REDOX AND FUNCTIONAL CHARACTERIZATION OF A SURFACE LOOP SPANNING RESIDUES 536 TO 541 IN THE FLAVIN MONONUCLEOTIDE-BINDING DOMAIN OF FLAVOCYTOCHROME P450BM-3 FROM BACILLUS MEGATERIUM

DISSERTATION

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ABSTRACT

Flavocytochrome P450BM-3 (BM3) displays many of the same characteristics as the mammalian microsomal cytochrome P450 system; however, the two diflavin reductases utilize different electron transfer mechanisms. The flavin mononucleotide cofactor (FMN) in BM3 appears to use the thermodynamically unstable anionic form of the semiquinone (SQ) species as electron donor to heme which contrasts with the hydroquinone (HQ) species utilized in the mammalian enzyme. Several distinctive structural features of the peptide loop flanking the re-face of the FMN in BM3 were noted. The loop is shorter than other related proteins due to the absence of a highly conserved glycine residue, adopts a hydrogen bond-stabilized Type I’ reverse turn, and contains a tandem proline sequence that has been proposed to rigidify the loop. In this thesis, the roles of these features in establishing the unique functional properties of BM3 were investigated. First, the tandem proline residues (Pro540 and Pro541) were replaced as a pair and individually by alanine. While the single replacements did not significantly affect the two-electron midpoint potential ($E_{OX/HQ}$) or FMN binding, some differences were observed in the rate of the anionic SQ formation and the reductase activity for the P541A variant. The P540A/P541A double variant did not bind FMN. Thus, the tandem proline pair appears crucial for proper cofactor binding and Pro541 may exert some
influence on catalytic activity. Secondly, the loop size was extended by the insertion of the conserved glycine residue that is missing in BM3. The $E_{SQ/HQ}$ was shifted to a more negative value along with the striking appearance of a relatively stable neutral SQ species like that found in other diflavin reductases. The insertion appears to disrupt a strong H-bond between the loop and the N5 atom of the oxidized FMN observed in the wild-type BM3 and lowers the cytochrome c reductase and hydroxylase activities. These results support the hypothesis that the shorter loop resulting from the absence of the conserved glycine residue in this enzyme may preclude a crucial redox-dependent conformational change that stabilizes the neutral FMN SQ in other related proteins and substantially establishes some of the unique properties of BM3.
Dedicated to my parents and sisters
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CYTOCHROME P450 IS A LARGE SUPERFAMILY OF MONOOXYGENASES (ENZYMES THAT CATALYZE THE INCORPORATION OF AN OXYGEN ATOM INTO THE SUBSTRATE) THAT CAN BE FOUND IN ALL DOMAINS OF LIFE, FROM BACTERIA AND ARCHAEA TO MAMMALS (1). THIS CLASS OF ENZYME WAS NAMED AFTER ITS CHARACTERISTIC SPECTRAL FEATURE WITH ABSORPTION MAXIMUM AT 450 NM UPON BINDING TO CARBON MONOXIDE IN THE REDUCED STATE (2, 3). CYTOCHROME P450 WAS FIRST DISCOVERED TO BE CAPABLE OF OXIDIZING XENOBIOTIC COMPOUNDS IN THE ENDOPLASMIC RETICULUM OF THE LIVER IN 1955 (4, 5). THE ENZYME WAS LATER DEMONSTRATED TO BE A B-TYPE HEMOPROTEIN WHICH CONTAINS A THIOLATE ANION PROVIDED BY A CONSERVED CYSTEINE RESIDUE AS THE FIFTH AXIAL LIGAND OF THE HEME IRON (6).

CYTOCHROME P450S CATALYZE THE INSERTION OF AN OXYGEN ATOM, DERIVED FROM MOLECULAR OXYGEN, BOUND TO THE HEME IRON INTO THE SUBSTRATE, LEADING TO THE FORMATION OF THE OXYGENATED PRODUCT AND A MOLECULE OF WATER (7). THEY HAVE BEEN KNOWN TO INVOLVE IN REMARKABLY DIVERSE RANGE OF REACTIONS, INCLUDING HYDROXYLATION, EPOXIDATION, DEALKYLATION, DEAMINATION, DEHALOGENATION, ISOMERIZATION, AND NO REDUCTION, ETC. (8, 9). THE OVERALL REACTION IS SUMMARIZED AS FOLLOWS (SCHEME 1):
Depending on the P450 species, the substrates for cytochrome P450s include numerous endogenous compounds (such as steroids, fatty acids, prostaglandins, bile acids and vitamins, etc.), as well as the foreign compounds (xenobiotics) (such as drugs, alcohols, procarcinogens, antioxidants, organic solvents, anesthetics, pesticides and petroleum products, etc.) (8, 10). Extensive studies over the years have generated a simplified catalytic cycle as shown in Figure 1.1 (11, 12). The initial step involves substrate binding, which induces an increase in reduction potential of the heme iron (step 1). This change in potential then triggers the first electron transfer to the ferric heme (step 2). Oxygen binds to the ferrous iron (step 3) and forms an oxy-ferrous complex accompanied by molecular conversion to a more stable superoxy-ferric complex (step 4). Following the second electron transfer (step 5), dioxygen would interact with two protons from the surrounding solvent, with one oxygen atom forming water and another incorporated into the substrate, forming the oxygenated product (ROH) (step 6). The enzyme then returns to its resting state after the product dissociates from its active site. The electrons required for catalysis are provided by NAD(P)H and delivered via one or more redox partners.

\[
\text{RH} + \text{O}_2 + 2\text{H}^+ + 2e^- \xrightarrow{\text{P450}} \text{ROH} + \text{H}_2\text{O} \quad \text{(Scheme 1)}
\]
Figure 1.1 The catalytic cycle of cytochrome P450 in human (Modified from Plant, N., 2003 and Gibson, G.G., 2001) (11, 12).
**P450 redox system**

Several distinctive types of P450 redox systems have been previously characterized and described as follows:

*Three-component redox systems*

Most of prokaryotic P450-containing redox systems consist of three soluble proteins: a NADH-dependent flavin adenine dinucleotide (FAD)-containing reductase which delivers electrons to cytochrome P450 from NADH via an iron-sulfur ferredoxin (13). Prokaryotic P450s play critical role in the catabolism of compounds used as carbon source, xenobiotic detoxification, fatty acid metabolism and antibiotic biosynthesis (14). A well-established example is the bacterial P450cam system from *Pseudomonas putida*, whose function involves degradation of the camphor molecule that is used as sole carbon source for cell growth (15). This bacterial system is composed of P450cam and its redox partners, FAD-containing putidaredoxin reductase and the iron-sulfur-containing putidaredoxin (16). The eukaryotic P450 system in the inner membrane of mitochondria also contains three components that are similar to those of bacterial P450s, although the redox partners and P450 are integral membrane proteins (17). One of the examples is adrenal cytochrome P450, which is involved in steroid hormone biosynthesis (18). The reaction requires an adrenodoxin-mediated electron transfer from adrenodoxin reductase to P450 (CYP11 family) in mitochondria of the adrenal cortex.

*Two-component redox systems*

The eukaryotic P450 redox system located in the endoplasmic reticulum has two membrane-associated components: cytochrome P450 and NADPH-dependent cytochrome
P450 reductase. This P450 system is involved in a wide array of critical physiological processes in human, including detoxification of xenobiotics, drug metabolism (19), hormone synthesis and breakdown (20), and the generation of lipid mediators for cellular signaling (21, 22). Deficiencies or modification of P450 enzymes have been linked to several fatty-acid metabolic diseases and human cancer (20, 23). Cytochrome P450 reductase contains two noncovalently-bound flavin prosthetic groups, FAD and flavin mononucleotide (FMN), as redox cofactors for electron transfer from NADPH to the cytochrome P450. Although multiple cytochrome P450 genes (humans have 57 sequenced genes) can be found in a single species (24), most organisms contain only a single-copy gene encoding of cytochrome P450 reductase (designated CPR throughout1), which supports the catalysis of numerous P450 reactions (25).

One-component redox systems and general properties of flavocytochrome P450BM-3

In recent years, several novel P450 redox systems have been discovered as P450-fusion proteins, where the P450 protein is fused with its redox partners (13). The best example of P450 fusion system is flavocytochrome P450BM-3 (designated BM3 throughout), an enzyme isolated from the cytosol of soil bacterium Bacillus megaterium (ATCC14581). BM3 (CYP102A1) was initially discovered with fatty acid hydroxylase activity (26) and identified as a cytochrome P450 component based on its CO-binding difference spectrum (27). It was later characterized as a single soluble 119-kDa polypeptide chain of 1048 amino acid residues containing equimolar amounts of
noncovalently bound heme, FMN and FAD as prosthetic groups (28, 29). BM3 was the first bacterial enzyme described as a fusion between a catalytic N-terminal oxygenase domain (designated BMP) and a C-terminal reductase domain (designated BMR) that closely resembles the mammalian CPR (28, 29). Regulation of BM3 expression is controlled by a repressor protein known as Bm3R1. Binding to barbiturate-like molecules will displace the repressor and lead to protein expression (13, 30, 31).

**Biological relevance and application**

BM3 generally catalyzes oxygenation of saturated fatty acids of carbon length C\textsubscript{12}-C\textsubscript{18}, at the ω-1 to ω-3 positions (13) and polyunsaturated fatty acids, such as arachidonic acid (32). It reportedly has the highest fatty acid oxygenase activity among all the P450 monooxygenases (e.g. ~17,000 min\textsuperscript{-1} toward arachidonate) (32). Due to its great catalytic efficiency, BM3 is widely applied as a biocatalyst of bio-industrial interest. Through rational design and/or directed evolution approaches, different BM3 variants with novel catalytic properties, including human P450-like activities have been successfully generated (33). The physiological role of BM3 remains elusive, however. Cryle et al. proposed that BM3 mediates the oxidation of branched chain fatty acids in the membrane of *B. megaterium* (34). Wolf’s group suggests that BM3 serves as detoxifying device against polyunsaturated fatty acids (35). Besides being a successful molecular template for biotechnological application and because it bears resemblance with eukaryotic P450 systems, yet is more accessible, BM3 has also become an attractive
model for studying the structure and functional relationship within the P450 superfamily (36).

**Amino acid sequence and overall structure**

To date, only the structures of the isolated BMP domain as well as a non-stoichiometric complex of the FMN- and heme-binding domains are available; however, a protease-sensitive linker region connects the FMN and heme domains is absent in this complex (37). Attempts to solve the structure of intact BM3 have not been successful. The crystallization of the full-length enzyme perhaps is complicated by its multi-domain nature and aggregation behavior (13). Recent studies suggest that BM3 might exist and function in dimeric form where electron transfer occurs between monomers (38-40). However, whether the dimerization has any physiological significance is not fully understood.

Although BM3 is a bacterial enzyme, it shares a stronger similarity with eukaryotic P450s than with other bacterial P450s. Sequence analyses reveal that the BMP domain of BM3 displays only \(~22\%\) similarity with bacterial P450\textsubscript{cam} (30), while exhibiting \(~28\%\) sequence identity and \(~49\%\) similarity with human cytochrome P450 family 4 (CYP4), which function as a major fatty acid \(\omega\)-hydroxylase (41). The x-ray crystal structures of the isolated BMP domain with and without substrate indicate that BMP contains both \(\alpha\)-helical and \(\beta\)-sheet regions and appears to undergo a conformational change upon substrate binding (42, 43). This change opens a long, narrow
channel leading from the protein surface deep into the heme active site, which accommodates the fatty acid substrate.

BMR and CPR belong to the growing family of diflavin reductases that utilize both the FAD and FMN cofactors during electron transfer (44). The other members of this family include nitric oxide synthase (NOS), methionine synthase reductase (MSR), bacterial sulfite reductase and human-cancer related novel reductase 1 (NR1) (45-48). BMR displays 33% amino acid sequence identity and approximately 50% similarity with human CPR (28, 49) with the most highly conserved regions involved in NADPH, FAD and FMN binding. These binding regions also appear to be conserved in other diflavin reductases (Figure 1.2). These reductases are thought to have evolved as a fusion of bacterial flavodoxin and NADP⁺-ferredoxin reductase (FNR) (49, 50). A common domain organizational structure is shared by the members of this family, where the FMN-binding module is located at the N-terminal portion and the C-terminal contains the NADPH/FAD-binding module (Figure 1.3A). The crystal structures from several different diflavin reductases confirmed that these proteins adopt similar FNR- and flavodoxin-like folds for NADPH/FAD-binding and FMN-binding domains, respectively (37, 45, 48, 51-54). Figure 1.3B shows an overlay of the tertiary structures of CPR, FNR, a flavodoxin, and the FMN-binding domain of BM3. Unfortunately, the structure for the NADPH/FAD domain of BM3 has not yet been established. The FMN-binding domains of BM3 and CPR share a flavodoxin-like fold which consists of a central five stranded parallel β-sheet surrounded by α-helices on either side (55). The FMN is positioned at the tip of the C-terminal side of the β-sheet as in the flavodoxin, which is shown also for
comparison. The NADPH/FAD domain of CPR is very similar to FNR and consists of an anti-parallel β-barrel for the FAD-binding domain, and a five-stranded beta sheet flanked by α-helices for the NADPH-binding domain (56). Based on sequence homology, it is expected that the FNR-like domain of BM3 would also adopt a similar topology with that of CPR (57). In fact, a reasonable homology model of this domain has been established in this laboratory (unpublished results).
Figure 1.2 Sequence alignment of members in the diflavin reductase family (From Wang, M., 1997)
Figure 1.3 (A) Domain organization of P450BM-3. (B) Overlay of the FMN domain of P450BM-3 (red), cytochrome P450 reductase (blue), flavodoxin from *Clostridium Beijerinckii* (green) and NADP⁺-ferredoxin reductase (brown). Flavin cofactors, FAD and FMN are shown in yellow and green. An additional connecting domain in CPR is thought to bring both flavin cofactors together (53). Figure was generated using PyMOL (100).
Redox properties of BM3 and comparison with other homologues

As mentioned above, BM3, like other diflavin reductases, transfer electrons from NADPH to its terminal electron acceptor using both non-covalently bound FAD and FMN as redox cofactors (structures shown in Figure 1.4). Flavin cofactors exist in three different oxidation states: oxidized (OX), one-electron reduced semiquinone (SQ) and two-electron reduced hydroquinone (HQ) states with different protonation states associated with each of them (Figure 1.5). Under physiological conditions, the oxidized state of the flavin is usually present in its neutral form, while both neutral and anionic forms of hydroquinone are commonly found in nature. The semiquinone can exist in a neutral (blue) or anionic (red) form, with a pK \(_a\) of 8.5. Each redox/protonation state has very distinct UV-visible absorption properties (58), thus making them easy to identify (Figure 1.6).

