Mutation of the B10 Tyrosine and E11 Leucine in *Giardia intestinalis* Flavohemoglobin

A thesis submitted to the Committee of Graduate Studies in partial fulfillment of the requirements for the degree of Master of Science in the Faculty of Arts and Science

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ABSTRACT

Mutation of the B10 Tyrosine and E11 Leucine in *Giardia intestinalis* Flavohemoglobin

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The flavohemoglobin in *Giardia intestinalis* (gFlHb) is the only known protozoan member of a protein class typically associated with detoxifying nitric oxide (by oxidation to nitrate) in bacteria and yeast. Mutants of the B10 tyrosine (Y30F) and E11 leucine (L58A), conserved residues thought to influence ligand binding, were expressed and studied using Resonance Raman (RR) spectroscopy. In the wild type protein, RR conducted using a carbon monoxide probe detects two distinct Fe-CO stretches associated with two different active site configurations. In the open configuration, CO does not interact with any polar side chains, while in the closed configuration, CO strongly interacts with one or more distal residues. Analysis of the Y30F mutant provided direct evidence of this tyrosine’s role in ligand stabilization, as it had only a single Fe-CO stretching mode. This stretching mode was higher in energy than the open conformer of the wild type, indicating a residual hydrogen bonding interaction, likely provided by the E7 glutamine (Q54). In contrast the L58A mutant had no effect on the configurational nature of the enzyme. This was unexpected, as the side chain of L58 sits atop the heme and is thought to regulate the access of distal residues to the heme-bound ligand. The similar spectroscopic properties of wild type and L58A suggest that any such regulation would involve rapid conformational dynamics within the heme pocket.

**Key Words:** flavohemoglobin, gFlHb, *Giardia intestinalis*, B10 tyrosine, E11 leucine, E7 glutamine, nitrosative stress, nitric oxide, resonance Raman spectroscopy, dioxygenase, nitrosylase, catalytic globin, heme protein, Hmp, Yhb, mutational study, peroxidase, push-pull mechanism, superoxide
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<th>Description</th>
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<tbody>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>bp</td>
<td>Base Pair</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CIP</td>
<td>Calf Intestinal Alkaline Phosphatase</td>
</tr>
<tr>
<td>DEA-NONOate</td>
<td>Diethylamine NONOate</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>FAD</td>
<td>Flavin Adenine Dinucleotide</td>
</tr>
<tr>
<td>FHP</td>
<td><em>A. eutrophus</em> Flavohemoglobin</td>
</tr>
<tr>
<td>FNR</td>
<td>Ferredoxin Nucleotide Reductase</td>
</tr>
<tr>
<td>gFlHb</td>
<td>Giardial Flavohemoglobin</td>
</tr>
<tr>
<td>Hb</td>
<td>Hemoglobin</td>
</tr>
<tr>
<td>HbN</td>
<td><em>Mycobacterium tuberculosis</em> Hemoglobin</td>
</tr>
<tr>
<td>Hmp</td>
<td><em>E. Coli</em> Flavohemoglobin</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl β-D-1-thiogalactopyranoside</td>
</tr>
<tr>
<td>NAD(P)H</td>
<td>Nicotinamide Adenine Dinucleotide and Nicotinamide Adenine Dinucleotide Phosphate</td>
</tr>
<tr>
<td>NOD</td>
<td>Nitric Oxide Dioxygenase Reaction</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethane Sulfonyl Fluoride</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions Per Minute</td>
</tr>
<tr>
<td>SAP</td>
<td>Shrimp Alkaline Phosphatase</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide Dismutase</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris/Borate/EDTA</td>
</tr>
<tr>
<td>TCEP</td>
<td>Tris(2-carboxyethyl)phosphine</td>
</tr>
<tr>
<td>Yhb</td>
<td>Yeast Flavohemoglobin</td>
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1. INTRODUCTION

1.1 Overview

*Giardia intestinalis* (syn. *G. lamblia*, *G. duodenalis*) is a highly divergent eukaryote deficient in several typical eukaryotic organelles, such as peroxisomes and mitochondria (1). The absence of a heme-reliant respiratory chain (as an amitochondrial organism), combined with the fact that Giardia does not possess any of the enzymes involved in heme biosynthesis or a known mechanism for importing the cofactor, led to the conclusion that the parasite does not utilize heme or heme-proteins (2). This was proven wrong upon the discovery that Giardia encodes several proteins with heme-binding domains (3, 4). This includes a functional flavohemoglobin (gFlHb), likely obtained through a lateral gene transfer from a bacterium, making Giardia the only known parasitic protozoan with such an enzyme (3). Given its structural similarities to other flavohemoglobin variants, gFlHb provides an excellent model for the study of this particular protein class.

1.2 Flavohemoglobins

1.2.1 Structure and Function

Flavohemoglobins have a well-established role in the detoxification of the radical species nitric oxide (NO) in bacteria, yeast, and fungi (5–7). Composed of a C-terminal reductase domain of the ferredoxin nucleotide reductase (FNR) family (containing highly conserved binding sites for electron carriers NAD(P)H and FAD) and an N-terminal globin domain (with a heme binding pocket), flavohemoglobins catalyze the reaction of
nitric oxide with molecular oxygen (O₂) to form the less harmful nitrate (NO₃⁻) (5, 7, 8).

Figure 1 provides a homology model of gFlHb based on the structure of *Escherichia coli* Hmp. The aforementioned reaction is summarized in Equation 1 below.

\[
NAD(P)H + 2O_2 + 2NO \rightarrow 2NO_3^- + NAD(P)^+ + H^+ \quad [1]
\]

Under anoxic conditions, flavohemoglobins have also demonstrated the ability to reduce nitric oxide to nitrous oxide (N₂O) (7, 9), a two-step process by which flavohemoglobin donates an electron to NO to form nitroxyl (NO⁻), followed by the release of NO⁻ into solution and its self-reaction to form N₂O and water:

\[
NAD(P)H + 2NO \rightarrow 2NO^- + NAD(P)^+ + H^+ \quad [2]
\]

\[
2NO^- + 2H^+ \rightarrow N_2O + H_2O \quad [3]
\]

The reductase reaction, however, takes place at only a fraction the rate of the NO-dioxygenation reaction (approximately 1%) and given other prominent and far more efficient mechanisms of NO removal under anaerobic conditions (*via* flavorubredoxin for example), it is unlikely that the reductase function of flavohemoglobins serve a physiologically relevant role (7, 10). Indeed, many obligate anaerobes do not possess flavohemoglobins at all (7).

**1.2.2 Regulation and Superoxide Production**

Flavohemoglobin expression is typically modulated in an NO-dependent manner, and while gFlHb protein and mRNA levels are up-regulated in Giardia on exposure to nitrosative stress the mechanism is unknown. Much more is known in the Gram- positive and -negative bacteria, where flavohemoglobins are regulated under the broad and complex NO-sensing adaptive system controlled by transcriptional factor NsrR (7, 11). The need to regulate flavohemoglobin, counterintuitive given its protective nature
**Figure 1:** Homology model of gFlHb based on the structure of E. coli Hmp. The globin domain is highlighted in yellow and the flavin reductase domain in cyan.

**Figure 2:** Homology model of gFlHb indicating conserved residues surrounding the heme group.
(discussed in further detail below), stems from the reducing potential of its flavin domain, which like many flavoenzymes is able to donate an electron directly to O₂, converting it to superoxide (O₂⁻), in the absence of NO (7, 12, 13):

\[
\text{NAD}(P)H + 2O_2 \rightarrow 2O_2^- + \text{NAD}(P)^+ + H^+ \quad [4]
\]

This can lead to O₂⁻/H₂O₂ derived oxidative stress within a cell. NsrR-null bacteria express elevated levels of flavohemoglobin and show a marked increase in O₂⁻/H₂O₂ associated cellular damage (7, 14).

1.2.3 Structure of the Heme Binding Pocket

Sequence alignments of 115 flavohemoglobins from Gram-negative bacteria, Gram-positive bacteria, and unicellular eukaryotes identify 12 residues surrounding the heme pocket that remain invariant across all studied members of the protein family (15). To date, the crystal structure of three flavohemoglobins have been solved: *E. coli* Hmp, *A. eutrophus* FHP, and yeast Yhb (16–18). These structures, along with modelling studies originating from them, provide the basis of the proposed orientations of these residues around the active site heme group. Figure 2 presents a homology model (based on the structure of Hmp) of the 12 invariant residues around the heme group in gFlHb.

When comparing the structures and position of homologous amino acid residues between two globin chains, one uses a convention in which residues are numbered according to their positions within the eight conserved alpha helices (denoted by the letters A-H) of this protein family. This permits unambiguous comparison of residues even when differences in chain length lead to homologous residues having different numbers. For example, the histidine that is the axial ligand to the heme in Hmp is His-85, corresponding to His-107 in gFlHb; in the globin nomenclature described above both of
these are His F8 (the 8th residue on helix F). In this thesis both descriptions will be used, as appropriate to the case, to denote specific amino acid residues.

With respect to the plane of the heme, the term *proximal* refers to the side on which the coordinating histidine lies. The other, *distal* side, is where exogenous ligands such as O2, NO or CO bind.

The architecture of the proximal heme pocket maintains a similar conformation in each of the solved flavohemoglobin structures (Figure 3). The proximal ligand to the heme iron is the imidazole side chain of His F8. Hydrogen bonded to the protonated nitrogen of this residue is the carboxylate side chain of Glu H22, which itself is held in place through a hydrogen bond with the hydroxyl group of Tyr G1 (4, 15, 16, 19). This hydrogen bonding network locks the proximal histidine ligand in a rigid orientation relative to the heme group and increases the electronegativity of its unprotonated nitrogen, which results in a stronger coordinate bond with the heme iron, moving it out of the porphyrin plane and making it difficult for weak ligands to bind. This creates an interaction more typical of heme enzymes such as peroxidases. In effect, flavohemoglobins, like other catalytic globins, have globin domains that bear active-site residues of heme enzymes superimposed on a structural platform that shares the fold of our own O2 transport and storage proteins (4, 19, 20).

The distal heme pocket contains many of the key residues believed important for ligand binding. Two polar residues are conserved in the distal pocket of all members of the protein class; Tyr B10 and Gln E7. Also notable is Leu E11 with its nonpolar side chain adjacent to the heme iron, in the position that is typically occupied by the histidine (His E7) used to stabilize bound O2 in oxygen transport globins (4, 15).
Figure 3: A) Conserved residues and hydrogen bonding network in the proximal heme pocket of gFlHb. B) Key residues in the distal heme pocket of gFlHb.
Tyr B10 has been previously implicated in the process of O2 binding. Mutation of this residue to phenylalanine in Hmp resulted in a ~80-fold increase in the O2 dissociation rate constant and a ~30-fold decrease in nitric oxide dioxygenation activity (21). Tyr B10 has a similar stabilizing effect on the iron-ligand bond in other types of catalytic globins as well, including those thought to carry out NO/O2 chemistry (19).

In many of these catalytic globins, including both Hmp and gFlHb, an ability to convert between an open conformation, where the bound distal ligand is not interacting with any polar groups, and a closed conformation, where the bound ligand is undergoing a strong hydrogen bonding interaction with one or more distal residues, is observed (19). Gln E7 has been implicated in this process, as mutation of this residue to leucine (which removes the ability to hydrogen bond while retaining the shape of the side chain) in Hmp resulted in only a single observed conformation, close but not identical, to the previously recorded open conformation (22). Whether this glutamine residue is directly involved in ligand stabilization via hydrogen bonding or its absence simply destabilizes the hydrogen bonding interaction of Tyr B10 is unclear; the side chain amide Gln E7 faces outward towards the solvent in Hmp, but faces inward towards the heme iron in yeast Yhb (16, 18). Notably, the coordination environment differs in these two cases; Hmp was crystallized in its ferric 5-coordinate form whereas Yhb was crystallized with an unknown ligand bound on the distal side. Collectively these observations indicate flexibility in the position of the E helix that allows Gln E7 to adopt different conformations, with the Yhb structure resembling the catalytically active closed conformation.
Unlike Gln E7 the position of Tyr B10 is unchanged in different ligation states. Structure-function studies on mutants at this position have not been reported in flavohemoglobins, but have been conducted in structurally similar bacterial globins, notably HbN of Mycobacterium tuberculosis and Ascaris suum Hb, which also conduct NO/O₂ chemistry (19, 23–25). In these cases, mutation of Tyr B10 to phenylalanine, which removes its ability to hydrogen bond exogenous ligands, resulted in proteins that displayed only the open, or near open, conformational state. While this demonstrates an important role for the B10 tyrosine in exogenous ligand stabilization, it also shows that a hydrogen bonding interaction between the E7 glutamine and the heme ligand is not required to perform this type of chemistry. Indeed, the E7 position is not occupied by glutamine at all in HbN, a truncated hemoglobin. Still, it is possible for proteins to accomplish the same chemistry using different processes and this by no means excludes a role for Gln E7 in flavohemoglobins.

