Fe$^{IV}$=O) equivalent to compound II. Compound I has never been isolated, as it is highly unstable, which makes peroxidase activity of the WT Mb very low. It was observed that, in the presence of H$_2$O$_2$, WT Mb converts only 14% (Figure 3.8) thioanisole to sulfoxide with the turnover rate of 0.25 min$^{-1}$ which matches with the literature reports (Table 3.2). But under identical conditions, H64DOPA Mb converts 97% (Figure 3.8) thioanisole to its sulfoxide with a turnover rate of 2.5 min$^{-1}$. Thus the turnover rate of sulfoxidation is 10 times higher in the case of the mutant H64DOPA Mb than that of WT Mb.
Figure 3.8 GC analysis of thioanisole and benzaldehyde oxidation. Conversion of thioanisole (a) to its sulfoxidation product (b), (A) WT Mb, (B) H64DOPA Mb. (C) and (D) show oxidation of benzaldehyde (a) to benzoic acid (b) by WT Mb and H64DOPA Mb respectively. The reaction mixture contained 5 µM Mb and 5Mm H₂O₂.
Furthermore, H64DOPA Mb converts 80% (Figure 3.8) benzaldehyde to benzoic acid with 2.5 min\(^{-1}\) turnover rate (Table 3.2) while under similar conditions WT Mb converts 2% (Figure 3.8) benzaldehyde to benzoic acid with 0.01 min\(^{-1}\) turnover rate (Table 3.2). The turnover rate of benzaldehyde to benzoic acid was 54 times higher by mutant H64DOPA Mb as compared to the WT Mb.

Table 3.2 Rate of sulfoxidation of thioanisole reaction (Rxn 1) and benzaldehyde oxidation reaction (Rxn 2) by WT and H64DOPA mutant Mb.

<table>
<thead>
<tr>
<th></th>
<th>Rates (min(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rxn 1</td>
</tr>
<tr>
<td>WT Mb</td>
<td>0.25</td>
</tr>
<tr>
<td>H64DOPA Mb</td>
<td>2.5</td>
</tr>
</tbody>
</table>

In addition to the above experiments, ABTS oxidation reaction assays of Mb and its H64DOPA mutant Mb in the presence of 5 mM H\(_2\)O\(_2\) were also carried out at pH 7.0 and at 4 °C. The kinetic values and turnover factor for the WT and the mutant H64DOPA Mb were calculated based on the data obtained. The H64DOPA mutant Mb exhibited a \(K_m\) of 0.67 \(\times\)10\(^3\) µM, while that for the WT Mb was 19.3 µM, which corresponded to the literature value. The \(V_{\text{max}}\) for the WT and the mutant was observed to be 25.6 and 15.2 µM min\(^{-1}\), respectively (Table 3.3) (100).
Table 3.3 *Peroxidase assay of WT and H64DOPA mutant Mb with ABTS in presence of H₂O₂.*
ABTS oxidation reaction with WT Mb and its H64DOPA mutant Mb in the presence of 5 mM H₂O₂. Kinetic values are based on the average of at least 2 determinations and the unit for rate is turnover per min. Reaction has been done at pH 7.0 and at 4 °C.

<table>
<thead>
<tr>
<th>Protein</th>
<th>$K_m$ (µM)</th>
<th>$V_{max}$ (µM min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT Mb</td>
<td>19.3 [a]</td>
<td>25.6 [a]</td>
</tr>
<tr>
<td>H64DOPA Mb</td>
<td>0.67 × 10$^{-3}$</td>
<td>15.2</td>
</tr>
</tbody>
</table>

[a] This value is in agreement with data previously reported.

Significantly enough, under similar conditions, epoxidation of styrene did not form substantial amounts of the epoxide product (result not shown). In order to explain the observed catalytic reactions and the non-formation of epoxides, we propose that the formation of compound I was achieved through general acid base catalysis. The hydroxyl groups of the DOPA facilitate the mechanism (Figure 3.9) as they are closer to the heme center and can form a hydrogen bond with H₂O₂. The extra hydrogen bond is instrumental in stabilizing the compound I species in the mutant H64DOPA Mb, which further generates the peroxidase activity in the mutant Mb.
In this chapter we report the incorporation of an unnatural amino acid to a specific place in the binding site of Mb. The mutant H64DOPA Mb is an active and stable protein. It could carry out the oxidation of two substrates, namely, thioanisole and benzaldehyde with 20 to 40 fold higher catalytic rate in mutant Mb as compared to WT Mb. In addition, our observations support the idea that the distal His is important for the formation of compound I. In order to exhibit peroxxygenase activity in Mb, the mutation of the distal His with an unnatural amino acid assisted in substrate binding at the active site and enhanced the compound I formation. In addition, the ligand binding studies demonstrate electron deficient ligands exhibit sharper Soret than the electron rich ones for the mutant protein.
CHAPTER 4

FAVOURABLE BINDING SITE ORIENTATION IN MYOGLOBIN FOR DIRECT ELECTRON TRANSFER, ELECTROCATALYSIS AND MICROARRAY INVESTIGATION

4.1. Introduction

Much attention has been focused in recent years on the immobilization of proteins on electrode surfaces (123-126). Such studies are driven by possible applications in a broad range of chemical, biological, and medical technologies spanning bioelectrocatalysis, biomaterials, and biosensors (127-129). Further, immobilized protein assemblies provide a versatile platform for designing mechanistic studies on heterogeneous electron transfer between a cofactor and an active center of a protein. However, direct electron transfer (DET) between proteins and the electrode surface, which can provide new mechanistic insights, is difficult to achieve, mainly due to the lack of good immobilization techniques. Most of the current immobilization techniques show denaturation of the protein on adsorption on the electrode surface. Unfavorable active site orientation of the protein during random adsorption process on the electrode also hinders DET. Strategies to circumvent these difficulties include the use of organic mediators (e.g., dye molecules, conducting polymers, etc) and protein films which again can lead to denaturing of the proteins (130-133). In the past, various immobilization methods have been employed to immobilize proteins on the electrode surface. For example, non-covalent attachment of proteins (134, 135), entrapment in porous matrix (136-138), nanoparticles (139, 140), and sol gels (141-143) have been extensively studied. There are also some reports of covalent immobilization of proteins on the surface of the electrodes, where either chemically modified protein (which often leads to protein denaturation due to harsh chemical treatment) or the functional groups (such as Cys, Arg, Lys, Asp, Glu, etc.) available on
the surface of the proteins have been utilized to covalently attach the protein onto the suitably modified electrode surface (123, 144-146).

Mb is a water soluble heme containing protein, which is present in all mammals and vertebrates, and has been used extensively for investigating electron transfer and electrocatalysis with the protein immobilized on an electrode surface. Its main function is to store oxygen and enhance diffusion of oxygen in the muscle. This globular protein has a single 153 or 154 amino acid chain as in sperm whale, with a molecular weight of ~17 kDa (147). It is a versatile protein with high tolerance for chemical and mechanical environments and can be easily expressed and purified in large quantities in E. coli. Another important feature is that it can accommodate various mutations without any adverse effects on its conformational and functional properties (19, 24, 148). Mb contains a single iron-porphyrin center (heme b) that can accommodate ferrous, ferric or ferryl oxidation states within this heme active site moiety. For these reasons Mb has become an accepted natural model for exploring redox functional properties and electron transfer reactions in electrochemistry (149-152). For example, co-adsorption of Mb with surfactants like sodium dodecyl sulfate or cetyltrimethylammonium bromide on various electrodes such as glassy carbon, pyrolytic graphite, and platinum was reported (150). These protein films were utilized for the electrocatalytic reduction of 1,2-dibromocyclohexane and trichloroacetic acid (150). In the case of immobilization of Mb, a self-assembled monolayer using L-cysteine (L-Cys) on the modified electrode surface exhibited DET between the protein active site and the electrode as well as good electrocatalytic activity toward ascorbic acid oxidation (151).