In aqueous solution, the free flavin exists as a mixture of oxidized and hydroquinone forms and the semiquinone state of the flavin is thermodynamically unstable (59). When bound to a specific protein, the physico-chemical properties of the flavin can change dramatically. Depending on the host protein, the semiquinone can be stabilized to different extent and the pK \(_a\) of N5 can shift dramatically. For example, the neutral form of semiquinone is thermodynamically stabilized in \(C.\ beikerinckii\) flavodoxin with the pK \(_a\) of N(5)H increased to >13 from the value of 8.5 for free FMN. The midpoint potentials for the OX/SQ and SQ/HQ couples are shifted from -238 and -172 mV for free FMN to -92 and -399 mV upon binding (60, 61).
A distinct characteristic of the flavin cofactor is its capacity to mediate reversible one- and two-electron oxidation-reduction reactions through the free radical state. This represents a key feature in many flavoprotein reaction mechanisms. This is especially true for the diflavin reductases which mediate electron transfer between the obligatory 2-electron reductant NAD(P)H and one-electron oxidants such as the metallocenters in heme and Fe-S clusters. Despite overall sequence and structural similarities, the redox properties of BM3 are rather distinct from CPR and other diflavin reductases, especially in terms of their one-electron redox chemistry. For example, BM3 is usually isolated with both flavin cofactors in the fully oxidized state, while a relatively stable neutral SQ state persists in CPR in the presence of atmospheric oxygen and can only be re-oxidized rather slowly to the fully oxidized form (62, 63). While BM3 stabilizes the neutral form of the FAD SQ species and has midpoint potentials for both one-electron couples of the FAD fallen within the ranges found for other characterized diflavin reductase enzymes (64) (Table 1.1), the redox properties of the bound FMN cofactor are substantially different. In the case of BM3, a mixture of two SQ species (neutral and anionic forms) has been detected during fatty acid oxygenation by EPR spectroscopy (65). Potentiometric analysis further demonstrated that the neutral SQ species resides on the FAD of BM3 while the FMN cofactor is present as anionic form of SQ (64). In human CPR (as well as other mammalian diflavin reductase), the midpoint potentials of the two one-electron couples of the FMN are well separated, with the OX/SQ couple having the highest value of the four flavin couples in this enzyme (E_{OX/SQ} = -43 mV and E_{SQ/HQ} = -280 mV for CPR) (Table 1.1). The neutral form of FMN{SQ} is thermodynamically stabilized (62). In contrast,
potentials for the one-electron couples for the FMN in BM3 are more similar, with the highest value assigned to the SQ/HQ couple \( (E_{OX/SQ} = -206 \text{ mV} \) and \( E_{SQ/HQ} = -177 \text{ mV} \)). Unlike in CPR, anaerobic titration of the isolated FMN-binding domain in BM3 with sodium dithionite shows that the reduction proceeds as two-electron reduction process from OX to HQ state. The FMN\textsubscript{SQ} is not thermodynamically stabilized but forms transiently as the anionic species which can only be detected during the rapid mixing with the reductant \( (64) \). The differences in the redox properties of BM3 have suggested that the bacterial enzyme may have evolved distinctly different electron transfer and/or regulatory mechanisms compared to other diflavin reductases.
Figure 1.4 Structures of flavin cofactors (From DeColibus, L., 2006) (66).
Figure 1.5 Three oxidation states of flavin. The figure was generated using ChemSketch (155).
Figure 1.6 UV-visible spectra of the flavin cofactor in different redox states.
<table>
<thead>
<tr>
<th>Protein</th>
<th>FAD</th>
<th></th>
<th>FMN</th>
<th></th>
<th>Reference</th>
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<td>$E_{\text{SQ/HQ}}$ (mV)</td>
<td>$E_{\text{OX/SQ}}$ (mV)</td>
<td>$E_{\text{SQ/HQ}}$ (mV)</td>
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<tr>
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<td>-315</td>
<td>-365</td>
<td>Neutral</td>
<td>-146</td>
<td>-305</td>
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</tbody>
</table>

Table 1.1 Redox properties for one-electron couple of FAD and FMN domains in diflavin reductase family.
**Electron transfer mechanism**

The first step of the electron transfer pathway in BM3 involves a hydride transfer from NADPH to the FAD cofactor forming the FAD$_{HQ}$. This is followed by the rapid electron transfer from the FAD$_{HQ}$ to the FMN$_{OX}$ to form the disemiquinone species (FAD$_{SQ}$ and FMN$_{SQ}$) of BM3 which is evident by both UV-visible absorbance and EPR spectroscopic analyses. This species is thought to serve as the electron donor to the heme during fatty acid hydroxylation (65). In the absence of fatty acid substrate, electron transfer from FMN to heme is thermodynamically unfavorable ([Figure 1.7](#)). In this case, two electrons are delivered from the FAD$_{HQ}$ to the FMN, leaving FMN in the fully reduced state (FMN$_{HQ}$) (65). Although more thermodynamically favorable than FMN$_{SQ}$, the FMN$_{HQ}$ appears to be incapable of transferring electron to heme, and the enzyme usually becomes catalytically inactive when “over-reduced” to this redox state (70). However, the reduction potential of the heme iron increases by more than 100mV upon binding of the substrate, which facilitates the reduction of the heme-iron by the FMN$_{SQ}$ ([Figure 1.7](#)) leaving the BMR domain in one-electron reduced state (FAD$_{SQ}$ and FMN$_{OX}$) (64) ([Figure 1.8](#)). A second round of electron transfer follows which initiates the P450 catalytic reaction that leads to fatty acid monooxygenation, and returns the enzyme to the fully oxidized state (Figure 1.1 and Figure 1.8) (64).

Like BM3, the electrons flow through CPR in the sequence NADPH $\rightarrow$ FAD $\rightarrow$ FMN $\rightarrow$ heme ([Table 1.1](#) and [Figure 1.7](#)), with FAD responsible for splitting a pair of electrons derived from NADPH and the one-electron transfer to the FMN. The electrons are then passed to the cytochrome acceptor in two one-electron transfer steps. Extensive
spectroscopic and stopped-flow transient kinetic studies indicate that the several catalytic steps appear to occur much more rapidly in BM3 compared to its eukaryotic counterparts (44) which, in part, contribute to the high catalytic activity of the enzyme. However, the regulatory mechanisms underlying these efficient electron transfer steps remain elusive. For example, despite similar thermodynamic redox properties and conserved residues essential for facilitating NADPH binding and hydride transfer that are found in the FAD domain of both enzymes, FAD reduction occurs at much slower rate in CPR than BM3 (20s\(^{-1}\) versus >200s\(^{-1}\), respectively) (63, 71, 72). Some studies suggest that different modes of interaction with NADPH might occur in these enzymes (73, 74) which perhaps affect the successive catalytic step.

While it holds true that only the anionic FMN\(_{SQ}\) is capable of delivering an electron to the heme in BM3, the actual electron donor in CPR to cytochrome acceptor is still in debate. Both the SQ and HQ states of the FMN are proposed to be capable of electron transfer to the cytochrome acceptor with early studies favoring the FMN\(_{HQ}\) as the primary donor (44). More recent pre-steady-state investigations and kinetic reevaluations suggest that only the neutral FMN\(_{SQ}\) redox species is kinetically competent to do so under steady-state turnover conditions (75). However, it should be pointed out that these experiments were carried out using cytochrome c as the electron donor which has a reduction potential of >200mV (75-77). Because cytochrome P450 has a lower reduction potential, it seems to be thermodynamically infeasible for this species to mediate the electron transfer to the heme iron (Figure 1.7) (64, 78).
Stopped-flow studies indicate that heme reduction in BM3 is far faster than the reduction of eukaryotic P450s by CPR (72, 79) perhaps because the oxygenase (heme) domain is covalently fused to the diflavin reductase domain in BM3 while the cytochrome and CPR are separate components which require the formation of a non-covalent electron transfer complex. However, it is likely that the regulatory mechanism of electron transfer might involve more complex processes in this enzyme, giving the suggestion that the dimeric form of the enzyme can also function as fatty acid oxygenase (39, 40). Such phenomena have been observed in several diflavin reductases, in which the oligomer forms of the enzyme participate in catalysis (44).
Figure 1.7 Midpoint potentials for redox species and electron flow in BM3 and CPR. The figure is modified from (64).
**Figure 1.8** Electron transfer mechanism of BMR. Notations are as follows: FAD, FADH• and FADH⁻ represent OX, neutral form of SQ and anionic form of HQ of the FAD cofactor, respectively; FMN, FMN•⁻, and FMNH⁻ represent OX, anionic form of SQ, and anionic form of HQ of the FMN cofactor, respectively. The electron is transferred in the direction NADPH→FAD→FMN→Heme.

modified from [http://www.uky.edu/Pharmacy/PS/porter/CPR_enzymology.htm](http://www.uky.edu/Pharmacy/PS/porter/CPR_enzymology.htm)
FMN-binding environment

One of the remaining puzzles lies within the FMN-binding domain, which plays essential roles in bridging both NADPH/FAD- and heme-binding domains and serving as the electron mediator during catalysis. Questions arise as to why BM3 has such distinct redox properties in this domain and why different redox species of the FMN cofactor appear to be utilized for electron transfer compared to CPR and other diflavin reductases. Do these unique thermodynamic properties play any physiological role in the efficient electron transfer? To gain a deeper understanding of how flavoproteins modulate the redox properties of the bound cofactor and their electron transfer mechanism, the focus of this study was initiated by first investigating the factors that are responsible for the relatively unique redox properties of FMN-binding domain in BM3.

It has long been recognized that the catalytic power of the flavin lies in its binding site, where the chemical properties of each redox state can be modulated through the a series of interactions with the apoprotein, including electrostatic interactions (both short- and long-range) (80-83), aromatic stacking interactions (83-85), sulfur-flavin interactions (86), hydrogen bonding interactions (61, 80, 87-90) as well as the interactions arise from conformational changes involve the peptide backbone or the flavin itself (61, 91, 92). Many of the interactions have been extensively characterized in flavodoxins from Clostridium beijerinckii and Desulfovibrio vulgaris (61, 81-83, 85-86, 88-89, 92). Flavodoxins are small electron transfer proteins (≤ 20kDa) found in microbes and eukaryotic algae. These proteins use a single, non-covalently bound FMN as the one-electron carrier in several important biological processes, including nitrogen fixation.
reactions (93). One of the characteristic features of flavodoxins is the large perturbation of the reduction potential of the bound FMN cofactor resulting in one of the most negative midpoint potential for SQ/HQ couple (< -400mV) in the flavoprotein family. This potential shift is attributed to the substantial thermodynamic stabilization of the neutral FMN\textsubscript{SQ} and the destabilization of the anionic FMN\textsubscript{HQ} species (94). Because the strong structural similarity shared with the members of diflavin reductase, flavodoxin is serving as an excellent model in guiding our understanding of how the protein-flavin interactions in these proteins regulate the properties of the cofactor.

Based on x-ray crystallographic studies, the majority of the FMN-binding site is formed by three peptide loops which contact the re face (inner) and si face (outer) of the isoalloxazine ring and the ribityl moiety of the FMN (Figure 1.9A). Two aromatic residues (Tyr536 and Trp574 in FMN\textsubscript{BM3}) flank either side of the isoalloxazine ring of the FMN in CPR, BM3 and flavodoxins with the exception of Clostridium beijerinckii flavodoxin, in which methionine is positioned on the re face of the ring (37, 52, 94). The indole ring of Trp574 positioned nearly coplanar with the outer face of the isoalloxazine ring and Tyr536 is slightly tilted with respect to the plane of the ring (Figure 1.9B) (37). This configuration places the flavin in a relatively electron-rich environment that is sequestered from polar solvent, which has been demonstrated to be at least partly responsible for the destabilization of anionic HQ species in flavodoxin (83, 85). This unfavorable situation may be further accentuated by placement of the dianionic phosphate group of the FMN in an unusual binding subsite that does not contain compensating positive charge(s) but instead binding is stabilized by multiple hydrogen
bonds provided by the side-chain of peptide loop in these enzymes (37, 52). The effect of the charge on the phosphate moiety has been demonstrated in the flavodoxin to play a rather minor role, however (95).

Although sharing several general features among these enzymes, the FMN-binding domain of BM3 displays several unique structural characteristics. For example, the FMN environment in both flavodoxin and CPR is dominated by negative electrostatic potential provided by a cluster of negatively charged residues located near N1 of FMN (52, 96). In contrast, BM3 lacks the acidic cluster with the binding pocket consisting mainly of neutral and hydrophobic residues (37). The general negative electrostatic environment in the FMN-binding site has been shown contributing to the relative stability of the anionic HQ species in flavodoxin (82). However, perhaps the most notable difference relative to both CPR and the flavodoxin involves the re face FMN-binding loop, which is composed of residues \(^{536}\)Tyr-Asn-Gly-His, that is situated immediately adjacent to the N5/C4O edge of the isoalloxazine ring. Structure alignments clearly indicate that this loop in BM3 is the shortest among the three (Figure 1.10). The highly-conserved glycine residue found in the flavodoxin (Gly57 and Gly61 in Clostridium beijerinckii and Desulfovibrio vulgaris, respectively), CPR (Gly141), and several other diflavin reductase enzymes is absent in BM3 (Figure 1.10). In the flavodoxin, a redox-linked conformational change involving this glycine residue plays a crucial role in the modulation of the flavin potentials and the thermodynamic stabilization of the neutral flavin SQ through the formation of a hydrogen bond with the N5H of the FMN (61, 97, 98). A similar role for the equivalent glycine residue has also been proposed in CPR and
nitric oxide synthase (45, 99). However, unlike CPR and flavodoxin, the shorter loop in \text{FMN}_{BM3} \text{ places the backbone amide NH of Asn537 in a position to serve as a hydrogen-bond donor to the N5 of the FMN in the OX state (Figure 1.9B) (37). The loop in BM3 has also been proposed to be more rigid than in the proteins. This rigidity could result from the fact that unlike the others, the loop adopts tight four-residue type I’ turn conformation containing a strong hydrogen bond between the backbone amide and carbonyl group of the tyrosine and histidine (Figure 1.9B). The loop also contains a conserved and somewhat unusual tandem Pro-Pro sequence not found in CPR (Figure 1.11). Both features seem to alter the interactions with the FMN cofactor and/or preclude the type of conformational changes observed in the flavodoxin that are responsible for the highly stabilized neutral SQ species. Thus, it seem very plausible that these distinct structural features and protein-flavin interactions of the re face FMN-binding loop might contribute to the unique redox properties in this enzyme. And the roles of the tandem proline residues as rigidifying elements in regulating the redox potentials of BM3 will be investigated in detail through alanine replacements in Chapter 2. On the other hand, Chapter 3 will focus on the influence of the shortness nature of the loop in redox regulation via a glycine insertion and the functional consequence of this mutation will be examined in Chapter 4.
Figure 1.9 (Panel A) Structure of FMN-binding domain in BM3 (PDB: 1BVY, F chain). Three peptides in contact with the FMN cofactor are indicated by arrows. Figure was generated using PyMOL (100). (Panel B) Interactions of the FMN cofactor within the FMN-binding site (From Sevrioukova, I.F., 1999) (37).
Figure 1.9
Figure 1.10 Structural alignment for the re face FMN-binding loops of *D. vulgaris* flavodoxin (PDB code: 3fx2; green), human CPR (PDB code: 1b1c; blue), and BM3 (PDB code: 1bvy, F chain; orange). The flavin cofactor is shown in yellow. Note that the loop in BM3 is the shortest one among the three with tandem proline residues situated at the end. The orientation of the carbonyl groups of the conserved glycine residues in CPR and flavodoxin (Gly141 and Gly61, respectively) are indicated by arrows. Figure was generated using PyMOL (100).
**Figure 1.11** Amino acid sequence alignment for the binding region flanking the re-face of the FMN cofactor for six members of the diflavin reductase family including flavocytochrome BM3, human cytochrome P450 reductase (CPR), rat neuronal nitric oxide synthase (nNOS), *E. coli* sulfite reductase (SR), human methionine synthase reductase (MSR), and human novel reductase 1 (NR1). Note that the conserved glycine residue highlighted in red is missing in BM3.
CHAPTER 2