Though the exact nature of these conformational shifts remains unclear, a third conserved residue on the distal side, Leu E11, will likely play a key role, particularly in generating the open, non-hydrogen binding conformer. In Hmp crystallized in the ferric ligand-free form, Leu E11 sits directly over the heme iron binding site, restricting its access to O₂ and NO (15, 16). For an exogenous ligand to bind to the heme, Leu E11 would first have to rotate out of this position, and, even upon ligand binding, the residue may still control the access of distal residues to the substrate. The structure of Yhb appears to demonstrate this movement, as Leu E11 is shifted outwards towards the porphyrin scaffolding in the ligand-bound structure (18).
The shift between open and closed conformers likely requires the concerted movement of the three residues described above as part of a larger movement within the protein. Both Tyr B10 and Gln E7 are a significant distance from the ligand binding site in the ferric structure of Hmp, with the phenol ring of Tyr B10 in the second layer of the distal pocket and thought to require a water bridge to interact with the ligand (16, 26). Consequently, it is possible that a shift in position of the alpha helices of the globin domain itself, instead of just the individual movement of Leu E11, is the root of the conformational flexibility in the protein family. The globin domain of Yhb, for instance, has shown remarkable flexibility within its helices to bind large ligands such as econazole (18). A similar series of shifts could also be involved in small ligand binding in flavohemoglobins. This ability of several catalytic globins to exist in more than one conformational state, in addition to the trend towards a strong proximal iron-histidine bond relative to that of oxygen carriers, suggests that these characteristics are crucial for some globins to perform enzymatic functions (19).

1.2.4 “Push-Pull” Mechanism

As previously stated, the heme pockets of flavohemoglobins share several characteristics with that of peroxidases. These characteristics are central components of the latter’s widely accepted “push-pull” mechanism of action, a process thought to be utilized in several classes of heme enzymes to cleave oxygen-oxygen bonds (18, 19). In this mechanism, the proximal histidine ligand forms a strong hydrogen bond with a proximal residue bearing a carboxylate side chain (a glutamate in flavohemoglobins, aspartate in peroxidases) which imparts upon the proximal histidine a strong anionic character, pushing electron density towards the distal ligand. At the same time, hydrogen
bonding residues in the distal heme pocket (a distal arginine in peroxidases is mirrored by the B10 tyrosine and/or E7 glutamine in flavohemoglobins) stabilize the distal ligand, pulling electron density towards them. Both processes weaken the O-O bond, priming it for heterolytic cleavage. Given the structural similarities between the active sites of peroxidases and flavohemoglobins, it is likely that flavohemoglobins implement an analogous mechanism to cleave the O-O bond of dioxygen, prepping it for reaction with the second substrate (18, 19). Figure 4 visualizes this hypothetical mechanism in flavohemoglobins.

![Proposed “push-pull” mechanism of flavoHbs. Adapted from El Hammi et al.](image)

**Figure 4:** Proposed “push-pull” mechanism of flavoHbs. Adapted from El Hammi et al.
1.2.5 Flavohemoglobin reaction mechanisms: Dioxygenation vs. Nitrosylation

Two mechanisms for the catalytic action of flavohemoglobins have been proposed, dioxygenation and nitrosylation, which differ mainly on which reactant binds heme first. Both reactions begin with flavohemoglobin in the ferric state. NAD(P)H is the initial source of electrons, though as an obligate two electron donor it requires an FAD cofactor bound to flavohemoglobin to receive those electrons and donate them individually to reduce the heme iron to its ferrous state. This is where each mechanism diverges. In the dioxygenation model, FAD reduces the heme iron to its ferrous state allowing O₂ to bind. O₂ is then quickly reduced forming an Fe(III)-O₂⁻ complex. NO binds the O₂ molecule and the resulting complex undergoes isomerization to form nitrate (4, 5, 7, 18). In the nitrosylation model, NO is the first ligand to bind the heme iron, doing so in either the ferric or ferrous states. When FAD reduces the heme iron (if it has not done so previously) NO is quickly reduced to nitroxyl, forming an Fe(III)-NO⁻ complex. O₂ then reacts with the nitroxyl group and isomerizes to form nitrate (7, 27, 28). In both mechanisms, FAD can reduce the heme iron a second time to restart the catalytic cycle. Figure 5 details the steps of both mechanisms.

The debate over which model best fits the experimental evidence continues to be contentious. The dioxygenation model is the most widely accepted of the two proposed mechanisms. In support of this mechanism is the fact that nitric oxide, at high concentrations (O₂:NO ratios of 100:1), inhibits the enzyme, likely because NO competes with O₂ at the heme binding site (7, 29). Likewise, mutation of the B10 tyrosine has marked effect on O₂ binding, but very little impact on NO binding (7, 21). The nitrosylation model is also less compatible with much of the structural data compiled
Figure 5: Catalytic cycles of the dioxygenation and nitrosylation mechanisms.
about the heme pocket. For instance, the “push-pull” mechanism seemingly incorporated in flavohemoglobins is most associated with the heterolytic cleavage of O-O bonds. Even if this process could be incorporated into cleaving one of the N-O double bonds in nitric oxide (should it bind before O₂), the nitrogen would bind closest to the heme iron. This would mean that the protein is activating a bond for cleavage that does not need to be (or possibly cannot be) cleaved in order to form nitrate; or, that O₂ would need to bind next to the heme iron, away from the stabilizing hydrogen bond(s) and in a manner inconsistent the proposed binding pocket in yeast (18).

In favour of the nitrosylation model is the fact that NO binds to heme in flavohemoglobins at far greater affinity than O₂. Hmp has O₂ and NO dissociation constants (Kₐ) of 0.012 µM and 8 x 10⁻⁶ µM respectively, preferring NO to O₂ by a factor of 1500 (21, 28). Given this, the argument can be made that under physiological conditions (and several experimental conditions purporting dioxygenase activity), NO concentrations will be high enough to outcompete O₂ in binding to the protein (28). Direct experimental evidence in favour of the nitrosylation model is somewhat more limited. Steady-state spectra measured under certain NO concentrations were entirely in the ferric or ferrous NO bound states (27, 28). Likewise, when the flavohemoglobin-NO complex is generated in an anaerobic environment, introduction of even low micromolar concentrations of O₂ will quickly result in the production of nitrate (7, 27). However, these experiments were conducted at a relatively low temperature of 10 °C (temperature can effect binding constants) and in the absence of exogenous FAD, making it difficult to draw direct physiological relevance. Regardless, typical models of dioxygenase activity classify flavohemoglobin-NO complexes as inactive (5). This sort of evidence indicates
that nitrosylation is at least a viable mechanism, and if not the primary method of nitrate generation, could serve as a secondary method in situations of low \( O_2 \) availability.

1.3 *Giardia intestinalis*

1.3.1 Biology and Metabolism

*Giardia intestinalis* is a fresh water protozoan parasite that infects the digestive tracts of humans, wildlife, and livestock, and is a prominent contributor to the widespread scourge of diarrheal diseases throughout the world (30). Infection occurs upon ingestion of Giardia’s environmentally resilient cyst form, which, when present in the digestive tract of its host, differentiates into flagellated free-swimming binucleated trophozoites that attach and feed off the epithelial lining of the small intestine, causing symptoms such as diarrhea, abdominal cramps, and nausea. New cysts formed in the lower intestine are excreted through the host’s fecal matter, contaminating water supplies and restarting the cycle (1).

Though Giardia lacks mitochondria, a reduced form of the organelle, called mitosomes, are present (1, 30). However, mitosomes are used primarily for the synthesis of iron-sulfur clusters (31). As such, Giardia lacks the respiratory chain synonymous with the mitochondria of aerobic eukaryotes and relies primarily on anaerobic pathways for its energy requirements (31, 32). Molecular oxygen then, often an important substrate in the production of ATP, becomes a dangerous free radical to Giardia. Nevertheless, the parasite is still tolerant to oxygen environments. To protect itself from oxidative stress Giardia employs multiple flavoproteins, such NADH oxidase and flavodiiron, which use the electron donor NAD(P)H to reduce \( O_2 \) to water (33, 34).
1.3.2 Effects of Nitric Oxide

Nitric oxide (NO) is a free radical species that is cytotoxic at high concentrations (µM or higher) and is used as a signalling molecule at low concentrations (pM to nM). Giardia trophozoites are susceptible to nitric oxide killing (35). In most species, the cytotoxic effects of nitric oxide are direct and due primarily to its strong propensity to bind the open coordination sites of heme proteins. In particular, NO is known to complex with the heme groups of cytochrome c oxidases within the mitochondrial respiratory chain, hindering their function (4, 36). However, since Giardia does not possess a respiratory chain, another mechanism of action must be causing the cytotoxic effect within the parasite. Nitric oxide also forms several other reactive nitrogen species that can prove damaging. Nitrosoniun ion (NO+) bonds with the thiol side chains of cysteine residues to form S-nitroso compounds, which inhibit the function of proteins with active-site cysteine residues (4, 6). This includes glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a key glycolytic enzyme (4, 37). Likewise, peroxynitrite (ONOO−), formed by the quick reaction of nitric oxide with superoxide (O2−), binds to and disrupts iron-sulphur clusters, such as those found in the ferredoxin class of electron transfer proteins and in aconitase, a key enzyme of the citric acid cycle (4, 6, 38). The cellular damage caused by nitric oxide and the molecules derived from it is known collectively as nitrosative stress.

Giardia trophozoites encounter nitric oxide from a variety of sources within its host’s gastrointestinal tract. The most prominent is from the host’s immune system, which utilizes inducible nitric oxide synthase (iNOS) to produce cytotoxic concentrations of nitric oxide (4). Macrophages, found in Peyer’s patches within the duodenum and capable of engulfing Giardia, have been well-demonstrated to kill phagocytosed microbes
using nitric oxide derived from iNOS (4, 39). iNOS is also expressed in intestinal epithelial cells, the same cells to which Giardia trophozoites adhere (4, 40). Apart from the immune system, other sources of NO exposure include the contents of the host’s gut. Diets high in nitrite can be significant source of NO, as the molecule can be derived from nitrite under the acidic conditions of the stomach (4, 41).

In addition to the cytotoxic effects of nitric oxide, the signalling role that nitric oxide plays within the host can also prove harmful to the parasite. Nitric oxide produced by neuronal nitric oxide synthase (nNOS) is thought to stimulate gut motility, making it more difficult for Giardia trophozoites to adhere to the epithelial lining. As evidence, nNOS knockout mice are far less efficient in clearing Giardia infections (42).

1.3.3 Giardial Flavohemoglobin (gFlHb)

Though its method of heme acquisition remains unknown (given the aforementioned absence of any previously characterized heme synthesis/import mechanism), Giardia’s flavohemoglobin (designated gFlHb) seems to be functioning within the parasite, performing the same NO dioxygenation chemistry typically associated with other characterized flavohemoglobins. *In vitro* studies of the protein show higher rates of NADH oxidation in the presence of NO, consistent with dioxygenation activity, and Giardia trophozoites placed under nitrosative stress experienced a 5-fold increase in the level of gFlHb protein expression, a 10-fold increase in gFlHb mRNA levels, and were able to metabolize NO at higher efficiency (3, 4, 43). Also notable, gFlHb mRNA expression seems to be slightly increased as Giardia undergoes encystation (specifically in the 7-22 hour post induction interval) before dropping ~4-fold (when compared to trophozoites) in the final cyst form of the parasite (44, 45).
The primary amino acid sequence of gFlHb is most similar to flavohemoglobins found in enterobacterial hosts, conceivable given both reside in the gastrointestinal tract. gFlHb shares ~40% sequence identity with Hmp and ~30% sequence identity with yeast Yhb, the two most studied flavohemoglobins from a structural and functional standpoint (4). This includes the 12 residues invariant in the heme pockets of all known flavohemoglobins, suggesting gFlHb is utilizing the same catalytic mechanism as other flavohemoglobins. Figure 6 shows the sequence alignment of gFlHb, Hmp, and Yhb.

A notable and unique feature of gFlHb are two insertions, one on the flavin domain and one on the globin domain, each ~25 amino acids in length, not present in any known flavohemoglobins to date (3). While the crystal structure of gFlHb has not been solved, homology modelling using Hmp as the template structure indicates that these face each other in the contact surface between the globin and flavin domains (see Figure 7 for this homology model, with sequence insertions highlighted) (3). The true function of these insertions remain unknown, but it is common for Giardial proteins to have amino acid insertions when compared to homologs in other species (46). Deletion mutants on different proteins in the parasite indicate that these could be for stability purposes (46).

1.4 UV-Visible Spectroscopy

UV-Visible spectroscopy takes advantage of the elastic scattering of light (also known as Rayleigh scattering); an incident photon is emitted from a molecule at the same energy in which it was absorbed. UV-Visible spectra can be used to determine several features of heme proteins. In flavohemoglobins, the Soret band (found in all heme proteins, produced by the transition of an electron from a \( \pi \) orbital to a \( \pi^* \) orbital in the porphyrin ring of the heme group) is associated with the oxidation state, coordination
**Figure 6:** Sequence alignment of gFlHb, E. coli Hmp, and Yeast Yhb. The sequence insertions on gFlHb’s globin and flavin domains are highlighted in green and orange, respectively.