Even though a wide range of reports are available for immobilization of proteins for carrying out DET and electrocatalysis with electrodes, a direct comparison involving active site orientation of monolayer and multilayer covalent attachment of proteins is absent. This is mainly due to the difficulty to achieve a site-directed covalent tagging of the native conformation of Mb on the surface with the full control over active site orientation. Furthermore, formation of both a
monolayer as well as a multilayer on the electrode surface is a challenging task. Herein we use a unique biochemical technique based on the incorporation of an unnatural amino acid \textit{in vivo} to generate a native Mb protein with additional functional groups (92, 153). NH$_2$Tyr was genetically encoded with the amber nonsense codon, TAG, by means of a previously evolved orthogonal tRNA$_{CUA}$/aminoacyl-tRNA synthetase pair that is specific for NH$_2$Tyr (154). The S3NH$_2$Tyr mutant Mb was immobilized by forming a benzoxazine ring through a Diels-Alder reaction in buffer (155). This provides an effective strategy for covalent immobilization of a single monolayer of Mb mutant on a gold electrode surface without impairing its electroactivity (Figure 4.1A). The random multiple covalent attachment of the WT Mb was achieved through 1-ethyl-3-(dimethylamino-propyl) carbodiimide (EDC)-catalyzed bioconjugation reaction. The DET characteristics and electrocatalytic behavior of such random multilayered assembly of covalently-bound WT Mb counterpart (Figure 4.1B) are compared in this study with a site-directed monolayer protein on the gold surface.

Due to its ability to perform total genomic analysis, DNA microarray has revolutionized the genomic biotechnology. Likewise, protein microarray has the potential to generate advanced bioinformatics platforms for proteomics and it could transform the field of drug discovery, medical diagnostics, and biochemical analysis. Development of functional protein microarray is more difficult than DNA, peptide, and antibody microarrays. Strong attachment of proteins on surface, native protein conformation, homogeneous protein monolayer, control over active site orientation, and retention of protein activity have been the major problem while dealing with protein microarray.

In order to understand the role of the amino acid residues which are located far from the active site such Ser 3 in ligand adduct formation, we have extensively formed and studied ligand adducts with various ligands such as CO, NO, O$_2$, CN$^-$, and N$_3^-$ in this chapter.
Figure 4.1 Graphical representation of WT and S3NH₂Tyr mutant Mb attachment on modified gold electrodes. (A) Ser 3 replaced with NH₂Tyr Mb, with its heme active site; which leads to site-directed covalent attachment, with full control over active site orientation, of S3NH₂Tyr Mb mutant on the modified gold electrode; (B) WT Mb (PDB: 1MBO) with all surface-exposed amino acids are shown, aspartate and glutamate in red color (with acidic side-chains) and arginine and lysine in blue color (with basic side-chains) as points of immobilization for the bioconjugated crosslinking of the WT Mb proteins. Orange ellipses represent random active site orientation (with limited substrate accessibility) of bioconjugated protein cluster.
4.2 Experimental procedures

4.2.1 Chemicals

L-Cys, tetraethyleneglycol diacrylate (TEGDA), sodium periodate (NaIO₄) and \( N,N\text{-di-iso-propylethyl amine (DIPEA)} \) were obtained from Sigma-Aldrich USA and were used without further purification. All chemicals were of analytical grade.

4.2.2 Preparation of WT Mb and S3NH₂Tyr mutant Mb constructs

The WT Mb expression construct (pBAD with ampicillin resistant marker), S3NH₂Tyr Mb gene expression vector (pBAD with kanamycin resistant marker) and aminoacyl tRNA synthetase (aatRNA S) with tetracycline resistant marker vector (PAC) were a gift from Dr. Peter Schultz (The Scripps Research Institute, La Jolla, CA USA). The S3NH₂Tyr Mb expression vector and tRNA synthetase plasmid were co-transformed in DH10B E. coli cells. The double antibiotic resistant colonies were picked, grown, and the cell stocks were stored at –80 °C prior to protein expression.

4.2.3 WT Mb and S3NH₂Tyr Mb protein purification and analysis

The S3NH₂Tyr Mb was expressed in DH10B and was grown in GMML containing ampicillin and tetracycline antibiotics and the unnatural amino acid, NH₂Tyr. The WT and S3NH₂Tyr Mb mutated proteins were expressed, purified, and analyzed as described in chapter 2 (Figure 4.2). The mass of mutant S3NH₂Tyr Mb protein mass was analyzed by MALDI-TOF and was observed to be 18446 Da (Figure 4.3), which matched with the theoretically calculated mass.
Figure 4.2 Coomassie-stained sodium SDS-PAGE analysis of S3NH₂Tyr Mb and WT Mb proteins. Lane 1, expressed WT Mb; Lane 2, expressed S3NH₂Tyr Mb mutant in presence of 1 mM NH₂Tyr; Lane 3, molecular weight standards as indicated in kDa unit.
Figure 4.3 Electrospray ionization-time of flight-mass spectrometry (ESI-TOF-MS) analysis of the incorporation of S3NH₂Tyr into S3 (TAG) Mb mutant showing a mass of 18446 Da (calculated mass, 18446 Da) for S3NH₂Tyr Mb.
4.2.4 UV-vis spectroscopy

UV-visible spectra were acquired on a Cary 50 Bio UV-visible spectrophotometer. The concentrations of the WT and mutant Mb were determined from the absorption spectrum of the protein, using an extinction coefficient of 170 mM$^{-1}$ cm$^{-1}$ at 410 nm.

4.2.5 AFM measurements

The experiments were performed with a Veeco MultiMode V SPM instrument. The cantilevers used in this experiment were made of P-doped n-type Si. The cantilever was oscillated in the constant excitation mode. Tapping mode Atomic force microscopy (AFM) images were acquired in the constant frequency shift mode using frequency modulation (FM) detection. Data analysis was done with the diNanoscope software 7.0.
4.2.6 Ligands adduct formation

The protein samples containing 65% glycerol (v/v) were taken in airtight cuvettes containing 0.1 M potassium phosphate buffer pH 7.0 and were degassed with the N$_2$ gas for ~2 hr at 4 °C and then reduced with minimal amount of Na$_2$S$_2$O$_4$ (in µL) from the stock of 20 mg/mL. In a chest freezer at ~35 to ~45 °C containing the above sample, pre-cooled O$_2$ gas was bubbled for 60 s and the UV-visible spectra were recorded. The ferrous-CO adducts were generated by gentle bubbling of CO in the deoxyferrous enzymes under N$_2$ for 30 s at 4 °C in 0.1 M potassium phosphate buffer pH 7.0 while ferrous-NO adducts were generated by µL addition of buffer saturated with NO gas. Ferric azide and cyanoferric adducts were prepared by addition of minimal amount of NaN$_3$ and potassium cyanide solution from their respective stocks.

4.2.7 Preparation of microarray

Clean glass slides were silanized, extended, and acryloyl functionalized. To obtain a pegylated linker, extended acryloyl glass slides were obtained from amine-coated glass slide by treating with 1 mM diisopropylethylamine followed by reaction with 1 mM tetra (ethylene glycol) diacrylate in dry dimethylformamide (DMF). In the presence of 100 µM NaIO$_4$, at room temperature, the mutant protein (3NH$_2$Tyr incorporated) was attached to the pegylated acryloyl glass slide, for 2 hr. The slides were washed in 100 mM potassium phosphate and water for 2 hr. At room temperature, carbonic anhydrase (CA) catalytic assay was carried out using p-nitrophenyl acetate (0.1 mM) in 15 mM Tris buffer (pH 7.6). Using Veeco Multimode V SPM instrument, AFM measurements were recorded for glass chip-bound proteins. To prepare microarrays, proteins were mixed with 0.1 mM NaIO$_4$ for 10 min prior to printing in a solution of 100 mM potassium phosphate buffer (pH 6.0). BioRobotics MicroGrid II 600 with an internal chamber humidity of 70% was used for printing. After printing, before being transferred to phosphate buffered saline (PBS) buffer (pH 7.5) for 48 hr of washing, proteins were allowed to bind for 4 hr at room temperature. With a 1:500 dilution in PBS buffer containing 1% bovine
serum albumin (BSA) and 0.1% Tween 20 for 1 hr, binding of HiLyte 647 fluorophore-tagged anti-6xHis tag rabbit Immunoglobulin G (IgG) was performed, followed by washing in PBS buffer containing 0.1% Tween 20 for 5 min twice. Using Coomassie bromophenol blue staining solution, Coomassie staining was done for 10 min, followed by 24 hr of washing in water.