ROLE OF PRO540 AND PRO541 IN MODULATING REDOX PROPERTIES OF
FLAVOCYTOCHROME P450BM-3

2.1 Introduction

Flavocytochrome P450BM-3 contains two proline residues (-Pro540-Pro541-) at the end of the surface loop flanking the re face of the flavin (re face FMN-binding loop) whereas only one proline residue (corresponding to Pro540 in BM3) was found in that of CPR (see Chapter 1). It has been proposed that the tandem proline sequence in BM3 could contribute to the “rigidity” of the loop and makes it unlikely to undergo the redox-dependent conformational change observed in flavodoxin and CPR (37, 61, 99). Examples of proline residues playing important structural roles as rigidifying elements in the biological functions have been noted in many proteins (101-105). Because of the steric constraints imposed by its cyclic pyrrolidine ring, proline residues usually have very restricted backbone conformation with its torsion angles constrained within a very small region on the Ramachandran plot (106). In the case of BM3, the torsion angles (Φ, Ψ) adopted by Pro540 and Pro541 are (-74, 150) and (-61, 161), respectively (37).
These values fall within the typical range of (-65, 150) in the β-sheet region for a proline residue (107). Furthermore, the crystal structure of FMN-binding domain of BM3 (FMN_{BM3} throughout) reveals that Pro540 serves to link between two adjacent β turns—a type I’ turn (^{536}Y-N-G-H) and the subsequent type I turn (^{541}P-D-N-A), with Pro541 occupying the first position in the second turn (Figure 2.1). And the peptide bonds linking Pro540 with these turns appear to be in the trans configuration in the crystal structure (37). Although Pro540 residue does not make direct contact with the flavin ring, the bulky side-chain appears to place constraints on the conformation of His539, the main-chain atoms of which are hydrogen bonded with those of Tyr536 (Figure 2.1) (37). Thus, it is quite plausible that these two proline residues serve to help maintain the His-Tyr hydrogen bonds necessary to stabilize the type I’ turn which makes important contacts with the cofactor.

How important is this tandem proline sequence to the function of BM3? Sequence comparisons indicate that, nearly all the diflavin reductase proteins possess at least one proline residue in the positions corresponding to Pro540 and/or Pro541 in BM3 (Figure 2.2). The tandem proline sequence is frequently found in this peptide loop across the family members. However, it is of interest to note that the second proline equivalent seems to be more highly conserved rather than the bridging proline. As mentioned the second proline equivalent occupies the first position in the second type I turn. This observation may reflect the overall tendency for prolines to occupy the first two positions of type I turns. In fact, both of these reverse turns in BM3 represent nearly “canonical” turns of their respective type (108). The first type I’ turn (^{536}Y-N-G-H) conforms very
well to turn tendencies with the strongest preferences for a Tyr at position $i$ and Asn at $i+1$, as well as the unusually high preference for Gly at position $i+2$. Charged or polar residues are favored at the final position (His in BM3). The second type I turn (P-D-N-A) also conforms to relatively strong positional preferences. The amino acids Pro (position $i$), Asp ($i+1$), and Asn ($i+2$) have among the highest positional frequencies for these locations. It is very plausible, then, that these two turns are highly stable and hold unique structural roles in BM3 and related proteins—the first making direct and important interactions with the FMN cofactor, the second assisting in positioning and rigidifying the first.

From a structure-function perspective, then, several questions arise. 1) How important are these turns in the overall function of the BM3 protein? 2) Does the “unique” –Pro-Pro sequence play a special role in forming the FMN-binding site? 3) Does either of these proline residues play any role in establishing the relatively unique redox properties of the FMN cofactor in BM3? 4) Is there any significance to the observation that some diflavin reductases contain only one proline residue and, if so, could this be related to differing loop size and structure in these proteins? It should be noted that for the diflavin reductases other than BM3 the first turn is a larger and “non-classical” β-turn which further raises the question of whether the unique redox properties of the FMN in BM3 are directly linked to the unique features of this turn that, again, interacts directly with the cofactor.

To address some of these questions, the tandem proline residues in the FMN-binding loop were replaced individually by alanine using standard site-directed
mutagenesis methods. Alanine was chosen because its small methyl side-chain is less likely to cause significant structural changes due to steric interference. Also, its local main-chain conformational restrictions are less tolerant compared to the glycine, but at the same time, should greatly relieve the conformational restraint exerted by the proline residue. Such replacements have been successfully applied to assess the conformational flexibility of the proteins in many studies (109, 110). Initially, the Pro to Ala replacements were introduced into FMNB_{M3} instead of the intact holoprotein to simplify the study since it has been shown to retain most of the redox properties of the holoenzyme (63). After the redox properties of the various variants were analyzed, the proline substitutions were introduced into the BMR and BM3 constructs for the analysis of the functional consequences on the reductase and hydroxylase activities of the flavocytochrome variants.
Figure 2.1 Structure of \( \text{FMN}_{BM3} \). The surface loop flanking the \( re \) face of the FMN ring is shown in green color. The hydrogen bonds within the main-chain atoms and with the N5 of the FMN (yellow) are indicated by dash lines. Note that for Tyr536 and Asn537 residues, only the main-chain atoms are shown in the figure. Figure was generated using PyMOL (100).
Figure 2.2 Sequence alignments of the re face FMN-binding loop among the diflavin reductase family. Sequences shown here are P450BM-3; Rat CPR, rat cytochrome P450 reductase; Rat nNOS, rat neuronal nitric-oxide synthase; Human iNOS, human inducible nitric-oxide synthase; SR$^1$, E. coli sulfite reductase; SR$^2$, Agrobacterium tumefaciens sulfite reductase; MSR, human methionine synthase reductase; NR1, human novel reductase 1; Yeast CPR, Saccharomyces cerevisiae cytochrome P450 reductase.
2.2 Material and Methods

2.2.1 Molecular cloning and site-directed mutagenesis

The recombinant forms of the FMN-binding domain (FMN<sub>BM3</sub>) (amino acids 471-649) as well as the diflavin reductase portion (BMR) (amino acids 471-1048) of the full-length wild-type flavocytochrome P450BM-3 (BM3) were each cloned separately into the EcoRI and BamHI restriction sites of the pT7-7 expression vector (Worthington Biochemical Corporation) and successfully over-expressed in <i>Escherichia coli</i> by a variation of previous reports (<i>64, 111, 112</i>). The wild-type BM3 construct (amino acids 1-1048) were cloned by PCR amplification using the genomic DNA from <i>B. megaterium</i> (ATCC 14581) as the template. The PCR primers also introduced unique EcoRI and BamHI restriction sites at the 5′- and 3′- end, respectively, for cloning into the pT7-7 expression vector (Worthington Biochemical Corporation). The following PCR primer pair was used:

5′-ATGGCTAGAATTCGCGAAATGCCTCAGCC-3′ for forward primer; and
5′-ACTCAAGAGGATCCCTACTACCCAGCCCACACGTCTTTTGCG-3′ for reverse primer.

The QuikChange™ site-directed mutagenesis method (Stratagene) was used to generate P540A and P541A variants in the FMN-binding domain, the reductase domain (BMR-P540A and BMR-P541A, throughout) as well as the full length BM3 (BM3-P540A and BM3-P541A, throughout). The primer sequences were as follows (only forward primer shown; mutated bases are underscored):
P540A: 5' - ATAACGGTCATGCGCCTGATAAAGC - 3'  
P541A: 5' - AACGGTCATCCGCTGATAAAGC - 3'

The nucleotide substitutions and the integrity of the entire open reading frame in all plasmid recombinants were confirmed by automated DNA sequence analysis (Plant-Microbe Genomics Facility at The Ohio State University).

2.2.2 Protein sample preparation

*Escherichia coli* cells, strain BL21 (DE3), were used to express the wild-type and variant proteins. Both the wild-type and mutations introduced in FMNBM3 were purified as described previously (111, 113). Fractions containing the protein of interest were pooled, concentrated by ultrafiltration, and subjected to gel permeation chromatography (111). The purification procedure for the wild-type BMR and BM3 as well as both P540A and P541A variants were adapted from Black *et al* (114) and Rock *et al* (115) with the following modifications. The cell pellets was resuspended in buffer A (50 mM Tris buffer, 0.05 mM DDT, 0.1 mM EDTA and 10% glycerol, pH 7.7) and lysed using a French Press. Two protease inhibitors, 1 μg/ml leupeptin and 1 μg/ml pepstatin A, were included in the cell lysis buffer to reduce proteolytic degradation of the more susceptible BM3 proteins (114). After centrifugation at 31,000 x g for 30 min, the supernatant was applied onto a 2',5'-ADP-agarose affinity column. The column was washed in turn with 10 volumes of buffer A and 10 volumes of (buffer A containing 5 mM adenosine, followed by elution of the flavoprotein with buffer A containing 10 mM 2'/3'-AMP.
(mixed isomer) and 100 mM NaCl. The protein was further purified by Sephacryl S-200HR gel permeation chromatography. Fractions were checked for protein purity using SDS-PAGE before pooling. Before activity measurement, the flavin content (FAD and FMN) of the BMR and BM3 preparations was determined by HPLC using a procedure similar to that described by Marohnic, et al (116).

2.2.3 UV-visible spectroscopic and midpoint potential analyses.

All ultraviolet-visible absorbance spectra were recorded on a Hewlett-Packard 8453 photodiode array spectrophotometer at 25 °C in 50 mM sodium phosphate buffer, pH 7.0. The anaerobic reduction of the protein samples by sodium dithionite and the determination of midpoint potentials for two-electron couple (E_{OX/HQ}) were performed as described previously (89, 111). Anthraquinone-2,6-disulfonate (E_m,7 = -184 mV) was used as indicator dye for establishing the system potential. The midpoint potential of the dye is reported versus standard hydrogen electrode at 25°C, pH 7.0 (117).

2.2.4 Determination of the dissociation constant for the FMN cofactor

The dissociation constant (K_d) for the oxidized FMN was determined by titrating flavin solutions with apoprotein that had been freshly prepared in buffer containing 10 mM β-mercaptoethanol (118) while monitoring the spectral changes by fluorescence spectroscopy. Apoprotein was prepared by TCA precipitation method as described by Haines, et al. (118). The K_d values were determined by nonlinear regression analyses of the plots of the fluorescence emission as a function of added apoprotein (86). The K_d
values for the HQ state, which cannot be determined directly, were calculated using the thermodynamic cycle linking the $K_d$ for the FMN complex in the oxidized state and the midpoint potentials for the OX/HQ couple of the bound and free FMN (Figure 2.3) (119).
Figure 2.3 Thermodynamic cycles linking midpoint potentials and dissociation constants for free and bound FMN.
2.2.5 Preparation of $^{15}$N-enriched FMN-reconstituted variants and $^{15}$N NMR spectroscopy

$^{15}$N-enriched FMN was extracted and purified from recombinant *Clostridium beijerinckii* flavodoxin expressed in *E. coli* cultured in minimal media containing $^{15}$NH$_4$Cl as previously described (88). The $^{15}$N-enriched FMN-reconstituted P540A and P541A variants were prepared by dissolving the lyophilized $^{15}$N-labeled FMN in a solution containing 4-5 fold excess molar ratio of apoprotein to ensure complete binding of the flavin. Sample reduction was achieved by the adding freshly prepared sodium dithionite solution to the anaerobic variant solution, prepared by flushing the protein-containing NMR sample tube with prepurified argon for 30-40 minutes. 1D $^{15}$N NMR experiments was performed on a Bruker DMX 600 MHz NMR spectrometer. 1D $^{15}$N NMR spectra were recorded at ambient temperature for the oxidized and reduced protein samples (1 mM each) in 100 mM sodium phosphate buffer, pH 7.0, containing 10% D$_2$O. The experiments were carried out with a 30-degree $^{15}$N flip angle, inverse gated $^1$H decoupling with a 100 µs $^1$H 90° pulse for the WALTZ-16 decoupling sequence, and a recycle time of 3.00 s. The $^{15}$N chemical shifts were referenced to an external standard of $^{15}$N-urea set at 76.0 ppm.

2.2.6 Pre-steady state kinetic measurement

Transient kinetic analyses were performed on a Hi-Tech Scientific SF-61 stopped-flow spectrophotometer. All kinetic experiments were conducted in 100 mM Tris-HCl buffer, pH 7.4. The final concentration, after mixing, of FMN-binding domain was 20 µM. The final sodium dithionite concentration was 400 µM to achieve pseudo first-order
conditions. Kinetic data were collected at 388 and 468 nm and the change in absorbance was recorded as a function of time. All the measurements were performed at least in triplicate. Kinetic parameters were established using the successive integration and/or Marquardt-Levenberg iterative non-linear regression analyses algorithms for each kinetic trace and are reported as the average value.

2.2.7 Steady state kinetic measurement

The catalytic activities toward different external electron acceptors, ferricyanide and horse heart cytochrome c (Sigma) were conducted for BMR and BM3. Measurements for ferricyanide reductase activity were initiated by adding the enzyme to a reaction mixture containing 100 μM NADPH and 500 μM potassium ferricyanide (final volume of 1ml) followed by monitoring the decrease in absorbance at 420 nm by ultraviolet-visible spectroscopy. The initial rate was calculated between 5-180 s and the turnover number was determined using a molar extinction coefficient of 1.02 mM⁻¹cm⁻¹ at 420 nm (120). Similarly, cytochrome c reductase activity was assayed by replacing ferricyanide in the above mixture with 65 μM of cytochrome c. The difference molar extinction for cytoc_red-cytoc_ox at 550 nm of 21 mM⁻¹cm⁻¹ was used to determine the rate of cytochrome c reduction (120). Activities were determined at several concentrations of the enzyme in the assay mixture. Steady-state fatty acid oxidation was determined by following the NADPH oxidation at 340 nm as described previously (32, 39). The measurements were carried out in 100 mM Tris-HCl buffer, pH 7.4 at 25 °C with various concentration of enzyme, NADPH (200 μM) and sodium laurate (500 μM, prepared in
ethanol). The rate of NADPH oxidation was calculated using extinction coefficient of 6220 M$^{-1}$cm$^{-1}$ for NADPH at 340 nm (32, 114).

2.2.8 Statistical analysis

Data from steady state kinetic measurements were analyzed using the two-tailed, unpaired Welch's t test (GraphPad Software). Statistical significance was accepted at $p < 0.05$.