<table>
<thead>
<tr>
<th>gFlHb</th>
<th>MTLSEDTLRAVEATAGLIAAQGIEFTRAFYERMLTKNEELKNIFNLAHQRTLRQPKALLD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hmp</td>
<td>-MLAQTIATXKVAPIPLVTGKPTLKTBFYRDTHNPELEFKPMQNLVRNGQREALFN</td>
</tr>
<tr>
<td>Yhb</td>
<td>-MLAEKTRSIILVPEEQTVGTIRTFYKMMLTEHTELINIFNRTNQKVRQPNALAT</td>
</tr>
</tbody>
</table>

| gFlHb     | SVAYALNIKRINELYELKGLPGPPHENALKELQGFSAEERVKHTSGFQPAQYQIV      |
| Hmp       | AIAAYASNIE---------NLFAVPAKEIAKHTSGFQIKEQYQYNIV               |
| Yhb       | TVLAAAKND----------DLVIMDHVKIQGKHRLAQKIEPRHYPV                 |

| gFlHb     | GAHLIATDEIR---TKKDIKAEKAYQFLADFLIKREEXIYATEGCKGQWRQTTRFR     |
| Hmp       | GEHLIATLDEMP---SQQVNLWAKGKAYVLAVNWAINFREAEYINENASKGMQKETRDRF |
| Yhb       | GEYLLKAIKELFAQATPEIAIWAGPEYQYTVNTHLRQENYQDALARHYSLC           |

| gFlHb     | VEERKVRNIEICKFRLVPAESEQAGVV--EHRPQYQLAFVRS--FEHFOHQIQIRQSYII |
| Hmp       | IVATPRSALESFEPFQ---DGQAVA--EYRQPQYLVWKLNP--EGFFQIEIRQYSLT    |
| Yhb       | ITAKEYVAISDEFTVKPK--FGSGIEELSFITTPQYTVNTHLRQENYQDALAHYSLC    |

| gFlHb     | SAPNSAYEIAVHERDEK----------GTYSYLVHDVSTGDLLLEVAPPYGDFFLRYIDEAEQA |
| Hmp       | RKPDQYQRAIEVARER---------GQVQSNKLMHANVPGVVLAVAPAFDFM          |
| Yhb       | SAGVQGRLAYVNEAENFPAGLSVLYLHKDAKVLKQGAPAGFAYAF                 |

| gFlHb     | RQDSQEQPEQMQQGAINPFAEKMTMPVILISGQIQPPLLMLAFQLAQKECKETARPFF  |
| Hmp       | ---------AVA---------DDTVLISAGQQTPLMAMLDTLAX---------AGTHAQVN |
| Yhb       | SACHIQNEVEPLVLSSGVVTP.LLAMEEQVK----------CNPNRPIY             |

| gFlHb     | WIHAANHRSFRAFKEVEYDAIRETPSLRVTVEFSEVRATDREGDYFDPCHRJLLDRQSE |
| Hmp       | WPHABGEINSVHAFDACVEKELQGS-1RFHATMYQPSEADAKQFQSEQMDGAKLEG    |
| Yhb       | WQIQQYDEKTAQAFKKHVDKE----------LAECNVIKIIIVHTDEFLNAFLKE    |

| gFlHb     | LTKLEA-DSNAPYHFYFFGPTGFMATAVEQKLTKSFVNSRIHFMGFFKAS---------- |
| Hmp       | --------AFSDPTQYFLCGVFQMQPTAKQLVLGDVGQENIHYECGFPHKVL--------- |
| Yhb       | --------K--SPABADVYVCGSLAMQAMIGHKELEHRRDIMHYEPFGPRMSTVQV      |

**Figure 7:** Homology model of gFlHb based on the structure of E. coli Hmp, with sequence insertions mapped on. The globin domain highlighted in green and the flavin domain in orange.
state, spin state, and bound ligands of the heme iron (3, 47). Fully-oxidized flavin (FAD) in flavohemoglobins is also active in the UV-Visible range, typically producing a low shoulder between 460-480 nm. This shoulder is lost in the fully-reduced state by electrons accepted from NADH to form FADH₂; as mentioned previously these electrons are subsequently donated to reduce the heme iron which enables it to bind O₂ (3, 47).

1.5 Resonance Raman Spectroscopy

1.5.1 Theory

Raman spectroscopy takes advantage of the occasional inelastic scattering of light, where scattered light leaves the sample at energies different from the incident beam. When this scattered light is of a lower energy than the incident beam, it is known as Stokes scattering; when of a higher energy, it is called anti-Stokes scattering. This small shift in the energy of the scattered light is attributed to a change in the vibrational state of a molecule. For instance, a molecule in a low energy vibrational ground state may absorb a photon and transition to a higher energy state, yet return to a higher energy ground state when the incident photon is emitted (Stokes scattering). The difference in energies of these two vibrational states correspond to different bending and stretching modes within the molecule, which fall exclusively in the infrared region of the electromagnetic spectrum (48).

Instances of these transitions however, are infrequent. Resonance Raman spectroscopy amplifies the effect by applying an incident laser at an energy of a well-known electronic transition. This has the added benefit of restricting the spectrum generated to a specific portion of the protein, a particularly useful approach for characterizing heme proteins. In this case, tuning the frequency of the laser to the
electronic transition associated with the heme group (the Soret band) focuses the spectrum towards this region of the molecule, allowing for detailed analysis of the active site of heme proteins (48, 49).

The high frequency region (1200-1700 cm\(^{-1}\)) of resonance Raman spectra contains modes susceptible to the oxidation, spin, and coordination states of the heme iron, as well as the stretching modes of heme vinyl and propionate groups, providing a useful probe of the local environment around the heme iron. The low frequency region (200-800 cm\(^{-1}\)) contains both the bending modes of heme vinyl and propionate groups and the stretching and bending modes of different iron-ligand bonds (both exogenous ligands and amino acid side chains provided by the protein that act as ligands), providing a direct probe of the binding properties of an enzyme (22, 49).

Typically, in resonance Raman experiments conducted on heme proteins, carbon monoxide (CO) is used as the probing ligand due to its high affinity to heme and sensitivity to steric and electrostatic interactions with nearby residues. Isotopic substitution is used to identify which spectral signals belong to CO, as the stretching modes of the Fe-CO and C-O bonds are both sensitive to atomic mass. In the low frequency (200-800 cm\(^{-1}\)) and very high frequency (1700-2200 cm\(^{-1}\)) regions, increasing the mass of CO with heavier isotopes shifts the Fe-CO and C-O lines, respectively, to lower energies, indicating which peak(s) is/are attributable to the ligand (49). In addition, the Fe-CO and C-O stretching modes fall along a well-known correlation line, whereby stronger Fe-CO bonds lead to weaker C-O bonds (19). This allows both modes to corroborate the other.
1.5.2 Resonance Raman on gFlHb

Overall, gFlHb retains similar spectral features to that of Hmp, the only other flavohemoglobin previously characterized using resonance Raman spectroscopy (19, 50). Figure 8 provides the high frequency spectra of ferric and ferrous gFlHb. The spin and coordination sensitive $\nu_2$ and $\nu_3$ markers indicate that each are 5-coordinate high spin species, consistent with a peroxidase-like proximal histidine discouraging the binding of weak distal ligands like water. The $\nu_4$ marker shifts from 1371 cm$^{-1}$ in the ferric spectrum to 1354 cm$^{-1}$ in the ferrous, an expected sign of higher electron density around the iron.

Figure 9 provides the low frequency spectra of the ferric and ferrous forms. Notable is the double peak in the ferrous $\nu_{Fe-His}$ marker (the iron-histidine stretch) at 235 cm$^{-1}$ and 257 cm$^{-1}$. This is a departure from Hmp, which had a single strong line at 244 cm$^{-1}$. The 244 cm$^{-1}$ stretching mode in Hmp and 257 cm$^{-1}$ stretching mode in gFlHb are consistent with the previously mentioned His-Glu-Tyr proximal hydrogen bonding network that gives the histidine ligand a strong imidazolate character (see Figure 3). The 235 cm$^{-1}$ line, unique to gFlHb, is likely attributable to a phenomenon similar to that found in type I/II peroxidases. In these proteins, tautomers form based on whether the shared hydrogen of the proximal histidine ligand and a nearby aspartic acid residue is closer to the histidine ($\nu_{Fe-His}$ of 230 cm$^{-1}$) or the aspartic acid (presumably glutamate-157 in gFlHb, with a $\nu_{Fe-His}$ near 240 cm$^{-1}$). The slight variation between gFlHb and Hmp suggests that there may be some difference in the geometry or protein dynamics of their proximal heme pockets (20, 50). It should be noted that the Fe-His stretch is not present in the ferric form. The reason for this is unknown, but is consistent across a range of histidine-coordinated hemeproteins. This may be caused in part by the wavelength of
Figure 8: High frequency resonance Raman spectra for the Fe(II), Fe(III), Fe(II)-CO and Fe(II)-O₂ complexes of wild type gFlHb. The excitation wavelength was 413.133 nm for the Fe(II), Fe(III), and Fe(II)-O₂ complexes and 441.565 nm for the Fe(II)-CO complex.

Figure 9: Low frequency resonance Raman spectra of the Fe(II) (413.133 nm excitation wavelength) and Fe(III) (441.565 nm excitation wavelength) complexes of wild type gFlHb.
laser used to excite the sample, or by the location of the iron atom relative to the
porphyrin plane (it is pulled towards the histidine in the ferrous form, perhaps making it
easier to detect).

Figure 8 also gives the high frequency ferrous CO spectrum. The Fe(II)-CO
complex is in the 6-coordinate low spin state, as indicated by ν3 and ν4 markers of 1372
cm\(^{-1}\) and 1500 cm\(^{-1}\). This is once again consistent with Raman spectra of Hmp (19).

Figure 10 gives the low and very high frequency spectra for the isotopic substitution of
CO in ferrous gFlHb. The low frequency spectrum has two lines attributable to the Fe-
CO stretch at 490 cm\(^{-1}\) and 541 cm\(^{-1}\), with corresponding C-O stretches in the very high
frequency region at 1964 cm\(^{-1}\) and 1910 cm\(^{-1}\), respectively. This falls along the typical
correlation line of Fe-CO and C-O stretches and is once more in line with that found in
Hmp (19). These lines, when compared to the Raman spectra of previously characterized
globins, form the experimental basis (along with similar stretches in Hmp) for the
aforementioned open and closed conformations proposed in flavohemoglobins, with the
490/1964 cm\(^{-1}\) stretches representing the open conformation and the 541/1910 cm\(^{-1}\)
stretches the closed conformation (50).

Finally, the high and low frequency Fe(II)-O\(_2\) spectra are given in Figures 8 and
11, respectively. The ν3 and ν4 markers of 1377 cm\(^{-1}\) and 1506 cm\(^{-1}\) in the high frequency
spectrum show that the O\(_2\) bound complex, much like the CO bound complex, is 6-
coordinate low spin. The 6 cm\(^{-1}\) upshift from the ferric form is consistent with the binding
of a strong π-acceptor ligand. Isotopic substitution in the low frequency region revealed a
single Fe-OO stretch at 549 cm\(^{-1}\). This is consistent with the O\(_2\) bound states of truncated
hemoglobins and other globins possessing extensive hydrogen bond networks to the
Figure 10: Low frequency (A) and very high frequency (B) resonance Raman spectra of the wild type gFILHb Fe(II)-CO complex, obtained using 413.133 nm excitation wavelength. $^{12}$C$^{16}$O minus $^{13}$C$^{18}$O difference spectra are included at each frequency region.
**Figure 11**: Low frequency resonance Raman spectra of Fe(II)-O\textsubscript{2} complex, obtained using a 413.133 nm excitation wavelength. A \textsuperscript{16}O\textsubscript{2} minus \textsuperscript{18}O\textsubscript{2} difference spectrum is also included.
ligand, again suggesting that distal residues are interacting with the bound substrate (50).
The lack of an open state conformation for the O$_2$ complex does not have a formal explanation, however the binding geometries differ between CO and O$_2$, with the former binding the heme iron in a linear geometry and the latter in a bent geometry (49).
Likewise, the terminal oxygen in O$_2$ is known to carry a partial negative charge, while the terminal oxygen in CO does not. As such, it is likely that the binding process in flavohemoglobins is optimized for O$_2$ (or NO, which also binds in a bent geometry), which is why two conformations can be observed when a non-physiological ligand like CO is bound, but not so with a functional ligand like O$_2$, at least under the timescales being probed (50).