4.2.8 Electrochemical instrumentation and procedures

Cyclic voltammetry (CV) was carried out using a three-electrode cell at 22 °C on a CHI720C electrochemical analyzer (CH Instruments, Austin, TX). The working electrode was polycrystalline gold (1 × 1 cm² in area, Alfa Aesar), the counter electrode was a platinum wire, and the reference electrode was Ag/AgCl (3.5 M KCl). The counter electrode and reference electrode were obtained from CH Instruments. All potential values below are reported with respect to the Ag/AgCl (3.5 M KCl) reference electrode. The working electrode surface was first polished on microcloth (Buehler No. 40-7212) with alumina slurry suspension (0.05 μm), then sonicated in ethanol, and finally rinsed thoroughly with Milli-Q water. The electrolyte solution was 0.1 M potassium phosphate buffer with 10 mM KCl solutions at pH 7.0. The solutions were deoxygenated by bubbling nitrogen prior to each experiment. The CV measurements were recorded at potential scan rates ranging from 0.05 Vsec⁻¹ to 0.5 Vsec⁻¹.

4.2.9 Electrode modification procedures

4.2.9.1 Au/L-Cys/WT Mb

The freshly polished and clean gold electrode was immersed in a 2 mM ethanol solution of L-Cys for 2 hr. It was then washed thoroughly and immersed in the 5 μM WT Mb solution in MES buffer at pH 5.0. To this solution, a freshly prepared aqueous solution of EDC was added and the electrode was incubated in this protein medium (2 mL) overnight at 4 °C. The electrode was then removed, rinsed thoroughly with Milli-Q water, and allowed to dry under nitrogen flow before use.
4.2.9.2 Au/L-Cys/TEGDA/ S3NH₂Tyr Mb

The freshly polished and clean gold electrode was immediately immersed in a 2 mM ethanolic solution of L-Cys for 2 hr. It was then washed thoroughly and immersed in 20 mM N,N-di-iso-propyl-ethylamine in dimethylformamide (2 mL). To this solution, 10 mM tetraethylene glycol diacrylate was added and the electrode was kept in it for 3 hr. The S3NH₂Tyr Mb was added in phosphate buffer solution and an aqueous solution of 100 µM NaIO₄ was added to it. The chemically-modified gold electrode was washed thoroughly to remove any traces of unreacted molecules and incubated in the mutant protein solution overnight at 4 °C. The electrode was then removed, rinsed with Milli-Q water thoroughly for 1 hr, and allowed to dry under nitrogen flow before use.

4.2.9.3 Electrocatalysis

Electrocatalysis was carried out in 8 mL of potassium phosphate buffer (100 mM at pH 7.0) containing 5 mM thioanisole dissolved in 30% t-butanol. O₂ was bubbled into the solution throughout the reaction time. Bulk electrolysis was then performed for 2 hr at a constant potential of −0.50 V. On completion of the catalytic cycle, the organic products were extracted in dichloromethane (3 × 2 mL). The solution was concentrated, and the products were analyzed by GC.

4.3. Results and discussion

4.3.1 Monolayer vs. multilayer covalent immobilization of Mb on Au

A central challenge in designing an Mb array for DET is that the electro-active heme centers are randomly oriented within the protein matrix. Further, conversion of heme protein into a protein film on the electrode surface may also lead to heme bleaching and protein denaturing (156-158). To address these challenges, a mild and native immobilization strategy was
developed. In this study, site-directed mutagenesis was used to incorporate an unnatural amino acid (Scheme 4.1) to immobilize Mb on the gold electrode as a monolayer using a unique Diels-Alder reaction in water or buffer at room temperature or 4 °C (155). The Ser 3 group replaced with an NH₂Tyr of the Mb provides an ideal basis for this reaction as it is solvent exposed at that position as well as it orients the active site away from the surface.
Scheme 4.1 Schematic representation of Au electrode modification for monolayered immobilization of S3NH₂Tyr Mb mutant on the Au surface; see text for details.
Incorporation of NH$_2$Tyr into the protein was carried out by using orthogonal tRNA$_{CUA}$/aminoacyl-tRNA synthetase pairs in bacteria. The mutant Mb was analyzed by SDS-PAGE (Figure 4.2) and MALDI-TOF (Figure 4.3). Surface modification of the gold electrode was initiated by modifying it first with an amine-functionalized thiol surface (Scheme 4.1). This chemically-modified surface was reacted with tetraethyleneglycol diacrylate to obtain the acryloyl moiety on the surface. Note that the tetraethyleneglycol portion serves to also provide a lipid-like environment to the covalently attached protein, thus protecting its native conformation on the surface. In the presence of mild oxidant NaIO$_4$, the NH$_2$Tyr on the surface of the S3NH$_2$Tyr Mb mutant was easily oxidized to form the o-iminoquinone. This intermediate, in turn, underwent a Diels-Alder cyclo-addition reaction with suitable alkenes to form a benzoxazine moiety. The entire process of the covalent attachment of mutant S3NH$_2$Tyr Mb onto the acryloyl derivatized gold electrode surface was carried out under ambient or 4 °C temperature. The mutation at Ser 3 with the unnatural amino acid, NH$_2$Tyr, enables full control of the orientation of the active site of the protein, when covalently immobilized onto the modified gold electrode (Figure 4.1A). Thus the catalytic and electro-activity of the heme can be easily achieved even when the mutant protein is covalently attached to the surface of the electrode.

To immobilize the WT Mb as a multilayer, a different strategy had to be employed (Figure 4.1B). The presence of carboxylic acid and amine groups on the protein surface can be utilized to form covalent cross linking bonds between these groups and also with suitably modified, i.e., amine-functionalized, gold surfaces. This bioconjugation reaction is facilitated by the amino acids with carboxylate groups, such as aspartate (20, 27, 44, 122, 126, and 141 positions), glutamate (4, 6, 18, 38, 41, 52, 54, 59, 83, 136, and 148), and amine groups, such as arginine (31, 45, 118, and 139) and lysine (16, 34, 42, 47, 50, 56, 62, 63, 77, 78, 79, 96, 98, 102, 133, 140, 145, and 147 positions). Compounds such as 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide possessing the carbodiimide functional group can be successfully used for crosslinking these amino acids to form amide linkages (159, 160). The EDC reaction chemistry
(Scheme 4.2) was exploited in this study to covalently bind the WT Mb to the amine-functionalized gold surface, as well as to bioconjugate the Mb as a polymer to generate Mb multilayer with disoriented binding sites. The random orientation of the binding sites hinders exposure of some of them to the solvent phase for catalytic or electrochemical activity.

Scheme 4.2 Schematic representation of multilayered WT Mb immobilization on the modified gold electrode surface using EDC catalyzed bioconjugation reaction. Inset: Graphical representation of WT Mb (PDB: 1MBO) with all surface-exposed amino acids. The aspartate and glutamate are represented in red color and arginine and lysine residues in blue color.
The surface topography of the covalently attached Mb assemblies were probed by AFM. Figure 2 compares the AFM images for a chemically-modified amine-functionalized Au surface before (Figure 4.2a) and after S3NH₂Tyr Mb mutant (Figure 4.2b) or WT Mb (Figure 4.2c) covalent attachment. While the pristine surface before protein attachment was featureless (Figure 4.2a), an average feature height of ~5 nm was observed in the case of the mutant protein monolayer, which correlated to the actual size of the protein based on the X-ray crystallographic structure of Mb (Figure 4.2b). On the other hand, covalently-attached WT Mb was noticeably rougher, with an average feature height of ~175 nm (Figure 4.2c). As a control experiment, the WT Mb was also immobilized on the acryloyl-derivatized gold electrode surface using NaIO₄ under the same reaction conditions as the S3NH₂Tyr Mb mutant. However, as expected by the absence of the α-iminoquinone moiety, the WT Mb did not form a monolayer on the modified surface (Figure 4.3) compared to the genetically modified S3NH₂Tyr Mb mutant (Figure 4.2b). Since the covalent linkage is formed only between the α-iminoquinone moiety of the protein and the acryloyl group on the modified electrode surface by the Diels-Alder reaction, the WT Mb does not get covalently immobilized on the electrode surface.
Figure 4.4 Tapping mode AFM topographic images for (a) chemically modified Au surface, (b) with immobilized S3NH₂Tyr Mb mutant and (c) and with WT Mb on the surface.
4.3.2 Electrochemical characterization

Cyclic voltammetry (CV) was used to study the DET electrochemistry of immobilized Mb on the Au electrodes. In this study, though L-Cys and cysteamine were used to modify the gold electrode surface (Figure 4.1 and Table 4.1), cysteamine-bound Mb failed to display reversible redox properties in CV measurements (data not shown). Both WT Mb and the S3NH2Tyr Mb mutant were immobilized on L-Cys functionalized surface to obtain the CV data. The results show well defined redox electrochemistry when examined by CV from scan rates 0.05 Vsec⁻¹ to 0.5 Vsec⁻¹ (Figure 4.4).