2.3 Results

2.3.1 Spectral properties and the midpoint potentials

Both P540A and P541A variants generated in the FMN$_{BM3}$ show similar visible absorbance spectra in the OX state with the presence of a broad absorbance band in the long wavelength region (550 – 700 nm) as observed for the wild type (Figure 2.4). This absorbance band is indicative of the formation of a charge-transfer complex between the flavin isoalloxazine ring and the indole ring of Trp 574. The similarity of the spectra among these proteins suggests that the overall flavin environment and solvent accessibility for the variants are not significantly perturbed.

During the anaerobic reductive titration using sodium dithionite as the reductant, no accumulation of flavin SQ species were detected in both proline variants as in wild type (Figure 2.5) (112). The spectral changes observed for both variants are consistent with a single two-electron reduction process in which the OX state is reduced directly to HQ state without any appreciable formation of the intermediate SQ species. This
conclusion is supported by the presence of a distinct isosbestic point at 350 nm throughout the titration. These results are similar to the wild-type protein. The midpoint potentials for the OX/HQ couple for both P540A and P541A variants were determined during similar reductive titrations with sodium dithionite at pH 7.0 except that the redox indicator dye, anthraquinone-2,6-disulfonate was included to establish the system potential throughout the process (Figure 2.6A and B). The P540A variant exhibits a midpoint potential value for the OX/HQ couple of -194 mV that is very similar to wild type (-192 mV), while P541A variant shows a slightly more positive value of -180 mV (64) (Figure 2.7A and B).
Figure 2.4 Absorbance Spectra of wild type (1), P540A (2) along with P541A (3) introduced in the FMN_{BM3}. Two absorption maxima are at 388 and 468 nm for P540A, and 387 and 467 nm for P541A which are similar to those of the wild type (390 and 469 nm).
**Figure 2.5** Ultraviolet-Visible spectral changes for wild type (Panel A), P540A (Panel B) and P541A (Panel C) during reductive titration with sodium dithionite under anaerobic condition. Spectral data were recorded in 50 mM sodium phosphate at pH 7.0 at 25 °C.
Figure 2.5
Figure 2.6 Determination of midpoint potential for two-electron couple ($E_{\text{OX/HQ}}$) in P540A (Panel A) and P541A (Panel B) variants. The measurements were performed under anaerobic conditions in 50 mM sodium phosphate at pH 7.0, 25°C in the presence of a standard indicator dye, anthraquinone-2,6-disulfonate ($E_{\text{m,7}} = -184 \text{ mV}$) and an electron mediator, benzyl viologen.
Figure 2.7 Determination of midpoint potential for the OX/HQ couple ($E_{OX/HQ}$) for P540A (Panel A) and P541A (Panel B) variants. Titration data were fitted to the linear version of the Nernst equation.
2.3.2 Dissociation constant measurement of FMN for P540A and P541A variants

Because the midpoint potential for each redox couple is established through the relative stability of each redox state, the strength of FMN binding to each variant was evaluated by determining the dissociation constant for the cofactor in the OX state. FMN binding was monitored by the quenching of the fluorescence of FMN solution upon addition of the freshly prepared apoprotein (Figure 2.8A and B). The P540A variant displayed a similar $K_d$ value of 43 nM with that of the wild type (41 nM), while P541A variant has a $K_d$ value of 138 nM, which is about 3-fold increase compared to the wild type (Table 2.1). Since the reduced form of the flavin is not stable in solution, the dissociation constant in the fully-reduced state was derived from the midpoint potentials ($E_{OX/HQ}$) of the bound and free FMN and the $K_d$ for the oxidized FMN-apoprotein binding complex based on the thermodynamic cycle shown in Figure 2.3 and described by Dubourdieu et al (119). The calculated $K_d$ value for the FMN$_{Hq}$ in each of the proline variants (18 nM and 20 nM for P540A and P541A, respectively) is similar to that of the wild type (15 nM) (Table 2.1). When translated into binding free energy, P540A exhibits the binding affinity toward the oxidized FMN by the same extent as wild type (-10.1 kcal/mol) and P541A forms a little less stable complex (-9.4 kcal/mol). On the other hand, the free energy for the fully-reduced FMN-binding complex were quite similar among the wild type, P540A and P541A (-10.7, -10.6 and -10.5 kcal/mol, individually). As with the wild type, both P540A and P541A variants show little binding preference between the OX state and HQ state toward FMN with the fully-reduced complex being more stable
than the oxidized complex by ~ 0.5-1.1 kcal/mol (Table 2.1). Nonetheless, these results suggest that the amino acid replacements for either P540 or P541 have rather small effects on the binding of the FMN cofactor.
Figure 2.8 Determination of the dissociation constant ($K_d$) of the complex of the FMN cofactor in the OX state with apo-P540A (Panel A) and apo-P541A (Panel B) by fluorescence titrations. $K_d$ for each variant was obtained by nonlinear regression analysis as described in Materials and methods (86).
<table>
<thead>
<tr>
<th></th>
<th>$E_{\text{OX/HQ}}$</th>
<th>$K_d$ (μM)</th>
<th>Gibbs Free Energy (kcal/mol)</th>
</tr>
</thead>
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<tr>
<td></td>
<td></td>
<td>OX</td>
<td>HQ</td>
</tr>
<tr>
<td>Wild type</td>
<td>-192</td>
<td>0.041</td>
<td>0.015</td>
</tr>
<tr>
<td>P540A</td>
<td>-194</td>
<td>0.043</td>
<td>0.018</td>
</tr>
<tr>
<td>P541A</td>
<td>-180</td>
<td>0.138</td>
<td>0.02</td>
</tr>
</tbody>
</table>

**Table 2.1** Two-electron midpoint potentials, dissociation constants and binding free energy changes for both OX and HQ states in the wild type, P540A and P541A variants.
2.3.3 One-dimensional $^{15}$N NMR spectroscopy in the oxidized and fully reduced state

1D-$^{15}$N NMR experiment was carried out to study whether the amino acid replacements have any effect on the flavin environment and hydrogen-bonding interactions. The chemical shifts of N1 and N5 atoms of the flavin in the oxidized state (represent pyridine-type nitrogen atoms) are quite sensitive to hydrogen bonding interactions. Upon hydrogen bonding, both atoms would display significant up-field shifts. On the other hand, N3, N10 in the oxidized state and all four atoms in the reduced state which are typical of pyrrole-like nitrogen atoms are less sensitive and would show small downfield shifts upon hydrogen bonding (121). By comparing the chemical shifts for variants in oxidized and fully reduced states with the wild type, and FMN in aqueous solution and TARF in chloroform (represents apolar environment), changes in hydrogen-bonding interactions caused by the amino acid replacements can be evaluated.

Previous NMR studies revealed several features of the flavin environment and intermolecular hydrogen-bonding interactions in both oxidized and the fully-reduced wild type FMN$_{BM3}$ (111). 1) In the oxidized state, both N1 and N5 atoms in the flavin displayed upfield shifts relative to the FMN in aqueous environment, consistent with the formation of strong hydrogen bonds at these positions with the protein backbone amide groups (Asp 571 and Asn 537) in the crystal structure (37) (Table 2.2). 2) In the fully-reduced state, the chemical shift of N5 atom displayed a large upfield shift relative to that of FMN in aqueous solution. This upfield shift might be due to a more sp$^3$-hybridized N5 atom and the flavin ring adopts a less planar configuration. The NMR spectra for the P540A and P541A apoproteins reconstituted with $^{15}$N-enriched FMN showed four well-
resolved resonance peaks represent N1, N3, N5 and N10 atoms for each variant in the oxidized and fully-reduced states and were assigned by analogy to those of the wild type (Figure 2.9 and Figure 2.10). The $^{15}\text{N}$-chemical shift values of each flavin nitrogen atom obtained for P540A were similar to that of the wild type in the oxidized state. While the chemical shifts of N1, N3 and N10 atoms for P541A variant remain similar to the wild type, the value of N5 atom was somewhat further upfield by 1.5 ppm relative to the wild type (Table 2.2). This upfield shift might be indicative of a stronger hydrogen bond at N5 atom of the flavin in P541A. On the other hand, the chemical shifts in the fully-reduced state for both proline variants also displayed similar chemical shift values as for the wild type. A 1.6 ppm upfield shift from the wild type at N5 atom was observed in P541A mutant. This might be explained as an increase in sp$^3$ character for this atom (Table 2.3). Overall, the NMR data for both proline variants suggests that these amino acid replacements did not significantly affect the interactions and flavin environments. However, some subtle changes were noted in P541A mutant.
Figure 2.9 $^{15}$N NMR spectra of the oxidized FMN bound to P540A (upper) and P541A (lower) variants. Both spectra were recorded in 100 mM phosphate buffer, pH 7.0.
Figure 2.10 $^{15}$N NMR spectra of the fully-reduced FMN bound to P540A (upper) and P541A (lower) variants. Both spectra were recorded in 100 mM phosphate buffer, pH 7.0.
Table 2.2 $^{15}$N chemical shift values for free and bound FMN in the oxidized state at pH 7.0.

<table>
<thead>
<tr>
<th>Atom</th>
<th>$^{15}$N Chemical Shift (ppm)</th>
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</thead>
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<tr>
<td></td>
<td>FMN $^a$</td>
</tr>
<tr>
<td>N1</td>
<td>190.8</td>
</tr>
<tr>
<td>N3</td>
<td>160.5</td>
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<tr>
<td>N5</td>
<td>334.7</td>
</tr>
<tr>
<td>N10</td>
<td>164.6</td>
</tr>
</tbody>
</table>

$^a$ From Vervoort et al (122); $^b$ From Kasim et al (111)
<table>
<thead>
<tr>
<th>Atom</th>
<th>$^{15}$N Chemical Shift (ppm)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>FMNH$_2^a$</td>
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<td>149.7</td>
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<tr>
<td>N5</td>
<td>58.0</td>
</tr>
<tr>
<td>N10</td>
<td>87.3</td>
</tr>
</tbody>
</table>

$^a$ From Vervoort et al (122); $^b$ From Kasim et al (111)

**Table 2.3** $^{15}$N chemical shift values for free and bound FMN in the fully reduced state at pH 7.0.
2.3.4 Detection of SQ formation by stopped-flow studies

Because FMN$_{SQ}$ only transiently exists during the reduction process, this species is not easily detected under the equilibrium condition. Therefore, pre-steady state kinetic study was carried out using a stopped-flow spectrophotometer to observe the possible SQ intermediate and to investigate the changes in relative stability of the redox species influenced by the mutations. Sodium dithionite in excess and the protein sample was rapidly mixed anaerobically at pH 7.4 while monitoring the absorbance change associated with reduction of flavin either at 468 or 388 nm. Our data shows that the wild type and variants formed anionic SQ as indicated by the spectral change at 388 nm during the reduction by sodium dithionite (Figure 2.11). The absence of spectra changes around 580 nm indicated that the neutral flavin radical was not formed during the reduction process. The observed rate constants were calculated by fitting the time course of absorbance change at 388 or 468 nm to a single or multiple exponential equations. A biphasic process representing the formation and decay of SQ species is observed during the time course in all proteins with $K_1$ is 10- to 250- fold higher than $K_2$ (Table 2.4). The formation of the anionic SQ ($K_1$) is 10-fold faster for P541A FMN (0.91 sec$^{-1}$) than that for P540A (0.09 sec$^{-1}$) and the wild type (0.11 sec$^{-1}$). The rate of the formation of HQ (or decay of SQ, $K_2$) is quite comparable among the wild type (0.006 sec$^{-1}$), P540A (0.007 sec$^{-1}$), and P541A (0.004 sec$^{-1}$). This suggests that the proline residues do not greatly influence the conversion of the SQ to the HQ. However, the higher rate of SQ formation observed in P541A suggests the OX species seems to become less stable and quickly reduced to SQ species in this variant.
Figure 2.11 Pre-steady state kinetic studies were carried out using stopped-flow experiments to detect possible formation of SQ. Changes in absorbance during the reduction were recorded at 468 and 388 nm. The data shown here is that for P540A variant as an example.
Table 2.4 Pseudo first-order rate constants for reduction of the wild type and variants with excess of sodium dithionite. \( K_1 \): the rate of SQ formation; \( K_2 \): the rate of SQ decay.
2.3.5 Steady-state turnover measurements

To investigate the effects of the amino acid replacements on functional activity of the enzyme, the P540A and P541A replacements were also introduced into the BMR and BM3 constructs which, unlike the isolated FMN domain, retain enzymatic activity. Because these two proteins have a tendency to lose the FMN cofactor during purification, the flavin content in purified samples were determined by HPLC. The P540A replacement in both constructs retained about half as much FMN as FAD (52% and 36% for BMR and BM3 variants) while the P541A variant was observed to contain only ~ 10% of the expected FMN (Table 2.5). External electron acceptors including ferricyanide and cytochrome c were used to evaluate the reductase activities of the variants using NADPH as the electron donor (120). Steady-state measurements of fatty acid hydroxylation were also carried out using sodium laurate as substrate. 5-fold excess of FMN were incorporated in the assays to make sure any changes in the functional activities were not due to the loss of FMN.

Ferricyanide reduction: The ferricyanide reductase activity of diflavin reductases has long been thought to involve the transfer of electrons from the reduced pyridine nucleotide to the ferricyanide via the FAD cofactor and, thus, has been used to assess the functional properties of the FAD domain (123). Table 2.6 shows the ferricyanide reductase activity of the BMR variants in the absence and presence of FMN compared to the wild-type BMR. BMR-P540A exhibits the higher reductase activity (both in the absence and presence of FMN) compared to the wild-type BMR. On the other hand, the reductase activity of BMR-P541A appears to be affected by the FMN deficiency (only ~
13% FMN bound, as purified) and exhibits ~40% of the wild type activity in the absence of FMN. But this variant was able to retain ~70% of the activity after FMN incubation. There seems to be no direct correlation between FMN content and ferricyanide reductase activity; however, the changes in the reductase activity might be due to the impairment of the electron transfer from NADPH to ferricyanide via FAD influenced by the amino acid replacement and FMN deficiency as documented elsewhere (120).

*Cytochrome c reduction:* Unlike the ferricyanide reductase activity, cytochrome c reduction requires the FMN cofactor, which is widely accepted to be the electron donor to the heme-iron of the oxygenase domain (50, 123-125). Cytochrome c reductase activity was determined for both BMR and BM3 (Table 2.6 and 2.7). In the absence of FMN, BMR-P540A exhibits ~90% of the wild type activity, while BM3-P540A displays a somewhat lower activity (~40% of the wild type). After incubation with excess of FMN, BMR-P540A exhibited an increased reductase activity, which is even higher than that of the wild type by 1.5 fold (Table 2.6), while BM3-P540A has about ~70% of the wild type activity in the presence of FMN (Table 2.7). On the other hand, the P541A variant of the BMR and BM3 proteins, which contain ~10% of its complement of FMN as purified, display only about 5% of the wild-type activity in the absence of additional FMN. By adding the FMN, the activity of both constructs was recovered up to 45% of the wild-type activity.

*Fatty acid (laurate) hydroxylation:* Sodium laurate hydroxylase activity for wild-type BM3 and the proline variants were determined by monitoring NADPH oxidation at 340 nm. The BM3-P540A displayed a hydroxylase activity that was ~81% of wild type
activity even in the absence of FMN. Interestingly, the addition of excess FMN only slightly increased the hydroxylase activity (870 vs. 780 min\(^{-1}\)). As for BM3-P541A, \(~7\%\) and \(~34\%\) of the wild type activity were retained in the absence and the presence of FMN, respectively (Table 2.7).