1.6 Proposed Project and Rationale

The goal of my research was to produce and characterize two mutants of gFlHb: one with tyrosine-30 (corresponding to the B10 tyrosine) mutated to phenylalanine (Y30F) and the other with leucine-58 (corresponding to the E11 leucine) mutated to alanine (L58A). Phenylalanine was chosen to replace tyrosine because it maintains the aromatic group of the residue while removing its ability to hydrogen bond. Alanine was chosen to replace leucine because it removes the functional group entirely. Figure 12 gives a picture of these mutations. By making each residue non-functional I hoped to gauge their roles within the protein. First, by characterizing their spectral features using UV-Visible and resonance Raman spectroscopy, and second, by measuring their reaction rates with respect to NADH oxidation, which can be followed by UV-Visible spectroscopy. While enzyme rate measurements on an Hmp mutant at the B10 position (in which phenylalanine replaces tyrosine) have been reported, resonance Raman
Figure 12: Mutations made in the Y30F and L58A variants, in comparison to wild type gFlHb.
spectroscopy has not yet been done on any such mutant flavohemoglobin. Resonance Raman spectroscopy provides a direct probe into the binding properties of a ligand and thus can be used to determine the exact effect the residue has on ligand binding. Furthermore, the E11 leucine has never been studied from a mutational standpoint. Resonance Raman spectroscopy, the technique used originally to propose the conformational changes that accompany catalysis in flavohemoglobins, is thus well suited to study the influence of this residue. The rate properties of each mutant were also measured to determine how each mutated residue effects catalysis, by monitoring the rate of NADH oxidation in the absence and presence of an NO-donor compound.

A secondary objective included characterizing the steady-state spectra of gFlHb and the aforementioned mutants during catalysis in order to test the dioxygenation model vs. the nitrosylation model. Given the sensitivity of UV-Visible spectra to the heme bound ligand, acquiring a spectrum under reaction conditions can determine whether the protein is in the O₂ or NO bound states during catalysis and, subsequently, whether mutation can affect that selectivity. Finally, I set out to measure superoxide production in gFlHb when NO is absent from the reaction, and furthermore, whether ligand stabilization from the B10 tyrosine or the conformational attributes proposed to be imparted by the E11 leucine effect those rates.
2. MATERIALS AND METHODS

2.1 Mutant Vector Construction

2.1.1 Overview

Mutant vectors were constructed using a previously constructed pJ401 expression vector containing the gFlHb gene (DNA 2.0, Menlo Park, CA). Figure 13 provides a visual description of this process. First, a segment of the wild type gene (containing the codons of the Y30 and L58 residues desired for mutation) was excised from the plasmid. The pJ401 vector possesses single cut sites for restriction enzymes NdeI and BglII that flank both of the desired mutation sites. NdeI/BglII digests of this vector resulted in a 544 bp fragment that left a 4832 bp backbone containing the spine of the pJ401 vector and the remnants of the gFlHb wild type gene (referred to henceforth as the pJ401 backbone). DNA inserts containing the mutated sequences (Tyr30→Phe and Leu58→Ala) were ordered from Bio Basic Inc., pre-cloned into pBluescript II SK+ ampicillin resistant vectors. These insert vectors possessed NdeI/BglII cut sites like that of the wild type pJ401 vector and digests using these restriction enzymes resulted in a 481 bp fragment that could be ligated into the previously cut pJ401 backbone. The difference in the size of the pJ401 excision (544 bp) and the insert fragments (481 bp) arose because the inserts did not incorporate a 21 amino acid polyhistidine-tag that was present in the wild type gene within the pJ401 vector. The ligation of the 4832 bp pJ401 backbone and the 481 bp fragment resulted in the 5316 bp mutant vectors used.
**Figure 13:** A description of the process used to construct the Y30F and L58A vectors. Both a wild type pJ401 vector containing the wild type gene and a pBluescript II SK+ vector containing an insert with one of the two desired mutations were digested using restriction enzymes NdeI and BglII. The 481 bp inserts were ligated into the 4832 pJ401 backbone using T4 DNA ligase to produce the final 5316 bp mutant vector.
2.1.2 Vector Cloning

To increase the quantity of plasmid available, the wild type gFlHb pJ401 vector and pBluescript II SK+ mutant vectors were transformed into HB101 *E. coli* cells and grown overnight at 37 °C on LB agar plates (with the appropriate antibiotic present; kanamycin for the pJ401 vectors and ampicillin for the pBluescript II SK+ vector). Colonies of the transformed bacteria were then used to inoculate 10 mL of LB media (with appropriate antibiotic) and grown overnight at 37 °C. Cells were harvested by centrifuging at 3000 g for 10 minutes. The plasmid DNA was extracted and purified using a Qiagen QIAprep Spin Miniprep kit and stored in a 10 mM Tris-HCl, pH 8.5 buffer. Plasmid DNA concentrations were estimated by measuring the absorbance at 260 nm on a Thermo Fisher Nanodrop spectrophotometer.

2.1.3 Isolation of pJ401 gFlHb Vector Backbone and Mutant Inserts

To ensure the complete digestion of the wild type pJ401 plasmid, NdeI/BglII digests were performed sequentially, one restriction enzyme at a time, allowing for the veracity of each cut to be confirmed by agarose gel electrophoresis before proceeding with the next step. This was of particular importance for the pJ401 vector, as the close relative size of the single cut plasmid to that of the double cut made them difficult to discern from each other. The single cut pJ401 vector recloses back onto itself at much higher efficiency than the insert ligates into the double cut backbone (the former a first-order reaction and the latter a second-order process), which can cause false positives when each are transformed into cloning strain bacteria, as both possess the same type of antibiotic resistance (and express similar looking protein). Since it had been established to work with the pJ401 vector, the same sequential digestion was used with the insert
vector (though cuts were not confirmed after each digestion, as the insert is easy to differentiate from the backbone).

NEBuffer 3.1 (50 mM Tris-HCl, 100 mM NaCl, 10 mM MgCl₂, 100 μg/mL BSA, pH 7.9) from New England Biolabs was used for digestions as both restriction enzymes were predicted to have 100% activity within the buffer. Plasmids were first incubated overnight at 37 °C in 70 μL solutions containing NEBuffer 3.1, 30 units of NdeI (0.43 units/µL), and 5 μg of the DNA to be cut (10 μg for the Y30F insert). An 8 µL addition containing 15 units of BglII (0.19 units/µL) and 10 units of NdeI (in case of any remaining uncut plasmid) was added the next day and the combined solution was again incubated overnight at 37 °C. Restriction enzyme digests should typically take place over much shorter time frames (usually 2 hours to start), to decrease the chance of non-specific cutting. In this case, longer incubations were utilized because previous experiments indicated that increasing the digestion time gave more complete cuts without intensifying the amount of nuclease or star activity. It should also be noted that sequential digestion is usually only appropriate if checking the success of the first restriction enzyme via agarose gel before adding the second. If this technique is not being used, a concurrent digestion (both restriction enzymes added simultaneously) would be recommended, as this decreases the incubation time and thus reduces the chance of non-specific cuts taking place, as discussed previously.

The resulting digests were run on a 100 mL 0.8% TBE agarose gel cast with the UV-active DNA-binding dye ethidium bromide (1 μg/µL) overnight at 15-25 V (Figure 14 gives the results of a typical gel used for excision). The low voltages/longer run times proved key, as they improved band separation and increased the accuracy of fragment
Figure 14: 0.8% TBE agarose gel showing the overnight digest of a wild type gFlHb pJ401 vector using the restriction enzymes NdeI and BglII. Lane 2 (NdeI Cut) shows the vector with a single cut, for reference to the double cut vector in lane 3 (NdeI and BglII Cut). The red square indicates the DNA extracted for ligation using a Qiagen gel extraction kit. A similar procedure was used to extract the mutant inserts from pBluescriptII SK+ vectors.
size compared to the DNA ladder used as the size marker (NEB 2-log DNA ladder). Bands corresponding to the 4832 bp pJ401 backbone and the 481 bp Y30F and L58A inserts were excised over a UV-box and purified using a Qiagen QIAQuick Gel Extraction Kit, with the products stored in 10 mM Tris-HCl, pH 8.5 buffer. DNA concentrations were estimated by measuring the $A_{260}$ on the Nanodrop spectrophotometer.

2.1.4 Dephosphorylation of the pJ401 Backbone

DNA ligase requires a 5’ phosphate on the terminal nucleotides of DNA fragments to complete a ligation. To ensure, as previously mentioned, that none of the pJ401 backbone recloses onto itself (as would occur if there were any single cut plasmid, which would have compatible cohesive ends, remaining) a dephosphorylation of the pJ401 backbone was conducted. With the backbone lacking any phosphate groups and the inserts maintaining them, ligation would only occur between the insert and the backbone reducing the number of false positives on transformation of the ligation product. Shrimp alkaline phosphatase (SAP) was used to complete this reaction. A 50 µL reaction mixture containing ~300 ng of the pJ401 backbone and 0.5 units of SAP (0.01 units/µL) in NEBuffer 3.1 was placed in a Techne Progene DNA Thermal Cycler and run at 37 °C for 1 hour, 65 °C for 20 minutes, and 25 °C for 15 minutes to complete the reaction. The use of SAP was significant. Prior to this, calf intestinal alkaline phosphatase (CIP) was used, which could not be inactivated by heating to 65 °C and not only required a second purification step to remove, but also did not work effectively; control transformations using CIP treated pJ401 backbone alone (i.e. without an added insert fragment) produced
several colonies on selective plates, indicating single cut plasmid had likely reformed on itself.

2.1.5 Ligation of Mutant Inserts into the pJ401 Backbone

As noted above, two ligation reactions were performed for each mutant; one reaction containing the pJ401 backbone and either the Y30F or L58A insert, and one control reaction containing the pJ401 backbone alone. New England Biolabs T4 DNA Ligase Reaction Buffer (50 mM Tris-HCl, 10 mM MgCl₂, 1 mM ATP, 10 mM DTT, pH 7.5) was used in each reaction. Reaction mixtures were 50 µL in volume and consisted of the insert and pJ401 backbone in a 5:1 stoichiometric ratio (~70 ng backbone to ~40 ng of insert) and 20 units of T4 DNA Ligase (0.40 units/µL). The control reaction omitted the mutant insert. Reaction mixtures were incubated overnight at 16 °C and then heat inactivated at 65 °C for 10 minutes.

2.1.6 Transformation of Ligated DNA into Chemically Competent DH5α E. coli

As with the ligation, two transformation were conducted for each mutant. The first, hereafter referred to as the mutant transformation, used the ligation products of the pJ401 backbone and mutant inserts. The second, hereafter referred to as the control transformation, used the ligation products of the pJ401 backbone alone. If a colony were to grow on a control plate, this would indicate the steps taken previously to prevent the backbone from reclosing on itself had failed and any colonies on the mutant plate would be called into question.

Ligation products were transformed into freshly prepared chemically competent DH5α E. coli cells. Two other E. coli strains were tried prior to this; HB101 grown in lab
and JM109 ordered from Promega. HB101 did not produce any colonies in any of the transformations. JM109 had a single successful transformation, but produced several colonies on the control plate and did not have a successful transformation thereafter. As such, DH5α is highly recommended as the cloning strain when transforming with DNA that comes from a ligation mixture.

Frozen 100 μL DH5α cell cultures were thawed on ice (typically taking 5 to 10 minutes). To the bottom of a pre-chilled 17 x 100 mm sterile polypropylene culture tube (not a microcentrifuge tube), 10 μL of mutant or control DNA was added (amounting to ~40 ng of DNA for the Y30F plasmid and control and ~30 ng for the L58A plasmid and control). The 100 μL DH5α cultures were then gently added to the culture tube using chilled pipette tips, without mixing. Cultures were subsequently incubated for 10 minutes on ice, heat shocked for 45 seconds in a 42 ºC water bath, and placed on ice again for 2 minutes. The transformed bacteria were then supplemented with 900 μL of LB media and incubated in a shaker for 1 hour to recover. Afterward, 100 μL of the recovered cells were plated onto a 50 μg/μL LB+kanamycin plate and incubated overnight at 37 ºC. The 900 μL remaining in each culture was centrifuged and resuspended in 100 μL of LB, then spread across a second LB+kanamycin plates and incubated overnight at 37 ºC.

2.1.7 Colony PCR Screening for Mutant Transformations

Colony PCR screening was used to verify that transformants were consistent with the pJ401 mutant vectors and that no truncations had occurred. Colonies from the mutant transformations were streaked onto a master plate and grown overnight at 37 ºC. Sterile toothpicks were used to inoculate 10 μL LB+kanamycin (50 μg/μL) in small PCR tubes with samples of the clones from the master plate, and were grown at 37 ºC in a thermal
cycler for 1 hour. From each culture, 1 µL of bacterial solution was added to a 49 µL PCR mix, detailed in Table 1.