Figure 4.5 CV response of L-Cys-modified, WT Mb and S3NH2Tyr Mb mutant immobilized gold electrode in nitrogen-purged 100 mM phosphate buffer with 10 mM KCl (pH 7.0) at room temperature at potential scan rate = 0.1 Vsec⁻¹.
Figure A: Cyclic voltammetry (CV) plots for Au-LCys-TEGDA-S3NH2Tyr Mb. The x-axis represents the potential (V vs. Ag/AgCl) ranging from 0.4 to -0.4 V, and the y-axis represents the current (A) ranging from 0.00005 to 0.00015 A. The CV curves are labeled with scan rates (V/sec) of 0.05, 0.1, 0.15, 0.2, 0.3, 0.4, and 0.5 V/sec.

Figure B: Plot showing the relationship between scan rate (V/sec) and current (A). The current increases linearly with increasing scan rate. The data points are differentiated into Cathodic (■) and Anodic (○) categories.

Caption: "Au-LCys-TEGDA-S3NH2Tyr Mb"
Figure 4.6 CV and peak current response of WT and mutant Mb. Mutant (A) S3NH$_2$Tyr Mb and (C) WT Mb immobilized gold electrode in nitrogen-purged 100 mM phosphate buffer with 10 mM KCl (pH 7.0) at room temperature. Potential scan rate ranges from 0.05 to 0.5 V/sec. Plot of peak current vs scan rate for mutant S3NH$_2$Tyr Mb (B) and WT Mb (D) immobilized on L-Cys modified gold electrode in nitrogen-purged 100 mM phosphate buffer with 10 mM KCl (pH 7.0) at room temperature. Potential scan rate ranges from 0.05 to 0.50 V/sec$^{-1}$.
It was observed that the peak currents were directly proportional to the scan rates, which signals that the proteins were immobilized to the electrode surface (Figure 4.4). The peak current, $I_p$, for a surface confined reactant is given by Eq. 1 (161).

$$I_p = \frac{n^2 F^2}{4RT} A \Gamma \nu$$  \hspace{1cm} (Eq. 1)

where, $n =$ number of electrons transferred, $F =$ Faraday constant, $R =$ universal gas constant, $T =$ absolute temperature in Kelvin ($T = 295$ K), $A =$ surface area of the electrode, $\Gamma =$ surface coverage or the concentration of the redox-sensitive covalently immobilized Mb protein in mol/cm$^2$, $\nu =$ scan rate. From equation 1, the slopes of the plots were given by $(n^2 F^2/4RT) A \Gamma$ and substituting the known values, such as the geometric area of the electrode surface ($A = 2.0$ cm$^2$) and the other constants, the estimated values of the number of molecules of the enzyme associated with the electrode surface ($\Gamma$) were $1.54 \times 10^{-10}$ mol cm$^{-2}$ for the S3NH$_2$Tyr Mb mutant protein immobilized on the modified gold electrode. Similarly, for the WT Mb protein, $\Gamma$ was $1.29 \times 10^{-10}$ mol cm$^{-2}$ on the modified gold electrode through the bioconjugation reaction. A comparison of the surface coverage of the monolayered S3NH$_2$Tyr Mb mutant and the multilayered WT Mb indicated that random orientation of the binding sites in the multilayered WT Mb leads to lesser number of redox-sensitive proteins on the surface. In contrast, the S3NH$_2$Tyr Mb mutant allows proper orientation of the active site, thereby exhibiting homogenously distributed redox-sensitive proteins on the electrode surface.

To investigate the influence of pH on the electron transfer between the active site of the immobilized protein and the gold electrode, CV was carried out in solutions of different pH ranging from 5 to 9. It was observed that with increasing pH, the reduction potential of the Fe-center exhibited a linear negative shift (Figure 4.5). These data support the notion that denaturation of the Mb protein did not occur within the pH range of 5 to 9.
Table 4.1 Comparison of heterogeneous electron transfer rates of Mb on gold electrodes.

<table>
<thead>
<tr>
<th>Surface modification used</th>
<th>Approximate theoretical length of anchoring tether (Å); molecule used</th>
<th>Heterogeneous electron transfer rate, $k_s$ (s$^{-1}$)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homocysteine</td>
<td>![Molecule Image]</td>
<td>5.35</td>
<td>(162)</td>
</tr>
<tr>
<td>L-Cys</td>
<td>![Molecule Image]</td>
<td>4.75</td>
<td>(151)</td>
</tr>
<tr>
<td>L-Cys</td>
<td>![Molecule Image]</td>
<td>4.75</td>
<td>This work</td>
</tr>
<tr>
<td>L-Cys-linked with</td>
<td>![Molecule Image]</td>
<td>26.66</td>
<td>This work</td>
</tr>
<tr>
<td>tetraethylglycol diacrylate</td>
<td>![Molecule Image]</td>
<td>2.26</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.25</td>
<td></td>
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</tbody>
</table>

81
Influence of pH on immobilized WT Mb and S3NH$_2$Tyr Mb

<table>
<thead>
<tr>
<th>pH</th>
<th>Potential/ V vs Ag/AgCl</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.00</td>
</tr>
<tr>
<td>5</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td></td>
</tr>
</tbody>
</table>

WT Mb

S3NH$_2$Tyr Mb

Figure 4.7 Plot of peak potentials vs pH for WT Mb and S3NH$_2$Tyr Mb. Proteins are immobilized on L-Cys-modified gold electrode in nitrogen purged 100 mM phosphate buffer with 10 mM KCl solutions (pH 7.0) at room temperature.

A wide variety of electrode systems have been used to explore the electrochemistry of Mb. A comparison of some of these systems is tabulated below (Table 4.2). Similar cathodic and anodic peak separation due to slow DET has also been reported in earlier literature. In this study, when employing L-Cys for the gold electrode surface modification, the electrochemical behaviour of the WT Mb and the S3NH$_2$Tyr Mb mutant was significantly different. Since the electrochemical reaction was quasi-reversible in nature, the formal reduction potential $E''$ was estimated from the midpoint potential $E_{1/2}$ [obtained from $(E_{pc} + E_{pa})/2$]. The WT Mb and the immobilized S3NH$_2$Tyr Mb mutant gave values of $E_{1/2}$ between 0.080 V and 0.103 V with respect to Ag/AgCl (3.5 M KCl) reference, respectively. These midpoint potentials of the heme center of the WT Mb are in accord with reported values in the literature.(151, 162) The rather large separation of the cathodic and anodic peaks indicated that DET to the protein was quite slow.