In order to obtain more accurate values for the specific activity, the variants were pre-incubated with free FMN in an attempt to compensate for the observed FMN deficiency. It was assumed that the FMN-binding sites of these variants would be fully saturated in the presence of excess FMN. The cytochrome c reductase activities in the absence and presence of FMN seem to correlate well with the FMN content for each BMR variant. The experimental value in the presence of FMN is in a reasonable agreement with the calculated value (in parentheses in Table 2.7) assuming the variant contains the full complement of FMN. However, somewhat lower activities compared to the calculated values in both cytochrome c reductase and even more so in fatty acid hydroxylase activities were observed for both BM3 variants. It is likely that these variants could not be fully reconstituted with FMN under the assay conditions. Furthermore, the lack of maximal activity for the fatty acid hydroxylation can also result from the hindrance of the electron transfer to the heme domain by the amino acid replacements (120).
### Table 2.5

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>nmol/nmol protein</th>
<th>Ratio (FMN:FAD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FMN</td>
<td>FAD</td>
</tr>
<tr>
<td>BMR-P540A</td>
<td>0.49</td>
<td>0.94</td>
</tr>
<tr>
<td>BMR-P541A</td>
<td>0.19</td>
<td>1.49</td>
</tr>
<tr>
<td>BM3-P540A</td>
<td>0.34</td>
<td>0.91</td>
</tr>
<tr>
<td>BM3-P541A</td>
<td>0.10</td>
<td>0.9</td>
</tr>
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Flavin contents of P540A and P541A variants of BMR and BM3 as determined by HPLC analysis.
<table>
<thead>
<tr>
<th>Electron Acceptors</th>
<th>Wild type</th>
<th>BMR-P540A</th>
<th>BMR-P541A</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>-FMN</td>
<td>+FMN</td>
</tr>
<tr>
<td>Ferricyanide</td>
<td>7300±370</td>
<td>13000±700*</td>
<td>10000±570*</td>
</tr>
<tr>
<td>Cytochrome c</td>
<td>4200±330</td>
<td>3700±270†</td>
<td>6500±810‡</td>
</tr>
</tbody>
</table>

Table 2.6 Steady-state catalytic activities of the wild-type, BMR-P540A and BMR-P541A variants in the absence and presence of FMN toward different electron acceptors. All measurements were performed in 100 mM Tris-HCl, pH 7.4 at 25°C. The number in parentheses is the estimated value assuming the variants contain the full complement of FMN. The statistical significance of variant values relative to wild-type BMR as established using the two-tailed, unpaired Welch's t test are as follows: * $p < 0.001$; † $p = 0.0013$; ‡ $p = 0.0117$. 

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<table>
<thead>
<tr>
<th>Electron Acceptors</th>
<th>Turnover number (min⁻¹)</th>
<th>BM3-P540A</th>
<th>BM3-P541A</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild type</td>
<td>-FMN</td>
<td>+FMN</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5900±590</td>
<td>2300±70</td>
</tr>
<tr>
<td></td>
<td>Cytochrome c</td>
<td>4000±490*</td>
<td>(6400)</td>
</tr>
<tr>
<td></td>
<td>Sodium Laurate</td>
<td>960±40</td>
<td>780±40*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>870±50†</td>
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<td>(2200)</td>
</tr>
</tbody>
</table>

**Table 2.7** Steady-state catalytic activities of the wild-type, BM3-P540A and BM3-P541A variants in the absence and presence of FMN. Fatty acid monooxygenase activity was determined using sodium laurate as substrate, and the rate of NADPH oxidation was measured. All assays were performed in 100 mM Tris-HCl, pH 7.4 at 25°C. The number in parentheses is the estimated value assuming the variants contain the full complement of FMN. The statistical significance of variant values relative to wild-type BMR as established using the two-tailed, unpaired Welch's t test are as follows: * *p < 0.001; † p = 0.0016; ‡ p = 0.0106.
2.4 Discussion

The differences in the redox properties of BM3 compared to CPR arise from the
differences in their flavin environment. The FMN-binding loop in BM3 which adopts a
type I’ turn conformation seems to be more rigid than its eukaryotic counterpart.
Different H-bonding pattern at N5 atom of the FMN ring were also noted in this protein,
where a strong hydrogen bond is donated by the backbone amide group to N5 position of
the FMN in the oxidized state. This is in contrast to CPR and flavodoxin, where the N5
hydrogen bonds are absent in the OX state but found in the reduced states in which the
N5H serves as H-bond donor a result of the redox-induced conformational change. This
H-bond interaction with N5 atom can contribute to the remarkable stabilization of the
flavin neutral radical in these proteins. The hypothesis is that the rigidity of the loop in
BM3 not only promote the formation of the N5 hydrogen bond, but restrict the structural
rearrangement observed in flavodoxin and CPR, a view long held in the literature (37).
The –Pro-Pro sequence situated at the end of the type I’ turn in BM3 is likely
contributing to this structural role. Interestingly, this tandem sequence also seems to be
well-conserved in other diflavin reductases (Figure 2.2). However, whether these proline
residues play any functional role was still poorly understood.

In an initial effort for this study, a double proline variant was generated through
alanine replacement in the FMN_BM3 construct (P540A/P541A) (unpublished results). The
loss of FMN cofactor during the purification process of this variant was observed,
suggesting these proline residues might be important for FMN binding. Why is that? In
BM3, the interactions with the re face of FMN mainly involve the type I’ turn (consist of
Y-N-G-H) of the polypeptide loop. Previous studies showed that disturbing the turn conformation by altering the glycine residue (usually found in the third position of type I’ turn) to alanine in this region led to the loss of FMN cofactor, suggesting that maintaining the type I’ turn conformation is essential for tight FMN binding (111). Although it is not known if the mutation would alter the turn conformation, the stability of the turn preceding the proline residues is likely affected by the double mutation. Two factors might play into this. It is plausible that introducing the alanine residue removed the steric constraint on His539 imposed by proline, and thus destabilizes the hydrogen bond interactions with Tyr536. Another possibility is that a greater degree of conformational freedom can be provided by alanine residues and therefore, destabilize the peptide loop.

The tandem proline sequence is also individually replaced with alanine residues. K_d measurements suggest that these replacements did not significantly affect the FMN binding. This is consistent with the results obtained from 1D \textsuperscript{15}N-NMR studies where both variants maintain similar H-bonding profiles of the wild type and the N5 hydrogen bond interactions in the OX state were not disrupted by the individual replacement of P540 and P541 residues. Unlike P540A, which exhibits similar FMN binding affinity of the wild type, and has a similar value of the two-electron midpoint potential for the OX/HQ couple, the potential value in P541A was found increased by ~ 12 mV and the binding in the OX state of this variant seems to be more susceptible to the amino acid replacement. A less stable binding complex was observed in the OX state (-9.4 kcal/mol) compared to that for the wild type (-10.1 kcal/mol), while the binding energy for P541A in the HQ state was only modestly increased by 0.2 kcal/mol relative to the wild type.
During the rapid mixing with sodium dithionite, transient absorbance changes near 388 nm were observed for both variants, suggesting that the reduction proceed through the same anionic SQ state as with the wild type. In P540A mutant, the rate of the FMN reduction in both first and second phases is similar to the wild type. Interestingly, the formation of the SQ species is ~ 10-fold faster in P541A than in the wild type, while the decay rate remains unaffected. The faster rate of SQ formation might imply the potential for the one-electron OX/SQ couple is shifted to a more positive value in this variant. The steady-state kinetic studies conducted in BMR and BM3 proteins show no effect on the functional activity for the P540A variant. However, the decreased reductase and hydroxylase activities were noted for the P541A variant, even in the presence of excess FMN. It seems like the higher rate of the SQ formation does not affect the overall catalytic activity. This is perhaps not surprising, however. Transient kinetic studies have suggest that flavin reduction during the fatty acid hydroxylation or cytochrome c reduction occur too rapid to serve as the rate-limiting steps in BM3 (72). It is plausible that some other steps which contribute to the overall catalytic rate are affected by the mutation located in the FMN-binding domain (e.g. electron transfer from FMN to heme/ cytochrome c) (44, 72).

As mentioned earlier, most of the diflavin reductase proteins contain at least one proline residue in the corresponding positions of Pro540 and Pro541 in BM3. The results of this study suggest that the proline residue in these proteins is needed for maintaining the stability of the FMN-binding loop; however, the tandem –Pro-Pro- sequence is not an essential requirement for FMN binding. Furthermore, the results show that P541A variant
displayed the most differences from the wild type. Multiple sequence alignments reveal
that the proline residue is more frequently found in the corresponding position of Pro541
than Pro540 across the diflavin reductase family (Figure 2.2). In BM3, Pro541 is part of
a type I turn consisted of $^{541}$P-D-N-A, and can accept a H-bond from the amide proton of
Ala544 (37). It is plausible that Pro541 associated with the $\beta$-turn places an additional
conformational constraint than Pro540, and therefore, is a more important structural
element in maintaining the local conformation. Finally, these results suggest that
although the proline residues play important structural roles in the stability of the loop,
particularly as the tandem pair, individually each may not be crucial in establishing the
redox properties of the FMN in BM3 as has been proposed.
3.1 Introduction

Despite sharing sequence and structural similarities with other diflavin reductases such as NADPH-cytochrome P450 reductase (CPR) and nitric oxide synthase, flavocytochrome P450BM-3 displays some unique redox and electron-transferring properties including the inability to thermodynamically stabilize the neutral semiquinone (SQ) state of the flavin mononucleotide (FMN) cofactor. Rather, the anionic SQ species is only transiently formed during rapid reduction. Why is this? The absence of a conserved glycine residue and, as a consequence, the shorter and less flexible cofactor-binding loop in P450BM-3 represents a notable difference from other diflavin reductases and the structurally-related flavodoxin. This difference may facilitate the formation of a strong hydrogen bond between backbone amide NH of Asn537 and the N5 of the
oxidized FMN, an interaction not found in the other proteins. In the flavodoxin, the conserved glycine residue plays a crucial role in a redox-linked conformational change that contributes to the thermodynamic stabilization of the neutral SQ species of the FMN through the formation of a hydrogen bond with the N5H of the flavin.

In this study, the FMN-binding loop in FMNBm3 was extended by introducing a glycine residue at a position corresponding to residue Gly141 in human CPR and Gly57 in C. beijerinckii flavodoxin. This insertion served three purposes. 1) The glycine insertion generates a loop sequence and size that is comparable to the other members of the diflavin family. 2) The insertion should disrupt the hydrogen bond with the N5 of the FMN in the oxidized state. 3) The expanded loop size and the unique structural feature of the glycine residue itself should provide for greater conformational flexibility that could lead to the stabilization of the neutral FMN SQ species through a hydrogen bonding interaction like that in the flavodoxin and CPR. Our results showed that the redox properties of this insertion variant (G537ins) were altered from that of the wild type. G537ins was observed to accumulate the neutral form of the FMN SQ species much like CPR. The one-electron midpoint potentials of the FMN in G537ins were reversed from those in the wild type. \(^{15}\)N NMR data provide evidence for the disruption of the hydrogen bond between the backbone amide group of Asn537 and the N5 atom in the oxidized state of the FMN. Molecular models suggest that the neutral FMN SQ could be stabilized through the hydrogen bonding with the backbone carbonyl group of the inserted glycine residue in a manner similar to CPR and the flavodoxin. These results demonstrate the
importance of the unique structural characteristics of the shorter loop in P450BM-3 in establishing the unique redox properties of the FMN in this protein.

3.2 Material and Methods

3.2.1 Molecular cloning and site-directed mutagenesis.

The recombinant form of the FMN-binding domain of the wild-type flavocytochrome P450BM-3 has been successfully cloned and over-expressed in *Escherichia coli* as described in chapter 2. The QuikChange™ site-directed mutagenesis method (Stratagene) was used to generate the glycine insertion variant in the FMN-binding domain (designated throughout as G537<sub>ins</sub>). The insertion was introduced between Tyr536 and Asn537 within the loop that flanks the N5/C4O edge of the FMN isoalloxazine ring using the oligonucleotide, 5’-GTAACGGCGTCTTATGGCAACGGTCATCCGCC-3’ (forward primer shown; inserted bases are underscored). The insertion and the integrity of the entire open reading frame in all plasmid recombinant constructions were confirmed by automated DNA sequence analysis.

3.2.2 Protein sample preparation

The wild-type FMN<sub>BM3</sub> and G537<sub>ins</sub> proteins were expressed in the BL21(DE3) strain of *Escherichia coli* and purified as described in chapter 2 except that G537<sub>ins</sub> were eluted from the DEAE-Sephacel column using a linear gradient of from 0 to 500 mM NaCl in 50 mM Tris buffer, pH 7.4 containing 0.1 mM EDTA, 0.05 mM DDT and 10%
glycerol. Typically, a significant loss of the FMN cofactor was noted for the G537$_{\text{ins}}$ variant during the purification procedure. The weaker FMN binding has been noted by others and may be related, in part, to the significantly higher $K_d$ value for the reduced FMN that was subsequently determined for this variant and our general observation that recombinant flavoproteins are often extracted initially from bacterial cells in the partially reduced state. However, the holoproteins could be reconstituted to various extents by incubation with excess of FMN on ice for at least 6 hours in dark. Excess flavin was removed by dialysis against 50 mM sodium phosphate buffer, pH 7.0.

3.2.3 Spectral analyses and determination of the one-electron midpoint potentials

All ultraviolet-visible absorbance spectra were recorded on a Hewlett-Packard 8453 photodiode array spectrophotometer at 25 °C in 50 mM sodium phosphate buffer, pH 7.0. The anaerobic reduction of the protein samples by sodium dithionite and the determination of midpoint potentials for both the OX/SQ and SQ/HQ couples were performed as described previously (111). Anthraquinone-2,6-disulfonate ($E_{m,7}$ = -184 mV) and anthraquinone-2-sulfonate ($E_{m,7}$ = -225 mV) were used as indicator dyes for establishing the system potential (117). Data analysis was performed using SigmaPlot (version 9.01, SYSTAT Software, Inc). The absorbance at 590 nm (the maximum extinction for the neutral SQ form of the flavin) was plotted as a function of the system potential at each point in the titration and after equilibrium had been established (no further spectral changes). Midpoint potentials for both couples were estimated by fitting
the plot to the following equation, which represents a two-electron redox process derived from the Nernst equation and the Beer-Lambert Law, as described elsewhere (62, 64):

\[
A_{590nm} = \frac{a10 \left( \frac{E_h - E_{1'}}{59} \right) + b + c10 \left( \frac{E_{2'} - E_h}{59} \right)}{1 + 10 \left( \frac{E_h - E_{1'}}{59} \right) + 10 \left( \frac{E_{2'} - E_h}{59} \right)} (1)
\]

where \( A \) is the total absorbance at 590 nm, \( a, b, \) and \( c \) are the component absorbance values for oxidized, semiquinone and reduced flavin, respectively, \( E_h \) is the system potential, and \( E_{1'} \) and \( E_{2'} \) are the midpoint potentials for the OX/SQ and the SQ/HQ couple, respectively.