Table 1: PCR mix used for colony PCR screening.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>dH₂O</td>
<td>35.98</td>
</tr>
<tr>
<td>10x PCR buffer</td>
<td>5.00</td>
</tr>
<tr>
<td>25 mM MgCl₂</td>
<td>6.00</td>
</tr>
<tr>
<td>*5’ Sense primer (11.82 µM)</td>
<td>1.06</td>
</tr>
<tr>
<td>*3’ Antisense primer (22.33 µM)</td>
<td>0.56</td>
</tr>
<tr>
<td>dNTPs (25 mM each)</td>
<td>0.20</td>
</tr>
<tr>
<td>Taq polymerase (BioShop)</td>
<td>0.20</td>
</tr>
<tr>
<td>Total</td>
<td>49.00</td>
</tr>
</tbody>
</table>

The PCR reaction was conducted in the same thermal cycler according to the parameters in Table 2.

Table 2: Duration and temperature in each step of the colony PCR screening.

<table>
<thead>
<tr>
<th>Step</th>
<th>Segment</th>
<th>Temperature (°C)</th>
<th>Duration</th>
<th># of Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>94</td>
<td>7 min</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>94</td>
<td>15 sec</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>53</td>
<td>30 sec</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>72</td>
<td>90 sec</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>4</td>
<td>HOLD</td>
<td>—</td>
</tr>
</tbody>
</table>

The 5’ sense (TGTCGCCCTTAATTGTGACG) and 3’ antisense (TGGTAGTGTGGGGACTC) primers used in the PCR mix were both located on the pJ401 vector backbone flanking the mutated gene site and amplified a hypothetical 1681 bp fragment (the bottom of Figure 13 gives the approximate locations). PCR products were run on a 0.8% TBE agarose gel along with a negative control containing the PCR mix without an added bacterial solution. A typical gel is shown in Figure 15. Based on the results of these gels, 3 colonies of proper size were chosen for each mutant. Colonies
Figure 15: 0.8% TBE agarose gel showing the PCR products from a colony PCR screening protocol of DH5α E. coli cells transformed with suspected gFIHb Y30F mutant pJ401 vector. The negative control consists of the PCR mix without an added bacterial colony.
were grown and plasmid purified using a slightly modified version of the method previously described in 2.1.2.

2.1.8 Sequencing of Mutant Vectors

The mutant vectors were sequenced at Mobix Labs (McMaster University) using the Sanger method. Each of the selected colonies were tested using both the 5’ sense and 3’ antisense primers utilized in the colony PCR screening, for double verification. Sequences were edited using CodonCode Aligner Software. All but one of the plasmids from the selected colonies possessed the desired mutation. The failed vector had a shortened read and thus it could not be determined if the mutation was present.

2.2 Expression and Purification of Y30F and L58A Mutants

2.2.1 Expression of Mutant Vectors

Mutant vectors were transformed into chemically competent BL21 E. coli cells in a modified version of the process described in 2.1.6. In this less stringent case, DNA was added directly to the BL21 cells and the transformation was conducted directly in the 1.5 mL screw cap tubes the cultures were frozen in. To each culture, 1 µL of the corresponding kanamycin resistant pJ401 mutant vector was added (amounting to 186 ng of the Y30F plasmid and 307 ng of the L58A plasmid). Cultures were incubated for 30 minutes on ice instead of 10 minutes before being placed in the 42 °C water bath. Finally, only a single 100 µL aliquot of the recovered cells were plated onto the 50 µg/µL LB+kanamycin plates and incubated overnight at 37 °C.

A single colony of the plated bacteria was selected and used to inoculate a 4 mL culture of 50 µg/µL TB+kanamycin media, which was incubated in a shaker overnight at
220 rpm and 37 °C. This starter culture was then added to a 2.8 L Fernbach culture flask containing 1 L of 50 µg/µL TB+kanamycin media and grown at 37 °C until it reached an optical density (absorbance at 600 nm) of 0.4-0.6. At this point, IPTG was added to a concentration of 0.1 mM to induce protein expression. The culture flask was incubated in a shaker overnight at 30 °C. Cells were pelleted at 6084 g for 30 minutes, collected, weighed, and frozen at -80 °C.

2.2.2 Lysis of Cell Pellets

Cell pellets were thawed at room temperature and resuspended in 4-5 volumes (v/w) of a 1x PBS buffer with 1 mM TCEP, 1 mg/mL lysozyme, 0.1% Triton X-100, 0.1 mM captopril, and a few flakes of added PMSF. The resulting cell suspension was mixed at 4 °C for 20-30 minutes until the mixture was homogenous with a viscous consistency. To lower the viscosity of the solution, 2.5 mM MgCl₂, 0.5 mM CaCl₂, 0.02 mg/mL RNase A, and 0.01 mg/mL DNase were added and mixed at 4 °C for another 30 minutes. The cell suspension was centrifuged at 17,210 g for 25 minutes to remove cellular debris. The resulting lysate was red in colour.

2.2.3 Ammonium Sulphate Precipitation

Ammonium sulphate was added slowly to the collected lysate over the course of 15 minutes, until 35% (w/v) saturation was reached, and then the solution was gently mixed for 20 minutes on a platform rocker to ensure the precipitation had occurred in full. The resulting solution was centrifuged at 4,200 g for 15 minutes and the supernatant collected. The concentration of ammonium sulphate within the collected supernatant was
subsequently brought up to 60% (w/v) and the remaining lysate centrifuged again at 4,200 g for 15 minutes. The resulting pellet was collected and supernatant discarded.

2.2.4 Protein Desalting

An 85 mL column of Bio-Gel P-6DG desalting resin was equilibrated with 3-5 column volumes of 20 mM Tris-HCl, pH 8.5 buffer. The protein pellet was resuspended in 5 mL of the same equilibration buffer (with a small amount of potassium ferricyanide added to track the position of the salt) and run through the column (with more equilibration buffer) to remove any remnant salt from the lysis and ammonium sulphate precipitations. The resulting eluate was centrifuged at 4,200 g for 30 minutes to remove any debris.

2.2.5 Anion Exchange Chromatography

Anion exchange chromatography was carried out using a 5 mL Bio-Scale Mini Macro-Prep High Q cartridge. Both the Y30F and L58A mutants have a weak association with the resin at pH 8.5. As such, the column was equilibrated with 5-10 column volumes of 20 mM Tris-HCl, pH 8.5 equilibration buffer, at a flow rate of 2-3 mL/min (the flow rate used in all steps of the process). The protein solution was then added and washed with equilibration buffer until the eluate was clear.

Due to their weak associations with the resin, the mutants themselves would occasionally elute without binding to the column. In these cases, the column was washed with the elution buffer (20 mM Tris-HCl, 500 mM NaCl, pH 8.5), re-equilibrated, and the mutant solutions added to the column a second time. Removing the proteins that bind the
resin at high affinity during the first addition seems to allow the mutants to associate upon the second addition.

In either case, a step-wise elution was used to separate each mutant from the column. The percentage of elution buffer was gradually increased with respect to equilibration buffer at increments of 5% to 10% (v/v) (using 10% increments seemed to increase the yield of protein). During each step, 6 mL fractions were collected until the eluate was clear. The purified mutants themselves were tracked using their red colour, with the reddest fractions being collected and used for SDS-PAGE. Typically, the mutants eluted at concentrations of elution buffer between 10-30% (50-150 mM NaCl).

2.2.6 Assessment of Purity Using SDS-PAGE

Collected mutant samples were diluted 20x and mixed with SDS loading dye. Each sample was heated at 90 °C for 5 minutes and pulse centrifuged to remove any condensation within the centrifuge tube. Samples were then loaded onto a 10% polyacrylamide gel (15 µL per lane) submerged in a 1x Tris-Glycine buffer. Electrophoresis was run at 150 volts for ~1 hour. The resulting gel was stained using Coomassie Brilliant Blue dye and destained overnight in water. Figure 16 gives an example of the typical purity obtained.

2.2.7 Oxidation of the Heme Group and Buffer Exchange

Mutants were oxidized to their ferric forms by adding a small spatula scoop of potassium ferricyanide and incubating at room temperature for 1 hour. The oxidized proteins were exchanged into a 20 mM Tris-HCl, pH 7.5 buffer using Amicon Ultra 15 mL Centrifugal Filters (30,000 molecular weight cut off) by centrifuging at 4,200 g until
Figure 16: SDS PAGE (10%) performed on samples of an L58A mutant of gFlHb having undergone a stepwise elution using a Q-Trap anion exchange column. Buffer A is 20 mM Tris-Hcl. Sample lanes are expressed in percentage of Buffer B (20 mM Tris-HCl, pH 8.5, 500 mM NaCl). Each fraction is 6 mL in volume. Samples are diluted 20x in each lane. The red square indicates the collected fractions.
the protein was concentrated to a volume of ~1 mL and then replacing with exchange buffer 3-4 times. As a consequence, this also removes the potassium ferricyanide and the residual NaCl from the elution buffer used during the anion exchange. Concentrations were determined on a Cary 400 Bio UV-Visible spectrophotometer using Beer-Lambert law by measuring the Soret peak of the ferric protein (assuming an extinction coefficient identical to that of the wild type, previously determined at 99.1 mM$^{-1}$ cm$^{-1}$).

2.3 Expression and Purification of Wild Type gFlHb

2.3.1 Immobilized Metal Affinity Chromatography

Wild type gFlHb was grown in BL21(DE3) E. coli cells as outlined in 2.2.1 and lysed using the method in 2.2.2. In this case, a pET-14b vector containing the wild type gene (DNA 2.0, Menlo Park, CA) was used in place of the pJ401 vector mentioned previously. Given that this vector confers ampicillin resistance, 100 µg/µL ampicillin was substituted for kanamycin. The wild type protein was purified using immobilized metal affinity chromatography to take advantage of the polyhistidine tag on its N-terminus. A 5 mL nickel was equilibrated with 25 mL of 50 mM Tris-HCl, 500 mM NaCl, pH 7.5 at a flow rate of 3 mL/min (used throughout). The crude lysate was added to the column and then washed with 25 mL of equilibration buffer and 25 mL of wash buffer (50 mM Tris-HCl, 500 mM NaCl, 25 mM imidazole, pH 7.5). The purified wild type protein was then eluted with 50 mM Tris-HCl, 500 mM NaCl, 250 mM imidazole, pH 7.5. The purity of the protein was verified using SDS-PAGE in a similar process to that described in 2.2.6.
2.3.2 Protein Oxidation and Desalting

The eluted protein was concentrated using an Amicon Ultra 15 mL Centrifugal Filter (30,000 molecular weight cut off) to a volume of ~1 mL and oxidized using potassium ferricyanide as detailed in 2.2.7. The protein was then exchanged into 50 mM Tris-HCl, pH 7.5 buffer using a 10 mL column of Bio-Gel P-6DG desalting resin (equilibrated with 5-10 columns volumes of the exchange buffer). Concentrations were once again estimated using Beer-Lambert law as described in 2.2.7.

2.4 Determination of Heme/FAD content and Stoichiometry

2.4.1 Bradford Assay

For the purposes of measuring heme and FAD equivalents, the concentrations of gFlHb and mutants were determined by Bradford assay. A standard curve was generated with 1.2 mL solutions of bovine serum albumin (at 0, 2, 4, 8, and 16 µg intervals from a 0.1 mg/mL stock solution), Bradford reagent (1 mL), and water. Standards were measured on a Cary 400 Bio UV-Visible spectrophotometer at 595 nm using disposable plastic cuvettes. Proteins were then diluted (20x for the wild type and Y30F mutant; 4x for the L58A mutant) and measured in similar 1.2 mL solutions to that of the standard (replacing BSA). The mass/absorbance relationship derived from the standard curve was used to solve for protein concentration (assuming a molecular weight of 52 kDa for each).

2.4.2 Pyridine Hemochrome Assay

The heme content of wildtype and mutant gFlHb was determined using the pyridine hemochrome assay (51). In basic solutions, heme proteins are denatured,
releasing heme, which binds two pyridine molecules as axial ligands. This pyridine-heme complex has distinctive peaks at 524 nm and 556 nm that allow for the concentration of heme to be determined (this assumes that all heme is protein-bound).

Each assay consisted of 500 µL of Reagent A (1 mL of 1 M NaOH, 2.5 mL of pyridine, and 1.5 mL of water) and 500 µL of 20 mM Tris-HCl, pH 7.5 (with the protein being measured), reduced with a few granules of sodium dithionite in a stoppered cuvette. The UV-Visible spectrum was measured between 500-700 nm and the heme concentration determined from the difference in absorbance at 556 nm and 540 nm \( A_{556-540} \) using an extinction coefficient of 22.1 mM\(^{-1}\) cm\(^{-1}\). Heme equivalents were determined by dividing the concentration of Heme by the concentration of protein determined by Bradford assay.