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Table 4.2 *Comparison of direct electrochemistry of Mb on various electrode systems.*

<table>
<thead>
<tr>
<th>Electrode</th>
<th>E_{1/2} values (V) [a]</th>
<th>ΔEp (V)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gold/L-Cys-linked with tetraethylglycol diacrylate and S3NH₂Tyr Mb mutant</td>
<td>0.103</td>
<td>~0.150</td>
<td>This work</td>
</tr>
<tr>
<td>Gold/L-Cys-WT Mb</td>
<td>0.080</td>
<td>~0.091</td>
<td>This work</td>
</tr>
<tr>
<td>Gold/L-Cys-WT Mb</td>
<td>0.086</td>
<td></td>
<td>(151)</td>
</tr>
<tr>
<td>Gold/Homocysteine-WT Mb</td>
<td>-0.012</td>
<td>~0.246</td>
<td>(162)</td>
</tr>
<tr>
<td>Gold nanopyramids-WT Mb</td>
<td>0.210</td>
<td>~0.060</td>
<td>(163)</td>
</tr>
<tr>
<td>Gold/azide-terminated alkane thiol-WT Mb</td>
<td>-0.335</td>
<td></td>
<td>(123)</td>
</tr>
<tr>
<td>NAF/Mb/IL/PtNPs/MWCNTs/GCE [b]</td>
<td></td>
<td>~0.140</td>
<td>(134)</td>
</tr>
<tr>
<td>Mb-CA/GCE [c]</td>
<td>-0.281</td>
<td>~0.102</td>
<td>(164)</td>
</tr>
<tr>
<td>Mb/DDAB–HIMIMPF₆ [d]</td>
<td>-0.261</td>
<td>~0.044</td>
<td>(165)</td>
</tr>
</tbody>
</table>

[a] All literature values for E_{1/2} have been converted to Ag/AgCl (3.5 M KCl) reference electrode, for clarity; [b] NAF/Mb/IL/PtNPs/MWCNTs/GCE = Glassy carbon electrode modified with multi-walled carbon nanotubes (MWCNTs), followed by platinum nanoparticles (PtNPs), ionic liquids (IL) and Nafion (NAF) with WT Mb; [c] Mb-CA/GCE = Glassy carbon electrode modified with calcium alginate and WT Mb; [d] Mb/DDAB–HIMIMPF₆ = Glassy carbon electrode modified with didodecyldimethylammonium bromide (DDAB), 1-hexyl-3-methylimidazolium hexafluorophosphate (HIMIMPF₆) and WT Mb.

The rate of heterogeneous electron transfer between the electrode and the immobilized protein was calculated using Laviron’s method ([161]). The value of the transfer coefficient (α) was determined using Eq. 2:

\[
E_{pc} = E^0 - \frac{RT}{\alpha nF} \ln \left( \frac{\alpha nF \nu}{RT_k_s} \right) = E^0 - \frac{RT}{\alpha nF} \ln \left( \frac{\alpha nF}{RT_k_s} \right) - \frac{RT}{\alpha nF} \ln \nu
\]

\[
E_{pa} = E^0 + \frac{RT}{(1-\alpha)nF} \ln \left( \frac{(1-\alpha)nF \nu}{RT_k_s} \right) = E^0 + \frac{RT}{(1-\alpha)nF} \ln \left( \frac{(1-\alpha)nF}{RT_k_s} \right) + \frac{RT}{(1-\alpha)nF} \ln \nu
\]  

(Eq. 2)

where \(E^0\) is the standard potential, \(k_s\) is the heterogeneous electron transfer rate constant, and \(n = 1\). The potentials for the cathodic (\(E_{pc}\)) and anodic (\(E_{pa}\)) peaks were plotted against \(\ln (\nu)\) and on analyzing the ratio of the slopes of the plots, an average value of the
transfer coefficient, $\alpha$, was found to be 0.54 for the S3NH$_2$Tyr Mb mutant, and 0.51 for the WT Mb immobilized electrode. The heterogeneous electron transfer rate constants for immobilized WT Mb and the S3NH$_2$Tyr Mb mutant were determined from the following equation (Eq. 3) at different scan rates:

$$\log k_s = \alpha \log(1 - \alpha) + (1 - \alpha) \log \alpha - \log \left( \frac{RT}{nFv} \right) - \frac{\alpha(1 - \alpha)nF\Delta E_p}{2.3RT}$$

$$\Delta E_p = \frac{2.3RT}{nF\alpha(1 - \alpha)} \left( \alpha \log(1 - \alpha) + (1 - \alpha) \log \alpha - \log k_s - \log \left( \frac{RT}{nF} \right) \right) + \frac{2.3RT}{nF\alpha(1 - \alpha)} \log v \quad \text{Eq. 3}$$

where $\Delta E_p$ is the peak potential separation. Using the respective values of $\alpha$ and from the plot of $\Delta E_p$ vs log $v$ (see Supporting Information Figure SI-6), $k_s$ was estimated to be 1.3 sec$^{-1}$ and 2.3 sec$^{-1}$ respectively for the immobilized S3NH$_2$Tyr Mb mutant and WT Mb on the chemically modified gold electrode. The S3NH$_2$Tyr Mb mutant monolayer has slightly slower electron transfer kinetics than the multilayered WT Mb presumably due to the varying length of the anchoring tethers in the two cases (see Table 4.1).

Table 4.1 compares surface modification chemistries based on anchoring molecules used for the immobilization of Mb on gold electrode surfaces and their associated $k_s$ values reported in two previous studies (151, 162) with the results from this study. It is observed that random immobilization of WT Mb leads to a decrease in the DET from 2.26 sec$^{-1}$ to 0.93 sec$^{-1}$ as the linker molecule length is increased from L-Cys (~4.75 Å) to homocysteine (~5.35 Å). Though the theoretical length of the tether is longer (~26 Å) in the case of S3NH$_2$Tyr Mb mutant, it facilitates faster electron transfer compared to homocysteine. This could be due to the tetraethylene glycol-linker providing a through-bond electron transfer environment from the electrode to the redox-sensitive heme center of the Mb (164, 166).
Figure 4.8 Comparison of immobilized Mb with hemin on modified gold electrode. CV response of hemin in solution, L-Cys modified, hemin immobilized, WT Mb and S3NH2Tyr Mb mutant immobilized gold electrode in nitrogen-purged 100 mM phosphate buffer with 10 mM KCl (pH 7.0) at room temperature and at a potential scan rate of 0.1 Vsec⁻¹.

An important aspect of the heme bleaching from the protein immobilized electrode surface was carefully investigated for both the S3NH2Tyr Mb mutant and the WT Mb protein. The redox responses of the free hemin (5 µM) in solution and when immobilized on the amine-functionalized gold electrode surface were observed to be quite different from those of the S3NH2Tyr Mb and the WT Mb-bound surface (Figure 4.6). From these data, it can be inferred that heme bleaching was absent from both the S3NH2Tyr Mb mutant and the WT Mb immobilized electrodes under the above mentioned experimental conditions.
4.3.3 Catalytic studies

Mb has often been utilized to explore the monooxygenase activity of heme proteins in presence of H$_2$O$_2$, in order to investigate the P450 catalytic shunt-pathway. A common substrate used in such studies is thianisole, which can be catalytically oxidized to its sulfoxide by high valent ferryl-porphyrin-π-cation radical species (also known as Compound I) of Mb. Hence, electrocatalytic conversion of thioanisole to its oxidized form provides an ideal comparison platform for the binding site orientation in these two modes of immobilization. Dioxygen was bubbled into the solution and bulk electrolysis was carried out for 2 hr with the covalently immobilized S3NH$_2$Tyr Mb mutant and the WT Mb (Table 4.3).

<table>
<thead>
<tr>
<th></th>
<th>Unreacted thioanisole (%)</th>
<th>Thioanisole sulfoxide (%)</th>
<th>TOF for the oxidized products (sec$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bare electrode</td>
<td>86</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>WT Mb</td>
<td>13</td>
<td>87</td>
<td>$1.9 \times 10^3$</td>
</tr>
<tr>
<td>S3NH$_2$Tyr Mb</td>
<td>19</td>
<td>81</td>
<td>$1.5 \times 10^3$</td>
</tr>
</tbody>
</table>

The GC results demonstrated that 87% conversion of the thioanisole to its oxidized form had taken place for the WT Mb immobilized electrode, while for the S3NH$_2$Tyr Mb mutant, 81% conversion was obtained. A reduced reaction time (1 hr) did not yield sufficient product formation in comparison to that of 2 hr catalytic reaction. A control experiment was also carried out for the electrocatalytic oxidation of thioanisole in the presence of bare Au. Only 14% conversion of the thioanisole to its sulfoxide was observed in this case after 2 hr. Considering
the amount of electro-active protein covalently attached onto the surface from Eq. 1, the turnover frequency (TOF) for the WT Mb was $1.9 \times 10^3$ sec$^{-1}$. Similarly, the TOF for the S3NH$_2$Tyr Mb mutant was $1.5 \times 10^3$ sec$^{-1}$. Significantly, the comparable catalytic activity observed for the multilayered WT Mb and the monolayered S3NH$_2$Tyr Mb mutant reiterates the importance of orientation of the active sites of both the proteins. The random orientation of binding site in the multilayered protein leads to loss of catalytic activity (considering the greater number of proteins on the surface), than the monolayered mutant S3NH$_2$Tyr Mb. Proper orientation of the heme active site in the monolayered S3NH$_2$Tyr Mb mutant assisted the catalytic and redox-activity of the protein, when covalently immobilized onto the modified gold electrode surface.