3.2.4 Determination of the dissociation constant and binding free energy for the FMN cofactor

The dissociation constant (\( K_d \)) for the oxidized FMN was determined at 25 °C by titrating flavin solutions with apoprotein that had been freshly prepared in 50 mM sodium phosphate buffer, pH 7.0, containing 10 mM \( \beta \)-mercaptoethanol while monitoring the spectral changes by ultraviolet-visible spectroscopy (118). The \( K_d \) values were determined by nonlinear regression analyses of the absorbance changes at 440 and 494 nm (from the difference spectra) as a function of added apoprotein (111). All titration data conformed to a hyperbolic binding isotherm involving a single binding site. The \( K_d \) values for the SQ and HQ states, which cannot be determined directly, were calculated
using the thermodynamic cycle linking the $K_d$ for oxidized FMN complex and the midpoint potentials for each couple of the bound and free FMN (119).

3.2.5 $^{15}$N and $^1$H-$^{15}$N HSQC NMR spectroscopy

Oxidized and reduced samples of the G537ins variant reconstituted with $^{15}$N-enriched FMN were prepared for both 1D-$^{15}$N and 2D-$^1$H-$^{15}$N HSQC NMR spectroscopy as described in chapter 2. Both 1D-$^{15}$N and 2D-$^1$H-$^{15}$N HSQC NMR experiments were performed on a Bruker DMX 600 MHz NMR spectrometer. 1D-$^{15}$N NMR spectra were recorded at ambient temperature for the oxidized and reduced protein samples (1 mM each) in 100 mM sodium phosphate buffer, pH 7.0, containing 10% D$_2$O. The experiments were carried out as described in chapter 2. Samples for 2D-$^1$H-$^{15}$N HSQC experiment contained approximately 500 µM of fully reduced protein in the same buffer as described previously, with 5 mM DTT to reduce protein aggregation or precipitation. The NMR data were acquired using an established protocol (88) except that a fast-HSQC pulse sequence (126) with $^1$H and $^{15}$N sweep widths of 9615 and 2432 Hz, respectively, was used for spectra acquisition and temperature calibration was performed using methanol. Proton chemical shifts were referenced externally to sodium 2, 2-dimethyl-2-silapentane-5-sulfonate (DSS).

3.2.6 Molecular modeling and computations

A molecular model of the G537ins protein was built using the Swiss-PDB Viewer™ software package initially on the basis of the 3D coordinates of wild-type
P450BM-3 (PDB code: 1BVY, chain F) (37) and the FMN-binding domain of human CPR (PDB code: 1B1C) (52). As a starting point, the polypeptide backbone configuration (phi/psi angles) was set to closely approximate the corresponding loop in CPR, which is of similar size to the corresponding loop in P450BM-3 after the glycine insertion. The geometry of the loop with the insertion was optimized using the AMBER molecular mechanical force field as implemented in the HyperChem modeling package (version 7.5, Autodesk, Inc.) using the Polak-Ribiere conjugate gradient algorithm. A distance-dependent dielectric parameter with a scale factor of one was used to simulate solvent effects. Also, 1-4 scale factors of 0.5 with no cutoff for the non-bonded interactions were selected in the calculation. A variety of initial loop structures were geometry optimized until a consistent convergent structure was achieved. The quality of the model was evaluated and compared with template structures using PROCHECK (version 3.5) (127) and WHAT IF (version 4.99) (128).

3.3 Results

3.3.1 Spectral and redox properties

The absorbance spectrum of G537ins after reconstitution with FMN showed two characteristic flavin absorbance maxima at 374 and 451 nm in the visible region (Figure 3.1). The absorbance maxima were blue shifted by 16-17 nm compared to the wild-type FMNB3 (390 and 468 nm). The shoulder near 480 nm and the broad absorption band between 550 and 700 nm that has been attributed to a weak charge transfer complex between the oxidized flavin ring and the flanking coplanar tryptophan residue were less
pronounced than for wild type (37). Such spectral shifts are indicative of greater solvent exposure of the isoalloxazine ring and/or a weakening of hydrogen-bonding interactions (129).

The spectral changes occurring during the anaerobic reductive titration of G537ins with sodium dithionite were substantially different from those for the wild type (Figure 3.2). Reduction of the wild-type FMNBM3 proceeded as a single, two-electron process from the OX to the HQ state, reflected by the appearance of a distinct isosbestic point at 348 nm and with no appreciable accumulation of either ionization state of the FMNSQ (Figure 3.2A) (112). The titration of G537ins with sodium dithionite under similar conditions resulted in absorbance decreases at both 451 and 374 nm. However, in marked contrast to wild type, an absorbance band between 520 to 700 nm was observed. This band, with a maximum around 590 nm, has the unmistakable spectral characteristics of the neutral form of the SQ (Figure 3.2B). The formation of the neutral SQ was fully reversible in that it accumulated to the same extent (approximately 55% of the maximum theoretical formation) when the fully reduced protein was re-oxidized with potassium ferricyanide (data not shown). The accumulation of the neutral SQ was a surprising outcome in that the wild-type P450BM-3 does not form a stable FMNSQ species, with the anionic form formed only transiently during reduction by dithionite in a stopped-flow spectrophotometer (63). There was no evidence for the transient formation or accumulation of the anionic SQ species in the insertion variant. Interestingly, the accumulation of the neutral form of the FMNSQ in the G537ins was more characteristic of the FMN-binding domain in the mammalian CPR (62).
The midpoint potentials of both one-electron couples for G537<sub>ins</sub> variant were determined by monitoring the spectral changes at 590 nm associated with the formation of the neutral SQ species during anaerobic reductive titrations. Appropriate redox indicator dyes were included to establish the system potential (III). The midpoint potentials established for each of the one-electron couples for G537<sub>ins</sub> were found to be reversed compared to those for the wild-type FMN<sub>BM3</sub> (64). While the E<sub>OX/SQ</sub> of the G537<sub>ins</sub> exhibited a value of -198 mV, which is similar to the wild-type FMN<sub>BM3</sub> (-206 mV), the E<sub>SQ/HQ</sub> shifted by over 65 mV to a more negative value of -245 mV compared that of the wild type (-177 mV) (Table 3.1). Interestingly, the midpoint potential for the OX/SQ couple in the G537<sub>ins</sub> variant remained significantly lower than for the human CPR (-43 mV) while E<sub>SQ/HQ</sub> became more comparable (-280 mV) (62). The potential difference between the one-electron couples of ~47 mV in the G537<sub>ins</sub> variant was in close agreement with that calculated from the stability constant (K<sub>s</sub>) for the neutral SQ based on its accumulation (~55%) in both in the reductive and oxidative titrations and the following relationship:

\[
K_s = \frac{[\text{FBD}_{SQ}]{^2}}{[\text{FBD}_{OX}][\text{FBD}_{HQ}]} = \exp\left[\frac{F}{RT} \left( E_{\text{OX/SQ}} - E_{\text{SQ/HQ}}^e \right) \right]
\]  \hspace{1cm} (2)

The extent of accumulation of the neutral SQ and the difference between the midpoint potentials of the two couples are substantially smaller than for those observed in human CPR and C. beijerinckii flavodoxin, ~240 mV and ~300 mV, respectively (61, 62).
Figure 3.1 Ultraviolet-visible absorption spectra of the wild-type FMN$_{BM3}$ (20 µM) (dashed line) and the G537$_{ins}$ variant reconstituted with FMN (solid line) both in the oxidized state. Both spectra were recorded in 50 mM sodium phosphate buffer, pH 7.0. The shoulder at ~500 nm (indicated by the arrow) and the charger transfer band (550-700 nm) observed in wild-type FMN$_{BM3}$ were less pronounced in G537$_{ins}$. 
Figure 3.2  Representative ultraviolet-visible absorbance changes during anaerobic reductive titrations of the wild-type FMN_{BM3} (Panel A) and G537_{ins} (Panel B) in 50 mM sodium phosphate buffer, pH 7.0. The arrows in both figures show the direction of the spectral change during the titration. A distinct isosbestic point that appeared at 348 nm (indicated by a star) for the wild-type protein was absent for the insertion variant. Inset: Plot of absorbance at 590 nm versus the system potential (E_h) obtained from several titrations for G537_{ins}. The midpoint potential for each one-electron couple was estimated by fitting the data to equation [1] as described in Material and Methods.
Figure 3.2
3.3.2 Binding of the FMN to G537\textsubscript{ins} in each oxidation state

A feature of the wild-type FMN\textsubscript{BM3} is the rather modest differences in the binding of each of the three oxidation states of the cofactor compared to many of the other FMN-binding domains. Binding free energies of -10.1, -10.8, and -10.7 kcal/mol were established for the OX, SQ and HQ states, respectively (Table 3.1). The dissociation constant (K\textsubscript{d}) for oxidized FMN for the G537\textsubscript{ins} variant was determined directly by spectrophotometric titrations to be 408 nM. This represents a ~10-fold increase compared to that for the wild-type FMN\textsubscript{BM3} determined here under similar conditions (41 nM) and the published value of 31 nM from Haines \textit{et al.} (118). This change represents a loss of about 1.4 kcal/mol of binding free energy. The K\textsubscript{d} values for the SQ and HQ states, which can not be established directly, were calculated from the experimental values for the midpoint potentials for each couple and the K\textsubscript{d} for the oxidized FMN based on a linked equilibrium analysis (119). The values were determined to be about 7- and 105-fold higher for the SQ and HQ states, respectively, than for the wild-type FMN\textsubscript{BM3} (Table 3.1). The free energy of binding the FMN in the SQ state was increased by 0.9 kcal/mol relative to the OX state for the insertion variant. The FMN\textsubscript{HQ} complex was substantially less stable (by ~2.7 kcal/mol) than for the wild-type protein and about 1.6 kcal/mol less stable than the SQ complex in G537\textsubscript{ins}. This observation contrasts with the wild type, for which there is little difference between the binding of the SQ and HQ species.
<table>
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<td>OX</td>
</tr>
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<td>Wild type</td>
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<td>-177^a</td>
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</tr>
<tr>
<td>G537_{ins}</td>
<td>-198 ± 11</td>
<td>-245 ± 14</td>
<td>0.41 ± 0.09</td>
</tr>
</tbody>
</table>

^aFrom Daff, et al (64). ^bK_d values in the OX state were determined by spectrophotometric titration of FMN with apoprotein and those in the SQ and HQ states were calculated as described in Material and Methods (119).

**Table 3.1** One-electron midpoint potentials, dissociation constants, and binding energies for each oxidation states of the FMN cofactor in the wild –type FMN_{BM3} and G537_{ins}.
3.3.3 $^{15}$N NMR studies of G537$_{\text{ins}}$

Previous studies in the flavodoxin have shown that while the N5 atom of the FMN is not hydrogen bonded in the oxidized state, a redox-linked conformation change permits the formation of such a bond between a protein backbone carbonyl group and the N5H of the flavin in both the neutral SQ and the HQ states (61). This interaction results in the thermodynamic stabilization of neutral FMN$_{\text{SQ}}$ and the higher potentials for the OX/SQ couple that characterize these proteins. X-ray crystallographic and NMR spectroscopic studies support a similar peptide backbone-mediated stabilization of these redox states in human CPR (52, 99). In contrast, a strong hydrogen bond between N5 atom of the flavin and the amide NH group of the peptide backbone is already established in the OX state in wild-type FMN$_{\text{BM3}}$ (37). With those observations in mind, 1D-$^{15}$N NMR studies were initiated to evaluate the effect of the glycine insertion on the changes in hydrogen bonding and the flavin environment in both OX and HQ states of the FMN$_{\text{BM3}}$. The 1D-$^{15}$N NMR spectrum of apoG537$_{\text{ins}}$ reconstituted with $^{15}$N FMN in the OX state is shown in Figure 3.3 along with the wild-type FMN$_{\text{BM3}}$ spectrum for comparison. Three resonance peaks were observed for the G537$_{\text{ins}}$ variant (Figure 3.3A). The $^{15}$N chemical shifts were assigned for the N1, N3 and N10 atoms by comparison to the wild-type FMN$_{\text{BM3}}$ (111) (Figure 3.3B) (Table 3.2). The resonance for the N5 atom was not as clearly established. A weak signal was occasionally observed in the 310 ppm region; however, the signal-to-noise in this region was poor and this chemical shift value was outside the typical range for the N5 atom in aqueous and apolar environments (122). It is also possible that the N5 resonance has been broadened beyond detection due to
conformational flexibility introduced in this region by the insertion, with the resonance at 
~310 ppm representing an artifact. Unfortunately, the extension of data recording times 
beyond 36 hours to improve the signal-to-noise ratio in this region resulted in protein 
precipitation. Nonetheless, the data suggest that the hydrogen bond and the environment 
of the N5 of the FMN have been significantly perturbed in the G537\textsubscript{ins} variant.

As for the wild-type FMN\textsubscript{BM3}, the $^{15}$N chemical shift of the N1 atom in G537\textsubscript{ins} 
was shifted upfield relative to FMN in aqueous solution (Table 3.2). For pyridine-type 
nitrogen atoms, such shifts are reflective of hydrogen bonding at the N1 of the oxidized 
flavin (121). Although pyrrole-type nitrogens atoms such as the N3 and N10 atoms of 
flavin in the OX state are rather insensitive to changes in hydrogen-bonding interactions, 
small downfield shifts do occur in response to such interactions (121). The $^{15}$N chemical 
shift for the N3 atom in the insertion variant shifted up field by ~2.6 ppm (to 157.9 ppm) 
relative to wild type (Figure 3.3 and Table 3.2), reflecting a weaker hydrogen-bonding 
interaction at the N3H in G537\textsubscript{ins}. The N10 atom of G537\textsubscript{ins} resonates at 159.8 ppm, 
which was upfield by ~ 2.8 ppm from those of FMN in aqueous environment and wild-
type FMN\textsubscript{BM3}, but downfield from TARF in CHCl\textsubscript{3} (Table 3.2). Because the N10 atom 
cannot form hydrogen bonds, this upfield shift must be explained in some other manner. 
The shift could result from a decrease in sp$^2$ hybridization if the atom was moved slightly 
out of the plane of the isoalloxazine ring (122) or from a change in the polarization of the 
isoalloxazine ring due to a weakening of the hydrogen-bonding interactions at C2O and 
C4O (130). However, these groups interact with the backbone amide NH of Gln579 and 
side-chain hydroxyl group of Thr577 in the wild-type domain which are located on an
adjacent loop (37). The upfield shift of the N10 resonance could also result from the disruption of the hydrogen bond at the N5 of the FMN (122, 130) as a result of the glycine insertion.