2.4.3 Determination of FAD Content

FAD was released by boiling protein samples at 100 °C for 3 minutes. Samples were then placed on ice for 3 minutes and centrifuged at 16,100 g for an additional 3 minutes, to remove protein debris. The supernatant of the solution was then measured in a 1 mL quartz cuvette with the spectrometer. FAD content was determined using the extinction coefficient of FAD at 450 nm (11.3 mM\(^{-1}\) cm\(^{-1}\)). FAD equivalents were determined by dividing the concentration of FAD by the concentration of protein determined by Bradford assay (3).
2.5 UV-Visible Spectroscopy

2.5.1 UV-Visible Spectra of Wild Type and Mutant gFlHb Complexes

UV-Visible spectra were measured on a Cary 400 Bio UV-Visible spectrophotometer (used throughout in all UV-Visible spectroscopic experiments) from 250-700 nm. Samples were run at a volume of 1 mL in a quartz cuvette of similar volume. A baseline of 20 mM Tris-HCl, pH 7.5, the buffer in which each sample was run, was subtracted from all spectra. Protein concentrations varied from ~4-6 µM. When applicable (i.e. the deoxy Fe(II) and Fe(II)-NO spectra), oxygen was removed from the sample buffer by running a gentle stream of argon through the liquid in a Parafilm-sealed container for 15 minutes. NO donor DEA-NONOate (Cayman Chemical) was used to generate the molecule. Table 3 gives the methods used to obtain each spectra.

Table 3: Method used to obtain each UV-Visible gFlHb complex.

<table>
<thead>
<tr>
<th>Complex</th>
<th>Method Used</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe(III)</td>
<td>Oxidized as described previously in 2.2.7.</td>
</tr>
<tr>
<td>Fe(III)-imidazole</td>
<td>250 mM imidazole was added to the ferric protein.</td>
</tr>
<tr>
<td>Fe(II) deoxy</td>
<td>A few granules of sodium dithionite were added directly into a cuvette containing deoxygenated buffer/protein to reduce the sample. The cuvette was then immediately stoppered.</td>
</tr>
<tr>
<td>Fe(II)-O2</td>
<td>100 µM NADH was added to a sample of the ferric protein (the Fe(II)-O2 complex was continuously being turned over in this case).</td>
</tr>
<tr>
<td>Fe(III)-NO</td>
<td>250 µM DEA-NONOate was added to the ferric protein.</td>
</tr>
<tr>
<td>Fe(II)-NO</td>
<td>A few small scoops of sodium dithionite was added directly to the sealed container of deoxygenated buffer. The buffer was then added to the cuvette along with the sample (thus reducing the protein) and 250 µM DEA-NONOate. The cuvette was then immediately stoppered.</td>
</tr>
</tbody>
</table>
2.5.2 Steady-State Spectra of NO Catalysis

A steady-state spectrum of NO catalysis was measured between 250-700 nm. Each assay was 1 mL in volume (measured in a quartz cuvette of similar volume) and contained 20 mM Tris-HCl, pH 7.5, 250 µM NADH, 1 µM FAD, and 25 or 100 µM DEA-NONOate. A steady-state spectrum free of DEA-NONOate (an Fe(II)-O₂ spectrum similar to that in 2.5.1) was also taken for comparison purposes. Protein concentrations were ~4-6 µM in each assay. Solutions were pre-incubated at 25 °C in the temperature controlled block of the spectrometer for 10 minutes prior to the addition of substrates NADH and DEA-NONOate and maintained at that temperature throughout the experiment. Spectra were measured immediately after substrate addition and mixing.

2.6 Resonance Raman Spectroscopy

2.6.1 Equipment and Calibration

Resonance-Raman spectroscopy was done at the laboratory of Professor Manon Couture, Department of Microbiology and Biochemistry, University of Laval, Quebec, QC. Wild type and mutant gFlHb were sealed into custom glass cuvettes at concentrations of ~50 µM. Samples were excited using the 413.133 nm line of a Kr-ion laser (Coherent, Santa Clara, CA) in the majority of redox and ligand-bound states. The 441.565 nm line of a He/Cd laser (Coherent, Santa Clara, CA) was used to excite the ferrous 5-coordinate state. Power levels were kept low (2-3 mW) and samples rotated continuously (1000 rpm) to prevent damage to the protein. Scattered light was collected at a 90° angle using an F#1 lens and refocused onto the entrance slit of a 0.75-m SpectraPro 750 spectrometer (Acton Research, Acton, MA) using an F#9.8 lens. A notch filter, placed in front of the entrance to the spectrometer, blocked Rayleigh scattered light.
at 441.6 nm (MK Photonics, Albuquerque, NM) and 413 nm (Kaiser Optical, Ann Arbor, MI). The slit of the spectrometer was set to 100 µm. Raman scattered light was detected using a liquid nitrogen-cooled CCD camera. Given this equipment, the spectrometer resolution was 1.37 cm\(^{-1}\). Spectra were acquired over the course of 30 minutes. Winspec data acquisition software (Roper Scientific) was used to remove cosmic ray signals and GRAMS software (Thermo Scientific) was used to analyze spectra. Indene was used to calibrate Raman spectra in the 150-1700 cm\(^{-1}\) region and ferrocyanide and acetone in the 1400-2300 cm\(^{-1}\) region.

2.6.2 Preparation of Samples

Ferrous gFlHb and its derivatives were generated by flushing samples with argon in a tightly sealed Raman cuvette followed by the addition of a freshly prepared sodium dithionite solution (in 20 mM Tris-HCl, pH 7.5 buffer). The Fe(II)-CO complex was generated by flushing sealed samples with 5\% \(^{12}\)C\(^{16}\)O (Praxair) and subsequently reducing the protein with freshly-prepared sodium dithionite solution. Isotopic substitution of the CO complex was accomplished by flushing the same CO-bound protein with argon (to remove remnant \(^{12}\)C\(^{16}\)O) and then injecting \(^{13}\)C\(^{18}\)O gas (99\%:95\%, Icon Isotopes).

2.7 UV-Visible Kinetic Assays

2.7.1 Rates of NO Activity as Measured by NADH Oxidation

The catalytic efficiency of wild type and mutant gFlHb was determined by tracking the rate of NADH oxidation at 340 nm. Assays were 2.5 mL in volume and run in a 3 mL quartz cuvette. Conditions for each reaction were 25 °C, 20 mM Tris-HCl, pH
7.5, 100 μM NADH, 1 μM FAD, and 100 μM DEA-NONOate, where applicable. The solutions were incubated at 25 °C in the spectrometer’s temperature controlled block for 30 minutes prior to the addition of reactants (NADH and DEA-NONOate) to ensure they reached thermal equilibrium. Rate measurements at 340 nm (the absorbance maximum for NADH) were taken over 3 minutes under aerobic conditions, with initial rates determined by a line of best fit generated by the Cary Enzyme Kinetics software. The velocity of NADH oxidation was standardized by dividing by the concentration of protein used (~0.4-0.6 μM), determined the day of each assay using the extinction coefficient of the ferric Soret peak. Each assay was performed in triplicate. The rate of NADH oxidation associated with NO consumption was calculated by subtracting the rate with NADH and FAD alone from the rate with NADH, FAD, and DEA-NONOate.

2.7.2 Rates of Superoxide (O$_2^-$) Production

The superoxide production of wild type and mutant gFlHb during NADH oxidation was tracked by measuring the rate of superoxide-dependent cytochrome $c$ reduction (cytochrome $c$ is reduced in the presence of superoxide, producing a characteristic absorption band at 550 nm). Rate measurements were done twice, with and without superoxide dismutase (SOD). The addition of SOD prevents the reduction of cytochrome $c$ by superoxide. Thus, subtracting the rate of cytochrome $c$ reduction with SOD from the rate without gives the amount of superoxide being generated by the protein.

Assays were 2.5 mL in volume and run in a 3 mL quartz cuvette. Each solution was temperature controlled to 25 °C (incubated in the spectrometer’s temperature control block for 30 minutes prior to substrate addition) and contained 20 mM Tris-HCl, pH 7.5,
~0.4-0.5 μM protein, 250 μM NADH, 25 μM cytochrome c, and 100 units of catalase (0.040 units/μL) to remove any H₂O₂ that may accumulate as a product of SOD. SOD containing solutions had 70 units (0.028 units/μL) of the enzyme. Assays were started upon the simultaneous addition and mixing of NADH and cytochrome c. Rate measurements were taken over 3 minutes. Initial rates were determined by a line of best fit in Cary Enzyme Kinetics software. Rates were again normalized by dividing by protein concentration (~0.4-0.6 μM). Each assay was performed in triplicate.
3. RESULTS AND DISCUSSION

3.1 Heme and FAD Content

Table 4 provides heme and FAD equivalents for the wild type, Y30F, and L58A proteins used in the experiment, as well as the literature value (3) for the wild type as a comparison.

**Table 4:** Heme and FAD equivalents measured in gFlHb variants.

<table>
<thead>
<tr>
<th>Protein Variant</th>
<th>Heme Equivalents</th>
<th>FAD Equivalents</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild Type gFlHb (Literature, (3))</td>
<td>0.65</td>
<td>0.3</td>
</tr>
<tr>
<td>Wild Type gFlHb (Experimental)</td>
<td>0.278</td>
<td>0.259</td>
</tr>
<tr>
<td>Y30F Mutant</td>
<td>0.375</td>
<td>0.379</td>
</tr>
<tr>
<td>L58A Mutant</td>
<td>0.305</td>
<td>0.297</td>
</tr>
</tbody>
</table>

Measuring with the pyridine hemochrome assay, heme equivalents ranged from 0.278 in the wild type protein to 0.375 in the Y30F mutant. While difficult to explain, these values are substantially lower than that of the literature value (0.65) for the wild type protein. Overall, this can be an issue of increased protein expression with stationary cofactor expression, decreased cofactor expression with stationary protein expression, or some combination of the two. There are several possible causes. One explanation, which perhaps also accounts for the slight differences between variants, is that each of the proteins had been through at least one freeze-thaw cycle before their heme content was characterized. The wild type protein was stored at -80 °C the longest and had the lowest number of heme equivalents. Still, this would seem a severe consequence for a rather typical method of protein storage. Other explanations could include different growing conditions (literature cultures were not supplemented with IPTG to prompt protein expression) and a different method of lysing cells (sonication versus a lysozyme
solution). Since the calculation of heme equivalents assumes that protein concentrations determined by Bradford assay are entirely that of gFlHb, it is possible that differences in the purity of the protein (the mutants and wild type are purified differently) could also justify the lower value. This appears unlikely, however, as both the literature wild type protein and the wild type protein studied here were purified using IMAC, yet show the largest discrepancy between each other. Regardless the reason, protein concentrations were determined using the previously solved extinction coefficient for gFlHb, which itself is designed to take into account only the holoprotein. Thus, all experiments were standardized by heme content.

FAD equivalents were much closer to literature value (0.3), ranging from 0.259 in the wild type to 0.379 in the Y30F mutant. Heme and FAD equivalents seemed to vary with each other (i.e. lower levels of heme seemed to correspond to lower levels of FAD). This would suggest that values are highly dependent on the particular protein preparation. Given that each of the experimental variants were grown and lysed in the same fashion, deviations between preparations could have arisen simply from differences in bacteria cultures or, as previously mentioned, in the way in which they were stored.

3.2 UV-Visible Spectroscopy of Y30F and L58A Mutants

Table 5 provides the UV-Visible spectral characteristics of gFlHb and the associated mutants in different iron-ligand complexes and oxidation states.
Table 5: UV-Visible characteristics of gFlHb variants in a variety of iron-ligand complexes and oxidation states.