4.3.4 Microarray studies

Functionalization of glass surface with polyethylene glycol (PEG) was done by using the methods as previously described (167). To attach S3NH$_2$Tyr Mb on PEG linker, Diels–Alder reaction was used in mild conditions, such as in water (168). Since the protein will bind only through its unnatural amino acid, we have control of the active site in mutants. The immobilized protein on glass surface was analyzed by AFM, in which monomeric S3NH$_2$Tyr Mb showed expected dimensions of height and width (Figure 4.3) based on crystal structure.

4.3.4.1 Preparation of functional protein microarrays

A library of five proteins was printed on a single slide, to determine if our technique can be used for microarray studies (Figure 4.7). In order to understand orientation of the protein arrays and selective protein-protein interaction, we allowed HiLyte 647 fluorophore-conjugated anti-6×His tag rabbit IgG to bind with the proteins that were spotted on the surface. S3NH$_2$Tyr Mb and T365 (3NH$_2$Tyr P450 BM3) (Figure 4.7) were the only two proteins that show surface-exposed 6×His-tag chains when we observed the site of immobilization. Thus these were the
two proteins that showed the direct and efficient binding with IgG. However, all other proteins except the control were attached to the surface after the tagging reaction, as they were visible in coomassie blue staining. This experiment confirmed the interaction between IgG and the protein could be generated in a specific manner as well as control over the orientation of the protein.
Figure 4.9 A microarray of five proteins on a single slide displays. (a) T365 (3NH₂Tyr) P450 BM3. (b) P450 BM3 control without NaIO₄ (control shows that printed proteins were detached from the surface after washing). (c) T73 (3NH₂Tyr) CA. (d) D125 (3NH₂Tyr) CA. (e) D133 (3NH₂Tyr) EGFP. (f) S3 (3NH₂Tyr) Mb. Column 2 displays an image of excitation at 649 nm and emission at 666 nm.
Figure 4.10 Spectroscopic characterization of high spin, low spin and ligand complexes of WT and mutant Mb. Electronic absorption spectra of (A) ferric and ferrous, (B) CO, (C) NO, (D) oxyferrous, (E) cyanoferric and (F) N$_3$~ complexes of WT Mb and S3NH$_2$Tyr Mb. The spectra were taken at 4 °C and the samples were examined in 100mM phosphate buffer at pH 7.0.
4.3.5 Ligand binding studies

To understand the effect of different ligands upon replacement of residues which are away from the active heme center of the protein, we have replaced the serine 3 (S3) with 3NH$_2$Tyr, a redox amino acid. Figure 4.8 clearly indicates that S3NH$_2$Tyr Mb protein behaves more or less similar to that of WT Mb with respect to ligand-binding studies. Since position 3 is quite distant from the heme center, it has almost no role in influencing the electronic density of the heme center. As position 3 is quite away, it did not play a major role in determining the ligand-metal charge transfer, metal-ligand charge transfer, and in π backbonding (104, 119). We did not observe significant difference in the electron absorption spectra of mutant protein as compared to WT Mb (15, 31).

4.4. Conclusion

Though, previous research groups have observed facile electron transfer using specific organic molecules as promoters, no prior studies were reported using mutant protein that utilizes its unnatural amino acid moiety to specifically immobilize onto the electrode surface. In this chapter, I report monolayer and multilayer immobilization of Mb on to chemically-modified gold electrode surfaces using two unique immobilization techniques as described above. Further, electrocatalysis of thioanisole oxidation was carried out successfully with both the protein immobilized electrode cases, albeit at variant facility. These results underline that the S3NH$_2$Tyr Mb mutant monolayer exhibits well-defined catalytic and electro-activity because of proper orientation of its heme active site, in comparison to the random multilayered WT Mb case.

To investigate protein-protein interactions, five separate proteins P450 BM3, Mb, enhanced green fluorescent protein (EGFP), HRP, and CA have been covalently attached onto a solid support and analyzed. Functional protein microarray concept development was confirmed by a catalytic activity designed assay using spectroscopic characterization. This
concept developed microarray will bring novelty in the field of drug discovery and biomedical and proteomic research. The ligand binding studies showed that the S3 position being quite away from the active heme center, does not affect the metal-ligand binding to a greater extent.
5.1 Introduction

The heme group is the most common prosthetic group in the metalloproteins (1). The iron center, porphyrin, and axial ligand constitute the three covalently linked integral components of a heme protein. In the heme proteins, a wide variety of porphyrines are present such as heme a, heme b, heme c, heme d, heme d1, heme o, heme P460, chloroheme, and siroheme. Among the globins and cytochrome, heme b and heme c are the most common (Figure 5.1). A widely studied example is Mb (sperm whale), a small protein with 153 amino acids and molecular weight of 17.5 kD, with oxygen storage as its main function in vertebrates (2, 14, 169, 170). The presence of different varieties of amino acids around the binding site has always been a matter of interest for the understanding of heme ligand interaction and its catalytic activity (169, 170). With the tool of site directed mutagenesis divers, residues can be introduced to the proteins, and complexes formed with different ligands can be investigated. Among the heme protein, various axial ligands such as His, Cys, Met, Tyr, and Lys are found and they play a crucial role in their properties (14, 22). His present at 64 position plays a major role in stabilizing the ligand adduct by forming bonds to the ligands attached to the heme moiety. It has been proposed that with open and closed confirmation distal His can act like a gate for ligand adducts (1, 15, 29, 171, 172).

Figure 5.2 B shows the details of amino acid residues around the heme center in sperm whale Mb. It is also believed that the size of certain residues also contributes significantly to determining the heme ligand complex stability. But the role played by the electronic nature around heme center is not clearly understood. The introduction of noncannonical amino acids with electron rich functional groups such as pNO2Phe, will allow us to investigate the electronic nature of heme center, and its effect on ligand (CO, NO, O2, CN–, and N3–) binding to a greater
extent. For our further understanding of interaction of CO, NO, O₂, N₃⁻, and CN⁻ with Mb as a model for heme protein, I have created mutations at its distal and proximal sites (15). By site directed mutagenesis we have replaced the His with pNO₂Phe at His 64 and His 93 positions and compared the interaction of different ligands with these mutants, as well as to WT Mb (15, 88).

Figure 5.1 Structures of heme b and heme c.
Figure 5.2 *Schematic representations of WT and mutants Mb.* (A) Overall structure of WT Mb. (B) Enlarged binding site of WT Mb, (C) structure of H64pNO$_2$Phe and (D) structure of H93pNO$_2$Phe mutants.

5.2 Experimental procedure

5.2.1 Chemicals

The gasses (O$_2$, CO, and N$_2$) have been purchased from Air Liquide, USA while pNO$_2$Phe, sodium azide, potassium cyanide, and Na$_2$S$_2$O$_4$ were purchased from Sigma-Aldrich
USA. All chemicals were of analytical grade and have been used without any further purification (173).

5.2.2 Preparation of WT, H64pNO₂Phe and H93pNO₂Phe Mb mutant constructs

The WT Mb expression constructs H64pNO₂Phe and H93pNO₂Phe Mb gene expression vector and aminoacyl tRNA synthetase (aatRNA S) plasmids were donated by Dr. Peter Schultz (The Scripps Research Institute, La Jolla, CA USA). The H64pNO₂Phe and H93pNO₂Phe Mb expression vector were co-transformed with tRNA synthetase separately in DH10B E.coli. The bacterial colonies which are double antibiotic (ampicillin and tetracycline) resistant were picked, grown, and the cell stocks were stored at –80 °C prior to the start of bacterial cell culture for protein purification (15).