Both the $^{15}$N-$^1$H coupling constant and $^{15}$N chemical shift changes for the fully reduced state of the wild-type FMN$_{BM3}$ suggest a high degree of sp$^3$ character of N5H due to the out-of-plane puckering of the central flavin ring, perhaps to avoid the potential clash with the backbone amide group of Asn537 in the reduced enzyme (111). A similar investigation for the G537$_{ins}$ variant in the reduced state would be intriguing because of our hypothesis that this interaction was disrupted by the insertion. Unfortunately, we were unable to obtain satisfactory results for the reduced G537$_{ins}$ by 1D-$^{15}$N NMR analyses due to very weak signals typically associated with $^{15}$N NMR analyses, the tendency for the sample to re-oxidize, and the precipitation of the protein during extended data acquisition times. Alternatively, $^1$H-$^{15}$N HSQC NMR studies on the reduced G537$_{ins}$ were initiated because of its increased sensitivity, although this approach would only be responsive to the N3H and N5H moieties. Only the cross-peak for the N3H of the reduced G537$_{ins}$ was observed (data not shown). The absence of a signal attributable to the N5H was consistent with the 1D NMR results, again suggesting a rapid proton exchange with the solvent and/or signal broadening due to rapid changes in its environment. The temperature coefficient ($\Delta \delta / \Delta T$) for proton chemical shift has been used as an indicator of relative hydrogen-bonding strength in that the chemical shift of the amide proton that is exposed to solvent is more sensitive to temperature than those involved in intermolecular hydrogen bonding (131). This approach was applied here. The
temperature coefficient for N3H in the reduced G537_{ins} was found to be -4.4 ppb/K, a value similar to that for an amide proton involved in an intramolecular hydrogen bond (132), but that was 3-fold higher than determined for the wild type under similar conditions (-1.65 ppb/K). These results indicate that the hydrogen-bonding interaction at N3H was retained but significantly weakened by the glycine insertion, a conclusion consistent with the 1D NMR data. Unfortunately, the absence of an HSQC signal precluded a similar analysis for the N5H.
Figure 3.3 (Panel A) The 1D-$^{15}$N NMR spectrum of the G537$_{\text{ins}}$ variant reconstituted with $^{15}$N-enriched FMN in the oxidized state. The $^{15}$N chemical shift values of 187.1, 157.9 and 159.8 ppm for the three resonance peaks were assigned to the N1, N3 and N10 atoms of the flavin, respectively. A signal was occasionally observed at ~310 ppm as shown in this spectrum but was not conclusively assigned (see “Results”). For comparison, the $^{15}$N chemical shifts values assigned for the N5, N1, N3, and N10 atoms for the FMN bound to the wild-type FMN$_{\text{BM3}}$ are 321.5, 189.0, 160.5 and 162.6 ppm, respectively (Panel B) (111). Both spectra were recorded under similar conditions with the proteins dissolved in 100 mM sodium phosphate buffer, pH 7.0, containing 10% D$_2$O, and using $^{15}$N-urea as the external standard reference.
Figure 3.3
<table>
<thead>
<tr>
<th>Atom</th>
<th>(15)N NMR chemical shift (ppm)</th>
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<tr>
<td></td>
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</tr>
<tr>
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<td>334.7</td>
</tr>
<tr>
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\(^a\)From Vervoort et al (122).\(^b\)TARF, tetraacetylriboflavin in CHCl\(_3\).\(^c\)From pervious work (111).\(^d\)Chemical shift value could not be assigned from this work.

**Table 3.2** \(15\)N Chemical Shift Values for Free and Bound FMN in the oxidized state.
3.3.4 Molecular Modeling

To gain structural insights on how the re face FMN-binding loop of G537$_{\text{ins}}$ interacts with its flavin cofactor, a structural model of G537$_{\text{ins}}$ was generated. The x-ray crystal structure of the wild-type FMN$_{\text{BM3}}$ served as the initial template and, because of the general sequence homology of the two loops after the insertion, the FMN-binding domain of CPR was used as a guide (37, 52). As a starting point for the model, the backbone torsion angles of seven residues (534A-S-Y-G-N-G-H$^{539}$) flanking the re-face of the FMN isoalloxazine ring were initially adjusted to values similar to those of the corresponding residues in CPR with Swiss-PDB Viewer modeling program. The modeled loop was then subjected to multiple rounds of geometry optimization using the AMBER molecular mechanical force field and evaluated by structure validation software as described in “Material and Methods”. As a check, the initial structure of the loop was repeatedly adjusted and re-optimized to establish that the loop converged to the final structure reported here.

Compared to the wild-type FMN$_{\text{BM3}}$ structure, the final positions of the $\alpha$-carbon atoms of the loop in the modeled structure of G537$_{\text{ins}}$ were more similar to those of the human CPR with a RMS deviation of 0.83 Å (Figure 3.4A). The inserted glycine residue was situated at a position corresponding to Asn537 in the wild-type structure. Perhaps the most significant structural consequence of the insertion was the dislocation of the backbone amide nitrogen atom of Asn537 to a position that was at least 2.5 Å further away from N5 atom of the flavin (Figure 3.4A). This displacement would certainly disrupt the hydrogen bond between Asn537 and the oxidized FMN that is observed in the
wild-type structure (37). Furthermore, the amide NH group of the inserted glycine was positioned 3.7 Å away from the N5 atom, a distance quite comparable to Gly141 in human CPR (3.8 Å) and too large for the formation of a new hydrogen bond at this position in the oxidized state, just as observed for human CPR and the flavodoxin (52). However, the carbonyl group of the inserted glycine was within a suitable distance to serve as a hydrogen bond acceptor to the N5H in the reduced states of the FMN. The functional significance of this observation will be discussed below. The rest of the enlarged loop in G537<sub>ins</sub> appears to be stabilized by maintaining the two hydrogen bonds that are present between the backbone atoms of Tyr536 and His539 that help form a type I’ turn with Gly538 at the third position in the wild type (37). However, a different turn conformation was adopted that more closely resembles that of human CPR (Figure 3.4B). Two tandem proline residues, located at the end of the loop, remain in the similar backbone conformations as in the wild type. The side chain of Tyr536, which stacks on the re-face of the flavin ring and hydrogen bonds with the phosphate group in wild-type FMN<sub>BM3</sub> (37), was also in a similar position as in the wild-type structure, implying that these interactions can still be maintained in G537<sub>ins</sub>. 
**Figure 3.4 (Panel A)** The peptide loop of the G537<sub>ins</sub> model (cyan) flanking the *re*-face of the FMN cofactor (yellow) was superimposed with the analogous regions of the wild-type FMN<sub>BM3</sub> (PDB code: 1bvy, F chain; orange) and CPR (PDB code: 1b1c; blue). The structure of the loop region of G537<sub>ins</sub> closely resembles with that of the human CPR. Note that Asn537 in the wild-type structure was pushed away from N5 atom (highlighted in blue) in the modeled structure. Potential hydrogen bonds between N5 and the glycine residues in CPR (G141) and the modeled structure (G537*) are indicated by the dash line.
Figure 3.4 (Panel B) Superposition of the residues $^{536}_{538}$Y-G-N-G in the modeled structure (cyan) and the corresponding residues in CPR (blue). The carbonyl group of the inserted glycine residue (G537*) in the modeled structure was oriented toward the FMN and the potential hydrogen bond with N5 atom of the cofactor is indicated by the dash line. Both figures were generated using PyMOL (100).
3.4 Discussion

The unique properties of flavocytochrome P450BM-3 provide an excellent means to expand our knowledge on the mechanisms by which flavoproteins modulate the redox and physicochemical properties of the bound flavin cofactor, a long-term focus of this laboratory. The absence of a conserved glycine residue in the shortened loop of P450BM-3 when compared to the flavodoxin-like FMN-binding domains of several diflavin reductase enzymes such as the mammalian CPR (52, 53), nitric oxide synthase (45), novel reductase 1 (46), methionine synthase reductase (47) and bacterial sulfite reductase (48) (Figure 3.1A) served as the target for this study. The results obtained provide both general insights into the various mechanisms by which flavoproteins regulate the redox properties of the flavin cofactor(s) and into the electron transfer activities of diflavin reductases.

In the flavodoxin, the equivalent glycine is known to be crucial in establishing the redox potentials and properties of the bound flavin cofactor (96, 98, 133-135). A redox-linked reorientation of the backbone carbonyl group of this residue from a position in which the carbonyl oxygen is pointed away from the flavin ring (designated the “O-down” configuration) to the “O-up” configuration in the reduced states establishes a new hydrogen bond with the N5H of the reduced FMN (61). This structural rearrangement closely resembles a type II to type II’ turn transition for which a glycine residue is favored at the second position in the turn. Substitutions by amino acid residues with side chains even as small as the methyl group of alanine for the conserved glycine residues in the flavodoxins from C. beijerinckii and D. vulgaris (Gly57 and Gly61, respectively)
perturb the redox potentials of the FMN and reduce the stability of the SQ state (61, 88, 97). A compelling correlation has been observed between the $E_{\text{OX/SQ}}$ and the conformational energetics of the reverse turn further demonstrating the functional importance of this glycine residue (61, 92). NMR studies of the FMN-binding domain in human CPR in the three oxidation states suggest that a similar redox-linked conformational change involving Gly141 mediates the stabilization of the neutral SQ species in this reductase as well (99).

Molecular models suggested that the insertion of a glycine residue after the tyrosine within the short loop $^{536}\text{Y-N-G-H}^{539}$ in P450BM-3 should generate a loop structure more like that found in CPR (37,52) (Figure 3.4). The insertion was predicted to disrupt the type I' turn configuration and extend the protein backbone away from the flavin ring; leading to the weakening or elimination of the hydrogen bonding interaction with the FMN. This conclusion was supported by the loss the NMR signal for the N5 atom, the upfield chemical shift for the N10, the UV-visible spectral changes and an observed loss of $\sim 1.4$ kcal/mol in binding free energy for the oxidized state (Table 3.1). However, the most noticeable consequence of the insertion was the accumulation of the neutral form of the $\text{FMN}_{\text{SQ}}$ during the anaerobic reductive titration of G537$_{\text{ins}}$. This observation is in marked contrast to the wild-type protein which only transiently forms the anionic semiquinone. Thus, the glycine insertion has generated a FMN-binding domain in P450BM-3 that exhibits redox characteristics more like that of the mammalian CPR. When CPR is reduced by two electron equivalents, the FAD and FMN cofactors rapidly equilibrate with each flavin achieving the neutral SQ state, generating the so-
called disemiquinoid species that is characteristic of this reductase (62). The appearance of the neutral FMN$_{SQ}$ in G537$_{ins}$ signaled a significant change in the ionization state of the SQ species compared to wild type. The pKa of the flavin SQ in solution is reported to be 8.6 (136). In the wild-type FMN$_{BM3}$, the pKa of the bound FMN must be at least two pH units lower because only the anionic species is observed. For G537$_{ins}$, the characteristic absorbance spectrum for the neutral SQ persisted during rapid mixing of the partially reduced protein with buffers of increasing pH in a stopped flow spectrophotometer, indicating that the glycine insertion has caused the pKa of the FMN$_{SQ}$ to increase to a value $>$12 (data not shown). In the C. beijerinckii flavodoxin, the pKa of the neutral SQ has been estimated to be $>$13 (61). This substantial increase was attributed primarily to the strong hydrogen-bonding interaction between the N5H of the neutral FMN$_{SQ}$ after noting that the pKa was lowered by at least two pH units in the G57T variant which disrupts this interaction (61). The increase in the pKa of the FMN$_{SQ}$ in the G537$_{ins}$ variant can be rationalized in part by a disruption of the hydrogen bonding interaction between the amide NH of Asn537 and the N5 of the FMN if preserved in the SQ state of the wild-type protein. The expansion of the loop would also result in a greater solvent exposure of the flavin ring.

However, the rather dramatic increase in the pKa implied by the pH jump analyses implies that a new hydrogen bond is formed between the carbonyl group of the inserted glycine and the N5H of the neutral SQ species as observed in the flavodoxin. Our molecular model does show that the carbonyl group of the inserted glycine residue is orientated toward the flavin and within hydrogen bonding distance to the N5H (Figure
This interaction has been estimated to contribute up to 4.0 kcal/mol towards the stabilization of the relatively air-stable neutral SQ species in the flavodoxin (88). However, the degree of stabilization in the G537\textsubscript{ins} variant appears to be lower as reflected a narrower separation of the midpoint potentials between two one-electron couples and the lower levels of accumulation of the radical at equilibrium during reductive titrations. Just as for the wild-type FMN\textsubscript{BM3}, the binding free energy for the neutral SQ state was only modestly higher than for the OX state (by 0.9 kcal/mol) (Table 3.1) compared to a differential stabilization of the neutral radical in the flavodoxin of 3.3 kcal/mol (88). These observations suggest that should a hydrogen bonding interaction be formed with the N5H of the FMN\textsubscript{SQ} in G537\textsubscript{ins}, it is weaker than in the flavodoxin. This conclusion is supported by the $^{15}$N NMR evidence for a weaker hydrogen bond at the N3H compared to the wild-type protein studies of the variant in both the oxidized and reduced states. In the flavodoxin, changes in interactions with the FMN N3H can reflect those at the N5 atom (131).

A somewhat surprising outcome was that the glycine insertion had the greatest effect on the stability of the FMN\textsubscript{HQ}. The midpoint potential for the SQ/HQ couple became substantially more negative than for wild type and the OX/SQ couple in the variant reflecting a loss of binding energy of ~2.7 kcal/mol for the FMN\textsubscript{HQ} species relative to wild type (Table 3.1). This observation is not easily explained yet certain factors may contribute. At least a portion of this difference can be attributed to the reduction of the neutral SQ for G537\textsubscript{ins} rather than the anionic species in the wild-type protein. Reduction would be accompanied by differences in the charge distribution in
flavin isoalloxazine ring in each case. Computational studies suggest that the $E_{SQ/HQ}$ in the flavodoxin is not only dependent on the general electrostatic environment of the cofactor but also quite sensitive to the backbone configuration of the analogous loop in those proteins including the orientation of the backbone carbonyl group of the conserved glycine ($J37$). The marked sensitivity of HQ state to even relatively subtle changes in the structure of the cofactor binding site has been noted previously in the flavodoxin ($6I$). So, altering the local backbone environment by expanding the size and structure of the loop in the FMN$_{BM3}$ could certainly account for the lower stability of the HQ complex.