<table>
<thead>
<tr>
<th>Complex</th>
<th>Wild Type Literature Value (3)</th>
<th>Wild Type Measured Value</th>
<th>Y30F Mutant Value</th>
<th>L58A Mutant Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe(III)</td>
<td>403-405, 450-490 (s), 533 (s), 645</td>
<td>406-409, 480-485 (s), 533-540 (s), 638-645</td>
<td>403, 484 (s), 533 (s), 586 (s), 645</td>
<td>408, 485 (s), 533 (s), 645</td>
</tr>
<tr>
<td>Fe(III) - Imidazole</td>
<td>413-414, 540, 570 (s)</td>
<td>413, 540, 568 (s)</td>
<td>414, 540</td>
<td>414, 540</td>
</tr>
<tr>
<td>Fe(II) Deoxy</td>
<td>433</td>
<td>432-433, 556-557</td>
<td>432, 557</td>
<td>431, 557</td>
</tr>
<tr>
<td>Fe (II) - O$_2$</td>
<td>416, 545, 580</td>
<td>413-415, 544-545, 579-580</td>
<td>415, 545, 580</td>
<td>415, 547, 577</td>
</tr>
<tr>
<td>Fe (III) - NO</td>
<td>417, 486 (s), 534, 566</td>
<td>420, 486 (s), 533, 567</td>
<td>419, 485 (s), 533, 566</td>
<td>419, 533, 566</td>
</tr>
<tr>
<td>Fe (II) - NO</td>
<td>N/A</td>
<td>419, 547, 580 (s)</td>
<td>420, 553, 580 (s)</td>
<td>419, 547, 580 (s)</td>
</tr>
</tbody>
</table>

In general, the mutant variants retained near identical spectral characteristics to that of the wild type protein (3), suggesting that the integrity of their heme pockets remain intact. This is of particular importance, as the direct effects of the mutations themselves (due to the function-loss of a conserved residue) are what is desired from a measurement standpoint, not an indirect destabilization of the environment surrounding them. The exception to this uniformity is in the ferric spectrum of the Y30F mutant (shown in comparison to the wild type protein in Figure 17), which notably had a broader, more asymmetric Soret band (predominately at the lower wavelengths) than the native protein. The irregularities in the Soret band are likely attributable to the presence of 4- and/or 6- coordinate heme, caused by the slight destabilization of the proximal heme pocket, which is described in detail in Section 3.3.1 when discussing the resonance Raman spectra of this mutant.
Figure 17: UV-Visible spectra of the wild type and Y30F Fe(III) complexes. The Y30F has a broader, more asymmetric Soret band (favouring the lower wavelengths) than the native protein.
3.3 Resonance Raman Spectroscopy

3.3.1 Y30F Mutant (Tyrosine B10 → Phenylalanine)

Figure 18 provides the high frequency resonance Raman spectrum of the ferric and ferrous forms of the Y30F mutant. The heme environment of Y30F remains relatively undisturbed when compared to that of the wild type. The spin and coordination sensitive $\nu_2$ and $\nu_3$ markers of the ferric spectrum are found in identical positions to the wild type protein at 1571 cm$^{-1}$ and 1493 cm$^{-1}$ respectively, indicating the heme group is primarily 5-coordinate and high spin. The ferrous spectrum is also similar to that of the wild type, with identical $\nu_2$ and $\nu_3$ markers of 1563 cm$^{-1}$ and 1470 cm$^{-1}$, indicating a 5-coordinate high spin species, and a similar shift of the $\nu_4$ marker from 1370 cm$^{-1}$ to 1354 cm$^{-1}$, consistent with higher electron density on the heme iron.

Nevertheless, there were some differences between the wild type and Y30F spectra. A faint $\nu_3$ signal at 1476 cm$^{-1}$ in the ferric spectrum of this mutant indicates a small fraction of 6-coordinate high spin heme. Furthermore, the ferrous spectrum (when probed with the 413 nm laser) has a shoulder at 1366 cm$^{-1}$, which, when combined with a faint $\nu_3$ signal at 1504 cm$^{-1}$, is characteristic of 4-coordinate heme (52). Notably, the fact that these signals disappear when probed with the 442 nm He/Cd laser is actually consistent with the behaviour of this particular species, as its main excitation wavelength (the Soret band) should be shifted to lower wavelengths than that of the 5-coordinate species. These combined results would indicate that the loss of the Y30 residue in this variant slightly destabilizes the proximal heme pocket. The presence of a 6-coordinate species for instance (with water as the likely distal ligand), is symptomatic of a loss of the peroxidase-like functionality of the proximal heme pocket, of which the strong
Figure 18: High frequency resonance Raman spectra of the Fe(III) and Fe(II) complexes in the Y30F and L58A mutants. The excitation wavelength was 413.133 nm for all spectra with the exception of the first Y30F Fe(II) spectrum, which used a 441.565 nm wavelength.

Figure 19: Low frequency resonance Raman spectra of the Y30F and L58A Fe(II) complexes, obtained using a 413.133 nm excitation wavelength.
imidazolate character of the proximal histidine pulls the iron out of the porphyrin plane and discourages this sort of weak ligand binding. The loss of the proximal histidine ligand entirely in the 4-coordinate species is only further proof of this decreased stability. Whether this could promote some sort of functional role for the Y30 residue in proximal heme binding is unclear. It is possible, given the flexibility of the heme pockets in flavohemoglobins, that the residue could play a role, though indirect. However, it is difficult to differentiate any functional significance of Y30 from a general destabilization of the heme pocket due to the mutation, at least at present.

Nevertheless, the contributions of the 4- and 6-coordinate species are quite small in comparison to the native 5-coordinate state, which still predominates in the mutant, suggesting that the mutation has not significantly altered the heme environment. The low frequency spectrum of ferrous Y30F, given in Figure 19, again supports this notion. The distinctive double iron-histidine stretch remains present in the mutant, with lines at 234 cm\(^{-1}\) and 257 cm\(^{-1}\). In addition, the bending modes of the vinyl and propionate groups of heme remain similar to their native states.

Given this, changes in the ligand-bound spectra should be due entirely to the removal of the functional hydroxyl group on the B10 tyrosine. The CO-bound complex, like the wild type, was 6-coordinate, with a \(v_3\) at 1501 cm\(^{-1}\) (not shown). Figure 20 presents the low frequency Fe(II)-CO spectra with the isotopes indicated. In contrast with wild type gFlHb, which had Fe-CO stretches at 490 cm\(^{-1}\) (open configuration) and 541 cm\(^{-1}\) (closed configuration), the Y30F mutant has a single stretching mode at 515 cm\(^{-1}\), which downshifts to 500 cm\(^{-1}\) upon substitution to a higher mass CO isotope. This confirms that the Y30 residue is involved in a strong polar interaction with CO at the
Figure 20: Low frequency resonance Raman spectra of the Y30F and L58A Fe(II)-CO complexes, including $^{12}\text{C}^{16}\text{O}$ minus $^{13}\text{C}^{18}\text{O}$ difference spectra. The excitation wavelength was 413.133 nm for each measurement.
distal coordination site. This, combined with the marked effect on the kinetics of the protein when the residue is mutated, would seem to confirm that the B10 tyrosine plays an important role during catalysis, likely stabilizing and/or activating the distal ligand, either directly or through a water bridge \((21, 26)\).

Note that the Fe-CO stretch in the Y30F mutant is higher in energy \((515 \text{ cm}^{-1})\) than the open configuration of the wild type protein \((490 \text{ cm}^{-1})\). This indicates that the CO ligand is still experiencing some polar interaction with a distal residue. Given the geometry of the heme pocket, the most likely candidate for this interaction, as the only other hydrogen bond donor in the distal region, would be the E7 glutamine \((Q54)\). It is uncertain, however, whether this is a true association or simply \(Q54\) opportunistically bonding with the distal ligand in the absence of Y30. As previously mentioned, it has been reported that E7 glutamine mutants in Hmp demonstrated only a single configuration in the CO-bound state, similar in energy \((499 \text{ cm}^{-1})\) to that of the open state \((494 \text{ cm}^{-1})\) \((22)\). This, combined with the recently solved structure of Yhb, which has the side chain of the E7 glutamine pointed toward the interior heme pocket, make a physiologically relevant interaction plausible \((18)\). Indeed, comparable globins do not have this residual interaction; a B10 mutant of \textit{Ascaris suum} Hb, another protein involved in NO/O\(_2\) chemistry, also has a glutamine in the E7 position and presents only a single near-open conformation in the CO-bound state. Nonetheless, this would seem to have to involve dynamic rotation of the residue, given that its side chain is facing the solvent in the crystal structure of Hmp. Moreover, even if this side chain is positioned in the heme pocket, it may not be directly hydrogen bonding with a ligand, instead simply positioning the Y30 residue into a specific orientation to accomplish that task.
It is also noteworthy that the open and closed conformers present during ligand binding seem to be disrupted upon mutation of Y30. As discussed in the previous paragraph, it is possible that Q54 is not directly involved in stabilizing the distal ligand and is instead forming an opportunistic interaction with the bound CO in the absence of the B10 tyrosine. This could be converting the 490 cm\(^{-1}\) open configuration to a configuration at 515 cm\(^{-1}\). However, if Q54 interacts with the distal ligand under native conditions, two configurations should still exist in the Y30F spectrum (\textit{i.e.} the open conformer should still be present, in addition to the new configuration at 515 cm\(^{-1}\)). The fact that it does not could indicate that Y30 is an essential cog in the formation of gFlHb’s two dueling conformers and that the mechanism responsible for the conformational character of flavohemoglobins is more involved than the simple rotation of the E11 leucine (discussed in further detail in 3.3.2).

3.3.2 \textit{L58A Mutant}

Overall, the ferric and ferrous resonance Raman spectra of L58A, given in Figures 18 and 19, were similar to that of the wild type protein. The ferric spectrum \(\nu_2\) and \(\nu_3\) markers were slightly upshifted in comparison to the wild type (1496 cm\(^{-1}\) and 1573 cm\(^{-1}\)), but still maintain the profile of 5-coordinate high spin heme. The \(\nu_3\) and \(\nu_4\) markers in the ferrous spectrum are also identical to that of the wild type (1470 cm\(^{-1}\) and 1354 cm\(^{-1}\)), indicating a 5-coordinate high spin species. In the low frequency spectrum, the double Fe-His stretch is likewise still present at 234 cm\(^{-1}\) and 257 cm\(^{-1}\). Unlike the Y30F mutant, there was no evidence of 4- or 6- coordinate heme in the ferric and ferrous spectra, perhaps further confirmation of the B10 tyrosine’s effect on the proximal environment.
In contrast to the Y30F mutant, the Fe(II)-CO spectra of L58A, shown in Figure 20, retain the same characteristics of the wild type protein. Once again, two Fe-CO stretches are present at 493 cm\(^{-1}\) and 542 cm\(^{-1}\), very close to the open (490 cm\(^{-1}\)) and closed (541 cm\(^{-1}\)) conformers of the native spectrum. Given the position of the E11 leucine residue directly over the distal heme binding site in homology models (based on the ferric structure of Hmp), the absence of this bulky side chain in the mutant could provide easier access of CO to hydrogen bond donors Y30/Q54 which might reduce or eliminate the peak associated with the open configuration of the protein. That does not seem to be the case in L58A. Regression analysis of the area of under each of these peaks reveals a slight decrease in the level of the open conformer in comparison to the wild type, but given the interference inherent to the spectrum it is difficult to call the result significant. It would seem, over the time scale probed, that the removal of the E11 leucine’s side chain has little impact on the conformational changes that accompany ligand binding. Since the placement of leucine in the ferric structure of the protein would mean it would likely have to move for an interaction with a distal residue to take place, this would indicate that the conformational mechanics of gFlHb must be rapid in nature.

The lack of any substantial differences in the Fe(II)-CO spectral profile of L58A could also signify that the process of ligand binding is much more complex than the isolated rotation of a single residue. The demonstrated flexibility of the heme pocket in flavohemoglobins, particularly that of the B, E, and G helices (which encompass 6 of the 9 invariant residues in the distal pocket), suggest that L58 could simply be a small cog in a larger, more intricate, process, or not involved at all. This would help illuminate why mutation of the B10 tyrosine or the E7 glutamine results in the breakdown of the
conformational nature of the CO-bound protein; they may crucial parts of that larger mechanism.

3.4 Rate Measurements of NADH Oxidation

Table 6 gives the rates of NADH oxidation for wild type gFlHb and the associated mutants under different conditions.

**Table 6:** Rates of NADH oxidation under different NADH (100 µM), FAD (1 µM), and DEA-NONOate (100 µM) conditions as well as the rate of NADH oxidation associated with NO consumption.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Rate of NADH Oxidation (min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild Type</td>
</tr>
<tr>
<td>NADH, pH 7.5</td>
<td>2.8 ± 0.1</td>
</tr>
<tr>
<td>NADH, FAD, pH 7.5</td>
<td>16.7 ± 0.6</td>
</tr>
<tr>
<td>NADH, FAD, DEA-NONOate, pH 7.5</td>
<td>30.9 ± 0.4</td>
</tr>
<tr>
<td>NADH Oxidation Associated with NO</td>
<td>14.2 ± 1.0</td>
</tr>
</tbody>
</table>

Without supplemental FAD in the assay, the mutants consumed NADH slightly faster than the wild type. This is likely tied to the FAD content of the purified protein, as both Y30F and L58A were isolated with higher levels of the electron donor than the native protein. When exogenous FAD was added to the reaction mix, wild type levels of NADH oxidation increased above mutant levels (though given their respective errors it is possible that the true values of wild type and L58A could be the same), which demonstrates the importance of supplementing assays of flavohemoglobins with the cofactor. Overall, the rate of NADH oxidation associated with NO consumption was lower in both mutants when compared to wild type gFlHb. This is most prominent in the Y30F mutant, which, even with the accompanying errors still showed a slight decrease in the rate of NO consumption from wild type. L58A also showed a decrease, but its larger error, when coupled with the native protein’s error, mean that the difference in rate may
not be significant. Still, these data would suggest that the mutations impact the ability of the gFlHb to process NO.

While similar rates are observed for the wild type flavohemoglobin and the two mutants, the use of an NO donor complicates the interpretation of these results. The release of NO from DEA-NONOate averages ~0.1 μM/s over the initial time spans in which rate data was generated (see Appendix 1 for a calculation of NO release). Given average protein concentrations of ~0.4-0.6 μM this would likely be rate limiting in the assays in which the NO donor is included. Thus, while the decrease in NO consumption of each mutant, particularly the Y30F mutant, is revealing, it is possible that the scale of this decrease, in comparison to the wild type, is being underestimated by these data. Differences in rates under non-saturating conditions can increase by orders of magnitude under saturating conditions. Likewise, if the maximum rate of the native protein was capped by the speed of NO release while the rates of the mutants were not, an even starker contrast in NO consumption would be hidden.