5.2.3 Purification and analysis of WT Mb, H64pNO₂Phe and H93pNO₂Phe Mb

The H64pNO₂Phe Mb and H93pNO₂Phe Mb were expressed in E. coli bacteria which were grown in GMML containing 5% LB media and suitable antibiotics (ampicillin and tetracycline). Both the WT and mutated (H64pNO₂Phe and H93pNO₂Phe) Mb proteins were induced, expressed, purified, and analyzed as described in chapter 2.
5.2.4 Electronic absorption spectroscopy

UV-visible electronic absorption spectra of WT Mb proteins and the mutant H64pNO$_2$ and H93pNO$_2$ Mb proteins were taken using Varian Cary 50 Bio UV-visible spectrophotometer. The concentrations of proteins were determined from the absorption spectrum of the protein, using the molar extinction coefficient determined by the heme chromatogen method (96).
5.2.5 Preparation of deoxyferrous Mb

The WT Mb and mutants Mb proteins were taken in their respective airtight cuvettes containing 0.1 M potassium phosphate buffer, pH 7.0. The N\textsubscript{2} gas was passed for \(\sim\)2 hr at 4 °C to degas the sample. The minimal amount of Na\textsubscript{2}S\textsubscript{2}O\textsubscript{4} (in µL) was added from the stock of 20 mg/mL and the electronic absorption spectra were recorded by Varian Cary 50 Bio UV-visible spectrophotometer (15).

5.2.6 Preparation of oxyferrous complex

The oxyferrous complexes were prepared in a chest freezer at −35 to −45 °C. The protein sample was placed in a 100 mM potassium phosphate buffer (pH 7.0), which contained 65% glycerol (v/v). The ferric protein was first degassed with N\textsubscript{2} gas for 2 hrs. Then it was reduced to deoxyferrous by addition of Na\textsubscript{2}S\textsubscript{2}O\textsubscript{4} (20 mg/mL stock) under N\textsubscript{2} atmosphere in a sealed cuvette at 4 °C. Pre-cooled O\textsubscript{2} gas was bubbled into the cuvette for 60 sec and the UV-visible spectra were recorded (15, 31, 40).

5.2.7 CO and NO complex preparation

The ferrous-CO adducts were generated by gentle bubbling of CO in deoxyferrous enzymes for 30 sec at 4°C in 0.1 M potassium phosphate buffer pH 7.0. The ferrous-NO complexes were generated by micoliter addition of buffer saturated with NO gas in deoxyferrous enzymes under N\textsubscript{2} at 4 °C in 0.1 M potassium phosphate buffer pH 7.0. The saturated buffer was prepared by gentle bubbling of NO gas in a sealed cuvette containing 0.1 M potassium phosphate buffer pH 7.0 for 30 min at room temperature (15, 31, 32, 40).

5.2.8 N\textsubscript{3}– and CN\textsuperscript{−} complex preparation
WT Mb and mutants Mb $N_3^-$ complexes were formed by $\mu$L addition of NaN$_3$ solution from 40 mM stock solution. The NaN$_3$ stock solution and protein were in 100 mM potassium phosphate buffer pH 7.0 and the temperature was kept at 4 °C. The cyanoferic adducts were prepared from the deoxyferric enzymes by addition of minimal volumes ($\mu$L) of a 1 M KCN stock solution, prepared in 100 mM potassium phosphate buffer pH 7.0. The deoxyferric enzymes were prepared by bubbling of N$_2$ gas in the ferric enzymes for at least 30 min.

5.3 Results and discussion

Here we studied the ferrous and ferric electronic absorption spectra of Mb and its mutants (H64pNO$_2$Phe Mb and H93pNO$_2$Phe) in absence and presence of exogenous ligands such as CO, NO, O$_2$, N$_3^-$, and CN$^-$ using UV-vis spectroscopy. The electronic absorption spectra of the ferric form of the enzyme showed that there are significant differences in their character, particularly at 550-650 nm (Figure 5.4). The peaks around 539, 578, and 628 nm in ferric and around 662 nm in ferrous enzyme were not prominent in mutants when compared to the WT Mb. Due to the presence of the electron rich functional group ($p$NO$_2$Phe), the Soret around 409 nm in ferric and around 430 nm in ferrous were a little sharper in case of mutant enzymes (Figures 5.4 and 5.5).
Figure 5.4 Characterization of ferric WT and mutants Mb. Electronic absorption spectra of the H64pNO₂Phe (blue dashed-dot), H93pNO₂Phe (black solid line) and WT (red dashed line) of ferric Mb enzymes. The spectra were recorded in 100 mM potassium phosphate buffer, pH 7.0 at 4 °C with 5 μM protein.
5.3.1 Ferrous-CO, ferrous-NO and ferrous $O_2$ complexes of WT, H64pNO$_2$Phe and H93pNO$_2$Phe Mb

To understand the effect of different ligands upon replacement of distal and proximal His and electronic nature of the heme center, I have investigated the ferrous-XO (X= C, N, O) complexes of Mb and its mutants. As XO molecules have vacant $\pi^*$ orbitals which closely matched with filled $d\pi$ orbitals of the Fe (II) ion of porphyrin, it makes the heme group electronically more favorable to bind to CO, NO and $O_2$. Furthermore, this kind of arrangement was found to be optimal for the Fe-XO $\pi$-backbonding. The backbonding was responsible for shortening (strengthening) the Fe-X bond and lengthening (weakening) of the X-O bond (Figures 5.6 and 5.7) (40, 174-176).
Figure 5.6 *The heme carbonyl complex showing \(d\pi-p\pi^*\) backbonding.*

Figure 5.7 *Showing presence of electron rich functional group \((\text{NO}_2^-)\) at distal position.*
Table 5.1 The electronic absorption spectral features of the oxy, carbonyl, NO, CN\(^-\), and N\(_3\)\(^-\) complexes with H64pNO\(_2\)Phe Mb, H93pNO\(_2\)Phe Mb mutants and WT Mb.

<table>
<thead>
<tr>
<th></th>
<th>H64pNO(_2)Phe</th>
<th>H93pNO(_2)Phe</th>
<th>WT Mb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Soret (nm)</td>
<td>Visible (nm)</td>
<td>Soret (nm)</td>
</tr>
<tr>
<td><strong>Ferric</strong></td>
<td>408.9</td>
<td></td>
<td>Ferric</td>
</tr>
<tr>
<td><strong>Deoxyerrous</strong></td>
<td>430</td>
<td>558</td>
<td>Ferrous</td>
</tr>
<tr>
<td>Fe(^{3+}) - CN(^-)</td>
<td>422.9</td>
<td>537; 576</td>
<td>Fe(^{3+}) - N(_3)(^-)</td>
</tr>
<tr>
<td>Fe(^{3+}) - CO</td>
<td>424</td>
<td>539; 573</td>
<td>Fe(^{2+}) - O(_2)</td>
</tr>
<tr>
<td>Fe(^{2+}) - O(_2)</td>
<td>419.9</td>
<td>542; 580</td>
<td></td>
</tr>
<tr>
<td>Fe(^{2+}) - NO</td>
<td>419</td>
<td>545, 577</td>
<td></td>
</tr>
</tbody>
</table>
Ferrous-CO complexes of the H64 and H93 pNO₂Phe have shown higher intensity of absorbance compared to ferrous-NO and ferrous–O₂ complexes (Figure 5.8). This may be due to Fe-CO being linear adduct and both of its π* orbitals have empty shells. It allows dπ–π* orbitals to overlap in a perpendicular direction, which results in a prominent overlapping, and more backbonding giving rise to sharper Soret (119). However under the similar conditions, NO and O₂, occupies one and two π* electrons, respectively in their π* orbitals. In order to accommodate any π-antibonding interaction, the NO and O₂ adducts have to bend, which further lowers the possibility of overlapping and backbonding. This leads to less prominent Soret in NO and O₂ as compare to CO (Figure 5.8, 5.9 and 5.10). It is noteworthy that there were significant differences within the XO adducts of different proteins’ electronic spectra (Table 5.1).