A second consideration is that if the rates of more than one of the variants were limited by NO release, the concentration of protein used in each assay, typically standardized, would start to unnaturally influence the results. Normalizing rates by dividing by protein concentration requires that assays are free to reach their maximum rates. If they are not free to reach these rates and instead capped, standardizing by concentration make assays with higher amounts of protein look as though they produce lower rates (and vice versa), problematic when the small scale of NO activity differences are taken into account.
This logic can be applied to the experiment at hand. In the NADH, FAD, DEA-NONOate, pH 7.5 assay the concentration of wild type protein was 0.412 µM, Y30F 0.487 µM, and L58A 0.610 µM. The variant with the highest rates of NO consumption (the wild type) was also the protein with the lowest concentration in the assay. This would be consistent with concentration inappropriately affecting rate. However, the mutants themselves did not follow this trend; the Y30F mutant produced a lower rate with a lower concentration than the L58A mutant. This is somewhat encouraging, as it suggests that the calculated rate of the Y30F variant may be expressing its true value in the experiment; at a minimum it looks to have a lowered NO consumption rate compared to the native protein, though, as mentioned previously, the extent of that decrease is still open to question. Still, with the very slow release rate of the NO donor, if Y30F is demonstrating its true rate, that difference would likely be quite substantial. The L58A mutant is more difficult. If it, along with the wild type, were also limited by NO release, then it is difficult to yield any true insight into the impact of the mutation on NO activity. The present difference in rate, which is already within the error of the wild type, could simply be caused by the larger protein concentration. The true level of NO consumption in L58A could be higher, lower, or the same as that of the native protein.

It should be noted that the wild type kinetic measurements (and curiously the mutants as well) displayed significantly faster rates of NADH oxidation in assays supplemented with FAD than in the wild type literature values (3). The reason for this discrepancy is unclear. Kinetic assays in the literature also used DEA-NONOate as an NO donor, so it is possible that rate limits caused by the slow release of NO could be responsible. For instance, if higher concentrations of protein were used in the literature,
this could artificially make NO consumption seem slower. Still, this would not explain the different rates in assays that did not use DEA-NONOate.

3.5 Steady-State Kinetic Spectra

Table 7 provides the spectral features of the reduced O₂- and NO-bound forms of each gFlHb variant, as well as the steady-state spectral features under two types of reaction conditions, differing only in whether 20 µM or 100 µM DEA-NONOate was used.

Table 7: Spectral features of reduced O₂- and NO-bound forms of each gFlHb variant, as well as the steady-state spectral features under two types of reaction containing 20 µM and 100 µM DEA-NONOate.

<table>
<thead>
<tr>
<th>Complex/Assay</th>
<th>Wild Type</th>
<th>Y30F</th>
<th>L58A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe(II)-O₂</td>
<td>415, 545, 579</td>
<td>416, 545, 578</td>
<td>414, 545, 576</td>
</tr>
<tr>
<td>Fe(II)-NO</td>
<td>419, 547, 580 (s)</td>
<td>420, 553, 580 (s)</td>
<td>419, 547, 580 (s)</td>
</tr>
<tr>
<td>250 µM NADH, 1 µM FAD, 20 µM DEA-NONOate</td>
<td>415, 545, 579</td>
<td>416, 546, 578</td>
<td>414, 545, 575</td>
</tr>
<tr>
<td>250 µM NADH, 1 µM FAD, 100 µM DEA-NONOate</td>
<td>415, 545, 579</td>
<td>416, 546, 578</td>
<td>414, 543, 574</td>
</tr>
</tbody>
</table>

Steady-state spectra show gFlHb in the ferrous oxygenated state at both concentrations of DEA-NONOate. This supports the dioxygenation model as the mechanism of catalytic action in flavohemoglobins, though further analysis shows that it far from proves it the physiologically relevant process outright. Previous studies on Hmp by Hausladen et al., which purported the nitrosylation model, established that the steady-state spectrum of the protein was in the Fe(III)-NO complex only at O₂/NO concentration ratios of 6.25:1 and 2.5:1. At ratios of 12.5:1 the complex was a mixture of different species, including the Fe(II)-O₂ form (27). In the present experiment, conducted under
aerobic conditions, the O$_2$ concentration was 272 µM (see Appendix 2 for calculation). Given the slow rate of NO release from DEA-NONOate (~0.1 µM/s), realistically the O$_2$/NO ratio in the experiment was likely greater than 100:1. As such, these results do not contradict those in the literature favouring nitrosylation.

Adding further issue is the fact that both experiments were conducted using different methodologies, neither of which were ideal. The true mechanism depends largely on the temperature and concentrations of O$_2$ and NO under physiological conditions, which, given the range of species that contain flavohemoglobins, is a somewhat subjective point of debate (5, 27). For the two species under study however, neither the 10 ºC temperature point from the literature nor 25 ºC temperature point in this experiment reflect the true environment in which they find themselves. Given the dependence of relative $K_m$ on temperature, the ideal way to determine mechanism in these variants would be an experiment closer to 37 ºC. Other changes could also be made to make the experiments more comparable. Hausladen et al. used stopped-flow spectroscopy to gather measurements, which provides an increased level of sensitivity that would benefit the present experiment. In contrast, they did not add exogenous FAD to their assay, which is crucial for sustaining high enzymatic turnover and could be biasing their result.

3.6 Rates of Superoxide (O$_2^-$) Production

Table 8 provides the standardized rates of cytochrome c reduction with and without superoxide dismutase (SOD), as well as the overall activity attributable to superoxide generation by the protein.
Table 8: Standardized rates of cytochrome c reduction with and without superoxide dismutase (SOD), as well as overall activity attributable to gFlHb superoxide generation.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Rate of Cytochrome c Reduction (min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild Type</td>
</tr>
<tr>
<td>Without SOD</td>
<td>5.2 ± 0.2</td>
</tr>
<tr>
<td>With SOD</td>
<td>4.0 ± 0.2</td>
</tr>
<tr>
<td>Superoxide Generation</td>
<td>1.2 ± 0.4</td>
</tr>
</tbody>
</table>

These results indicate that gFlHb does produce superoxide in the absence of NO. As such, its regulation in an NO-dependent manner in Giardia is likely functionally important for preventing oxidative stress (43). The mutations seem to have reduced the rate of superoxide production, though given the associated errors it cannot be said that either are significantly different from wild type. It should be noted, however, that FAD, both exogenous and protein bound, is capable of reducing cytochrome c (53). This is why, upon addition of SOD, reduction activity did not cease. It is also why exogenous FAD, which as stated previously is important for sustaining high enzymatic turnover, was not added to any of the assays. As such, it is possible that the difference between wild type and mutant superoxide production is higher than stated in these results, as the latter had a lower measured FAD content than the two mutants.

The lowered superoxide production of the Y30F mutant is particularly interesting. As a stabilizing functional group in the distal heme pocket, a possible consequence of its mutation would be increased dissociation of bound superoxide into solution. Instead, the mutation of this conserved residue seems to hinder the process by which O₂ is reduced to superoxide in the first place, perhaps a further confirmation of Y30’s role as facilitator of a “pull” effect that reduces the distal ligand and activates its bond for cleavage.
3.7 Future Experiments

The most enticing prospect stemming from this research is the potential role for the E7 glutamine in the catalytic machinery of the heme active site. For that reason, an interesting follow up would be to mutate the Q54 residue and then conduct the same UV-Visible and resonance Raman experiments used here. Specifically, two mutants would be able to address some of the lingering questions left by the remnant interaction at 515 cm\(^{-1}\) in the low frequency CO-bound Y30F spectrum. First, a double mutant of the Y30 and Q54 residues (mutated to leucine to maintain a similar size but remove any ability to hydrogen bond) that would be able to determine whether the remaining signal is indeed caused by the E7 glutamine. Second, a single Q54 mutant, which would be able to probe whether the loss of function of this residue alone is able to disrupt both the ligand binding properties and conformational nature of the protein as the loss of function from the Y30 residue was able to. This work is currently in progress.

With these and the previous mutations in mind, another important experiment would be to measure the NO kinetics using the molecule itself instead of another NO-donating substitute. Results from kinetic studies to date have been subject to much ambiguity due in large part to the slow NO release time of DEA-NONOate. Although it requires more sophisticated equipment, such as a stopped-flow spectrophotometer and gas-handling apparatus to precisely control NO and O\(_2\) concentrations, direct measurement of the kinetics using this experimental design will be able to yield the true magnitude of each mutation on the catalytic efficiency of the protein. This experiment is currently being planned to supplement a manuscript in preparation.
Finally, it would be beneficial to determine the steady-state spectrum of wild type gFlHb using NO itself as well. Just as the slow release of DEA-NONOate limited the kinetic experiments, so too did it limit the collection of the steady-state spectra. Given the large emphasis on relative O₂/NO ratios for determining the suitability of the dioxygenation and nitrosylation mechanisms, more direct control over the concentration of NO is needed. This would likely also require a stopped-flow apparatus to be truly effective. Furthermore, the steady-state spectrum should be carried out as close to physiological conditions as possible, as this seems to be what is lacking to date in the literature. This would likely entail conducting the experiment at 37 °C and supplementing with exogenous FAD.

3.8 Conclusions

Mutation of the B10 tyrosine and E11 leucine in gFlHb yielded significant insight into the dynamics of flavohemoglobin active sites. The breakdown in the conformational nature of the Y30F mutant directly implicates the B10 tyrosine in providing a hydrogen bonding interaction with the distal ligand, likely prepping it for catalysis. The remnant signal at 515 cm⁻¹ in the CO-bound spectrum, above that of the open conformation of the wild type at 490 cm⁻¹, indicates that a residual hydrogen bonding interaction remains, probably provided by the E7 glutamine. Whether this is a true physiological interaction or the residue opportunistically forming a bond with the distal ligand in the absence of Y30 remains unclear. Moreover, the similarity of the CO-bound resonance Raman spectrum of the L58A mutant to the wild type suggests rapid conformational dynamics within the heme pocket and may signify a more complex and involved movement of the helices in forming the two states of flavohemoglobins.
Kinetic assays probing NO activity reveal decreased activity in the two mutants, but would benefit from experimentation using NO directly, instead of a donor. The same is true of steady-state spectra of gFlHb, where stricter control of NO concentration would be able to extract more compelling implications in the dioxygenation/nitrosylation debate. Finally, as observed in other types of flavohemoglobins, gFlHb produces superoxide in the absence of exogenous NO. The production of superoxide is marginally affected by the previously described mutations.
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APPENDIX

Appendix 1 – Calculation of NO Release from DEA-NONOate

Given first order dissociation and a 16 minute half-life at pH 7.4 and 25 °C, the decay constant can be calculated as follows:

\[ k = \frac{\ln 2}{t_{\frac{1}{2}}} \]
\[ k = \frac{\ln 2}{960 \text{ s}} \]
\[ k = 7.2 \times 10^{-4} \text{ s}^{-1} \]

Where \( k \) is the decay constant and \( t_{\frac{1}{2}} \) is the half-life.

The concentration remaining after different time points can then be calculated (using the 10 second time point as a sample):

\[ C(t) = C_o e^{-kt} \]
\[ C(10 \text{ s}) = (100 \mu M)e^{-(7.2 \times 10^{-4})(10 \text{ s})} \]
\[ C(10 \text{ s}) = 99.3 \mu M \]

Where \( C(t) \) is the concentration at a specific time point, \( C_o \) the initial concentration, and \( t \) the dissociation time.

DEA-NONOate releases 1.5 moles of NO per mole of parent compound. Thus, 0.108 µM NO has been released into solution after 1 second, 1.05 µM after 10 seconds, and 2.15 µM after 20 seconds. This suggests an average NO release rate of ~0.1 µM/s upon introduction of DEA-NONOate to each assay.
Appendix 2 – Calculation of O₂ in Solution under Aerobic Conditions

Under aerobic conditions (and ignoring other dissolved solutes), the concentration of O₂ in solution can be calculated using Henry’s Law. The Henry’s Law constant for O₂ at 25 °C is 769 atm/M and the partial pressure of O₂ in the atmosphere is 0.209 atm.

\[ C_{O_2} = \frac{P_{O_2}}{K_{H_{O_2}}} \]

\[ C_{O_2} = \frac{0.209}{769 \frac{atm}{M}} \]

\[ C_{O_2} = 272 \mu M \]

Where \( C_{O_2} \) is the concentration of O₂ in solution, \( P_{O_2} \) is the partial pressure of oxygen in atm, and \( K_{H_{O_2}} \) is the Henry’s Law constant for O₂ at 25 °C.