When axial His H93 attached to heme center, was replaced by pNO₂Phe (an electron rich functional group), the sharper Soret was recorded due to stretching one dπ-π* backbonding electron transition via push effect The pNO₂Phe increases the electron density near the Fe (II) center, which enhances the metal-ligand charge transfer and backbonding. Similar results were not observed when the distal His 64 was replaced by pNO₂Phe. The distal pNO₂Phe probably increases the electron density around the CO orbitals which favors ligand-metal charge transfer and lowers the backbonding. Since O₂ has two π* electrons, reverse characteristics have been observed in the oxyferrous complex (Figure 5.10). The different enzymes show different peaks in 550-700 nm regions (Table 5.1), which explains that they also have diverse, features in visible region (3, 176).
Figure 5.8 Comparative studies of carbonyl complexes of WT, H64pNO$_2$Phe, and H93pNO$_2$Phe. Electronic absorption spectra of CO complex of WT Mb (red dashed line), H64pNO$_2$Phe (blue dashed-dot) and H93pNO$_2$Phe Mb mutant (black solid line). The spectra were taken at 4 °C and the samples were examined in 100mM phosphate buffer at pH 7.0.
Figure 5.9 Comparative studies of NO complexes of WT, H64pNO2Phe, and H93pNO2Phe. Electronic absorption spectra of NO complex of WT Mb (red dashed line), H64pNO2Phe (blue dashed-dot) and H93pNO2Phe Mb mutant (black solid line). The spectra were taken at 4 °C and the samples were examined in 100mM phosphate buffer at pH 7.0.
Figure 5.10 Characterization of oxyferrous complexes of WT, H64pNO$_2$Phe, and H93pNO$_2$Phe Mb. Electronic absorption spectra of WT Mb oxyferrous (red dashed line), H64pNO$_2$Phe mutant Mb oxyferrous (blue dashed-dot) and H93pNO$_2$Phe mutant Mb oxyferrous (black solid line). The spectra were taken at −35 to −45 °C and the samples were examined in 60% glycerol, 100mM phosphate buffer at pH 7.0.

The stability of XO adducts are dictated by hydrogen bonding between the XO and distal protein moiety (figure 5.11). In WT Mb at the 64 position, His is present, which is primarily responsible for the H-bonding, hence the stability of XO adducts (176). When I replaced the His with pNO$_2$Phe, the possibility of H-bonding at distal pocket was reduced, which lowers stability of complex. Furthermore, the electron rich oxygen allowed more electronic distribution, which led to prominent ligand to metal charge transfer as seen in uv-vis spectrum. The replacement of the proximal His 93 with pNO$_2$Phe led to increased electron density, and backbonding was more prominent around XO orbital; it helped in forming the H-bond with distal His. Thus I
conclude that when we replaced the axial His with the $p$NO$_2$Phe, the XO adducts were more stable than in other cases.

![Figure 5.11 Proposed structure of (A) Fe (II) O$_2$, (B) Fe (II) CO and (C) Fe (II) NO.](image)

5.3.2 Ferric-cyanide and ferric-azide complexes of WT and H64pNO$_2$Phe and H93pNO$_2$Phe mutants of Mb

Characterization of anionic ligand adducts such as CN$^-$ and N$_3^-$ to ferric WT Mb and mutant Mb were important to understand how the electronic nature affects heme center. It gives us the idea of the impact of protein environment on heme center. Ligand to metal charge transfer are ideal for metals in high oxidation states such as Fe$^{3+}$ that are bound to electron rich, low electronegativity ligands such as N$_3^-$ and CN$^-$ (15, 175-177). The charge transfer from ligand to metal will cause the increase in Soret, but due to lack of backbonding, it was not as
prominent as in the case of the CO adduct. The charge transfer from ligand to heme center made it a low spin complex; the red shift was observed to high energy (Figures 5.12 and 5.13). As CN\(^{-}\) is a stronger field ligand, the Soret was less sharp in the ferricyanide complex.

The introduction of electron rich oxygen in \(\rho\text{NO}_2\text{Phe}\) at axial and distal positions has a significant impact on the electronic density of the heme center. When we replaced distal His with \(\rho\text{NO}_2\text{Phe}\) in electron density around \(\text{CN}^-\) and \(\text{N}_3^-\) ligands, orbitals increased. Consequently, there was more ligand-metal charge transfer, resulting in an increase in Soret and red shift. While we replaced axial His with \(\rho\text{NO}_2\text{Phe}\), the Fe center was more electron rich, which caused the red shift (\(\lambda_{\text{max}}\) 423, 436.9, 437.4, and 576). There was less possibility of metal to ligand charge transfer and backbonding, as the ligand was electron rich and its orbitals are filled, and therefore no significant increase in Soret (119). The adducts also showed quite a distinct feature in their 550-700 nm region (Table 5.1), which supports that the heme environment has a significant role on heme proteins in the determination of their catalytic and ligand binding properties.
Figure 5.12 Comparative studies of CN⁻ complexes of WT, H64pNO₂Phe, and H93pNO₂Phe Mb. Electronic absorption spectra of ferric CN⁻ complex of WT Mb (red dashed line), H64pNO₂Phe (blue dashed-dot) and H93pNO₂Phe Mb mutant (black solid line). The spectra were taken at 4 °C and the samples were examined in 100mM phosphate buffer at pH 7.0.
Figure 5.13 Characterization of $N_3^-$ complexes of WT, H64pNO$_2$Phe, and H93pNO$_2$Phe Mb. Electronic absorption spectra of ferric $N_3^-$ complex of WT Mb (red solid line), H64pNO$_2$Phe (blue dashed-dot) and H93pNO$_2$Phe Mb mutant (black solid line). The spectra were taken at 4 °C and the samples were examined in 100mM phosphate buffer at pH 7.0.

The H-bonding also plays a significant role in the stability of CN$^-$ and $N_3^-$ adducts (Figures 5.14 and 5.15). Any residue that strengthens the H-bonding will make a stronger adduct and vice versa. In this case, as pNO$_2$Phe reduces the possibility of H-bonding at the distal pocket, it leads to weaker adducts. But the size of the residues also plays a significant role in holding the ligands by not allowing them to leave the binding site environment.
Figure 5.14 Two canonical forms of $N_3^-$ bound to heme iron of WT Mb and H64pNO$_2$Phe Mb mutant.

Figure 5.15 Two canonical forms of CN$^-$ bound to heme iron of WT Mb and H64pNO$_2$Phe Mb mutant.
5.3 Conclusion

To summarize, the importance and significance of electron rich functional groups on the electronic nature of heme center had been explored. It has been found that the axial His attached to the heme center plays a crucial role in dictating the electron cloud near the heme center. When the axial heme is replaced by an electron rich residue like $p$-NO$_2$Phe, the electron density was higher near heme center, which contributes to higher energy and red shift of the spectra. This study also concludes that the oxidation states of metal centers and the natures of ligand (electron rich or electron deficient) play an important role in determining the backbonding and direction of charge transfer (from metal to ligand or ligand to metal). The redox nature and size of the distal residue also has a high impact on determining the stability of the ligand adducts. It is well known that the H-bonding plays an important role in the stability of ligand adducts, but what role the size of noncanonical (such as $p$-NO$_2$Phe) residues play in the stability of ligand adducts is uncertain.
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BIOGRAPHICAL INFORMATION

Subhash Chand the elder son of Mr. Keshaw Prasad Yadav and Mrs. Urmila Devi Yadav has obtained his high school education at Shri Shanker Ji Intermediate College, Pushpanagar, Azamgarh. Ever since the initial years of education, his interests were focused in subjects like Science and Mathematics. In his primary school days, he has been academically oriented, always stood out for his scholastic performance and earned his way into the top rank in his class. He received B.Sc. (Biology) degree from the Ewing Christian College, Allahabad, India and then M.Sc. (Botany) degree from University of Allahabad, Allahabad, India. His respectable educational credentials landed him in the Indian Institute of Technology, Kharagpur (IIT Kharagpur), the prestigious science and technology institute of India, to pursue Master of Technology in Applied Botany (Biotechnology). After receiving his Masters degree he joined Meerut Institute of Engineering and Technology and served as Lecturer in Department of Biotechnology and Applied Sciences. He soon realized that higher education plays an important role in advancing one’s professional life and moved to USA to join University of Texas at Arlington so as carry out doctoral research. He joined Dr. Roshan Perera's group to obtain his Doctoral degree in Biochemistry. There he conducted stimulating research to incorporate unnatural amino acids in genetically modified myoglobin mutants so as to mimic the catalytic activities of various heme proteins.