In conclusion, the results of this study support the hypothesis that the shorter loop that flanks the re-face of the FMN in flavocytochrome P450BM-3 promotes the formation of a strong hydrogen bond to the N5 of the FMN in the oxidized state and that this interaction was maintained upon reduction to the anionic SQ. The expansion of this loop by the insertion of a glycine residue at a position where such a residue is highly conserved disrupted this interaction and promoted the formation and accumulation of the neutral form of SQ as observed in CPR, the flavodoxins, and other flavoproteins. These results also support our hypothesis on the importance of the structural role of the FMN-binding loop underlying the unique redox properties of P450BM-3 compared to CPR. Finally, the results of this study further expand our understanding of the specific structural features that are responsible for establishing the redox properties of flavoproteins.
4.1 Introduction

In previous chapter, we demonstrated that the structural characteristics of the reface FMN-binding loop are critical for tuning the redox properties of BM3. This includes the shorter and seemingly more rigid nature of the loop flanking the N(5)/C4O edge of the FMN cofactor as compared to its structural homologues. Furthermore, different hydrogen bonding pattern also seems to play an important role. In BM3, the loop lacks a highly conserved glycine residue as a H-bond acceptor upon reduction. Instead, a hydrogen bond is provided by the peptide backbone amide group (Asn537) to the N5 of the flavin in the oxidized state. By expanding the loop through the insertion of a glycine residue at the position corresponding to Gly141 in human CPR (designated as G537ins), our molecular modeling strongly suggests that the FMN-binding loop of G537ins will have a backbone conformation more similar to that in CPR. As described in the last chapter, our experimental results indicated that the midpoint potentials of OX/SQ and
SQ/HQ couples of G537\textsubscript{ins} were reversed compared to the wild-type P450BM-3, with $E_{OX/SQ}$ being more positive than $E_{SQ/HQ}$. Furthermore, the blue neutral FMN SQ species was thermodynamically stabilized, appearing and persisting throughout the anaerobic redox titration as observed for CPR. This was the first instance in which any form of the FMN SQ was observed to accumulate in this protein, which provided us with the opportunity to explore in more detail the effect of this change on the overall enzymatic activity of the intact monooxygenase. Give the novel redox properties of G537\textsubscript{ins}, it was of great interest to us to establish the effect, if any, of the insertion on the functional activity of the reductase and hydroxylase activity of this enzyme. Thus, the glycine insertion variant was generated in both BMR and BM3 (designated BMR-Gly\textsubscript{ins} and BM3-Gly\textsubscript{ins}, respectively). Steady-state turnover measurements toward cytochrome c reduction and fatty acid hydroxylation were evaluated and compared to CPR and the wild-type protein.

4.2 Material and methods

Information about the sequence of the primer used to generate the BMR-Gly\textsubscript{ins} and BM3-Gly\textsubscript{ins} constructs can be found in Chapter 3. All the experimental procedures were followed as described in Chapter 2.

4.3 Results

4.3.1 Protein purification and spectral properties
BMR-Gly\textsubscript{ins} and BM3-Gly\textsubscript{ins} were successfully expressed in \textit{E. coli} and purified on a 2',5’-ADP affinity resin followed by size exclusion chromatography. The eluted fractions were subjected to SDS-PAGE and UV-visible spectral analysis. Concentrated proteins were stored at -80°C. BMR-Gly\textsubscript{ins} exhibited typical flavin spectra with two absorbance peaks at 382 and 452 nm. Both were slightly blue shifted relative the wild-type BMR (\(\lambda_{\text{max}}\) at 385 and 459 nm) (\textbf{Figure 4.1}). The ratio of absorbance at 277 nm (primarily due to protein) to 452 nm (flavin) for the purified variant (11.57) is higher than that of the wild type (5.94) (see inset in \textbf{Figure 4.1}). The spectral shift and higher ratio are likely the result of some loss of the FMN cofactor in BMR-Gly\textsubscript{ins} (123). However, it should be mentioned that the changes in the hydrogen bonding environment could also contribute to the blue shift (138). On the other hand, the UV-visible absorbance spectrum observed for the purified BM3-Gly\textsubscript{ins} was quite similar to that of the wild-type BM3 (\textbf{Figure 4.2}). Both exhibit a heme Soret band at \(\sim\)419 nm and \(\alpha\) and \(\beta\) bands at 567 and 533 nm, a characteristic of a hemoprotein (114). The broad absorbance at either side of the Soret band is associated with the flavin cofactors (139). Because the absorbance properties of BM3 are dominated by the absorbance of the heme cofactor which has relatively high extinction coefficient compared to the flavins, it is difficult to detect reliably any spectral changes due to the loss of the flavin. However, the flavin content of the purified samples as determined by HPLC analysis indicated that while both BMR-Gly\textsubscript{ins} and BM3-Gly\textsubscript{ins}, contained nearly stoichiometric amounts of FAD, nearly all the FMN cofactor was absent in each variants (\textbf{Table 4.1}). The loss of FMN from the BMR-Gly\textsubscript{ins} and BM3-Gly\textsubscript{ins} proteins is consistent with the higher \(K_d\) value that was determined.
directly for the FMN$_{BM3}$ variant and the loss of the FMN cofactor during its purification (see Chapter 3). It should be noted here, however, that all these proteins retain, at least in part, the ability to bind the FMN cofactor based on reconstitution studies described later.
Figure 4.1 UV-visible absorbance spectra of purified wild-type BMR (solid line) and BMR-Gly_ins (dash line). Both spectra were recorded in 50 mM Tris-HCl buffer, pH 7.7. Inset: UV-visible spectra of the same protein samples ranging from 250 nm to 560 nm. All spectra were normalized to ~10 µM of total flavin in the samples.
Figure 4.2 UV-visible absorbance spectra of purified wild-type BM3 (solid line) and BM3-Gly$_{\text{ins}}$ (dash line) in the oxidized state. Both spectra were recorded in 50 mM Tris-HCl buffer, pH 7.7 and normalized based on the total concentrations of heme and flavin cofactors present in the samples (~ 1.4 µM).
<table>
<thead>
<tr>
<th>Enzyme</th>
<th>FAD (nmol)</th>
<th>FMN (nmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMR-Gly&lt;sub&gt;ins&lt;/sub&gt;</td>
<td>2.0</td>
<td>&lt; 0.03</td>
</tr>
<tr>
<td>BM3-Gly&lt;sub&gt;ins&lt;/sub&gt;</td>
<td>2.2</td>
<td>&lt; 0.03</td>
</tr>
</tbody>
</table>

**Table 4.1** Flavin content of the BMR-Gly<sub>ins</sub> and BM3- Gly<sub>ins</sub> variants as determined by HPLC analysis. All values have been normalized to the amount of protein samples analyzed (~2 nmol).
4.3.2 Electron transferring activity of G537<sub>ins</sub> in the reductase domain

The electron-transferring characteristics of the diflavin reductase domain (BMR) of flavocytochrome P450BM-3 has been studied extensively and compared to those of the mammalian CPR. Thus, the glycine insertion was initially introduced at the level of BMR to assess its effects on the cytochrome reductase activity. Unlike for the wild-type BMR prepared in the same way, very little of the FMN cofactor was found to bind to BMR-Gly<sub>ins</sub> as purified. It should be noted, however, that even the recombinant wild-type BMR has a tendency to lose some of the FMN during purification. However, the BMR-Gly<sub>ins</sub> holoenzyme can be partially reconstituted by incubation of the purified protein with a 5-fold excess FMN in dark followed by dialysis. Flavin analysis revealed that reconstituted BMR-Gly<sub>ins</sub> retained only 0.17 mol of FMN per mol of enzyme (and FAD) under these conditions. These results are corroborated by the activity measurements below and are also consistent with the inability to fully reconstitute FMN<sub>BM3</sub> with FMN.

The reasons for the incomplete reconstitution of the glycine insertion variants are not known at this time. It is quite possible that the apoprotein fraction either is incorrectly folded, partially denatured, and/or that the insertion has generated alternative conformations of the binding pocket that are incapable of interacting with the FMN cofactor. Other aspects will be discussed later.

Steady-state turnover measurements were initially performed for BMR-Gly<sub>ins</sub> using cytochrome c as an external electron acceptor, in the absence and the presence of free FMN. Although horse heart cytochrome c is obviously not the physiological oxidant, this cytochrome has been used extensively by investigators studying diflavin reductases.
and the consensus is that cytochrome c is a good experimental surrogate in the study of the reductase activities of these proteins (46, 116, 120, 140, 141). As purified and in the absence of added FMN, BMR-Gly\textsubscript{ins} displayed <1% of the cytochrome c reductase activity of the wild-type BMR (11 ± 1 vs. 4200 ± 330 min\textsuperscript{-1}, respectively) (Table 4.2). This result was not surprising given the near absence of FMN in this preparation. Previous studies have suggested that cytochrome c reduction requires the presence of both FAD and FMN cofactors (120, 123). The specific activity did increase by at least 23-fold upon incubation with excess of FMN (to a value of 260 ± 81 min\textsuperscript{-1} based on the total BMR-Gly\textsubscript{ins} protein added to the assay). But, this still only represents an apparent recovery of about 6% of the wild type reductase. Does this reduced specific activity represent a lower catalytic efficiency of the BMR-Gly\textsubscript{ins} variant or are other issues at play? The full recovery of cytochrome c reductase activity seemed unlikely because of the incomplete reconstitution of BMR-Gly\textsubscript{ins} protein with FMN (approximately 17%) as described above. However, the specific activity still seems to be lower than wild type. To gain a better estimation of the actual specific activity of the reconstituted variant, the cytochrome c reductase activity was assayed under more controlled conditions as follows. A series of solutions containing a fixed concentration of free FMN were preincubated for at least one hour on ice with increasing levels of the BMR-Gly\textsubscript{ins} protein (as purified lacking the bound FMN) and prior to assaying for cytochrome c reductase activity. The idea here was to titrate a known amount of FMN cofactor with increasing amounts of the protein such that all of the protein that was capable of being reconstituted contained bound FMN. As expected, the cytochrome c reductase activity was observed to increase.
in proportion to the amount of protein present until a maximal activity was achieved, presumably when all the FMN was bound and no further formation of the holoenzyme was possible (Figure 4.3). However, this level was first attained when the total protein concentration was ~9-fold that of the added FMN. Under these conditions, with the FMN concentration ~10-fold higher than the $K_d$ value of 410 nM (see Chapter 3), it was expected that if all the protein was capable of binding FMN, maximal activity would be achieved when the concentration of the added protein was equal to that of the FMN in solution. This data provides persuasive evidence that only about 11% of the BMR-Gly$_{ins}$ is capable of binding FMN and, thus, regaining reductase activity. In light of this, the specific activity of the reconstituted holoprotein was calculated to be 1900 ± 130 min$^{-1}$ or about 45% of wild-type BMR assayed under similar conditions.

While the presence of both FMN and FAD is required for the reduction of cytochrome c, it has been shown that the reduction of ferricyanide depends only on FAD domain in wild-type P450BM-3 (123). Thus, the ferricyanide reductase activity was also determined for BMR-Gly$_{ins}$ to assess the effect of the insertion on the function of the FAD domain. The FMN-deficient BMR-Gly$_{ins}$ was found to exhibit 34% of the wild-type ferricyanide reductase activity in the absence of free FMN (2500 ± 120 and 7300 ± 370 min$^{-1}$ for BMR-Gly$_{ins}$ and wild type, respectively). Interestingly, the insertion variant exhibits slightly decreased ferricyanide reductase activity (2000 ± 100 min$^{-1}$) in the presence of 2-fold excess of FMN (Table 4.2). It should be noted here that it is unlikely that the reduced reductase activity results from a competition between the free FMN and the ferricyanide for electrons from the reductase, since the amount of free FMN added to
the assay was very small relative to the ferricyanide (>6000-fold excess over added FMN). To ensure the exogenous FMN can not effectively compete for the electrons, NADPH oxidation was measured in the absence of ferricyanide under the same assay conditions. These results showed that there was no detectable oxidation of NADPH in the presence of the reductase and excess FMN beyond a very small baseline rate seen in the absence of FMN (i.e. no detectable reduction of the small amount of free FMN added).

4.3.3 Steady-state turnover measurements of BM3-Gly$_{\text{ins}}$ toward cytochrome c and sodium laurate

Similarly to BMR-Gly$_{\text{ins}}$, flavin analysis revealed that the purified BM3-Gly$_{\text{ins}}$ variant lacked the bound FMN cofactor (Table 4.1). As expected, the cytochrome c reductase activity of the purified BM3-Gly$_{\text{ins}}$ was <1% of the wild-type (11 and 5900 min$^{-1}$ for the variant and the wild type, respectively). To obtain a better estimate of the actual specific activity for the fully-reconstituted enzyme, the same approach was employed for both cytochrome c reductase and fatty acid hydroxylase activity as described in Section 4.3.2. That is, the assay samples containing increasing amounts of purified BM3-Gly$_{\text{ins}}$ where preincubated in the presence of a fixed concentration of free FMN. Like BMR-Gly$_{\text{ins}}$, both cytochrome c reductase and fatty acid hydroxylase activities (using sodium laurate as substrate) were found to increase proportionally with the amount of added BM3-Gly$_{\text{ins}}$ protein before reaching the maximal activity (see Figure 4.4 as an example). The specific activity of the reconstituted holoprotein toward cytochrome c was estimated to be 2500 ± 180 min$^{-1}$ which is about 42% of the wild-type
activity, while the hydroxylase activity of the variant was about 25% of the wild type (240 ± 13 vs. 960 ± 40 min\(^{-1}\)) (Table 4.3). Maximal activities were attained when the total protein concentration was 6-11-fold that of the added FMN. These results suggest that only up to ~ 16% of the BM3-Gly\(_{\text{ins}}\) is capable of binding FMN.
<table>
<thead>
<tr>
<th>Electron acceptors</th>
<th>Turnover number (min⁻¹)ᵃ</th>
<th></th>
<th>BMR-Glyins⁻</th>
<th></th>
<th>CPRᵇ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Wild type</td>
<td>- FMN</td>
<td>+ FMN</td>
<td></td>
</tr>
<tr>
<td>Ferricyanide</td>
<td>7300 ± 370</td>
<td>2500 ± 120*</td>
<td>2000 ± 100*⁺</td>
<td>4300</td>
<td></td>
</tr>
<tr>
<td>Cytochrome c</td>
<td>4200 ± 330</td>
<td>11 ± 1*</td>
<td>1900 ± 130*⁺</td>
<td>2300</td>
<td></td>
</tr>
</tbody>
</table>

ᵃ Values were determined from at least three independent measurements under the assay conditions described in Material and Methods. ᵇ From Kurzban et al. (142). ᶜ Reactions were performed in the presence of 2-fold molar excess of FMN over BMR-Glyins. ᵈ From the average maximal rate obtained under conditions described in Figure 4.3).

**Table 4.2** Steady-state kinetic measurements of wild-type BMR and BMR-Glyins toward electron acceptors. The statistical significance of variant values relative to wild-type BMR as established using the two-tailed, unpaired Welch's t test are * p < 0.001.
Figure 4.3 Reconstitution of cytochrome c reductase activity for BMR-Gly<sub>ins</sub>. Increasing levels (24 nM – 1.92 µM) of the BMR-Gly<sub>ins</sub> protein (as purified lacking the bound FMN) were preincubated for one hour on ice either in the absence (triangles) or presence of a constant concentration of free FMN (120 nM) (solid circles) prior to assaying for cytochrome c reductase activity in 100 mM Tris buffer, pH 7.4 at 25 °C as described in Material and Methods. Each data point represents the average (± SD) of at least three independent determinations in the presence of FMN. The specific reductase activities based on total protein assayed were derived from the slope for each set of enzyme assays (lines 1 and 2; 11 ± 1 and 200 ± 5 mol/min/mol protein<sup>−1</sup>, respectively). The specific activity of the fully-reconstituted BMR-Gly<sub>ins</sub> was estimated to be 1900 ± 130 mol/min/mol holoenzyme based on the maximum activity that was attained when the FMN apparently becomes limiting (line 3) and a maximal level of holoenzyme was formed. Maximal activity was reached when a 9-fold excess of BMR-Gly<sub>ins</sub> protein was added (intercept of lines 2 and 3) suggesting that only ~11% of the protein could be reconstituted with FMN under these conditions.
Figure 4.3
<table>
<thead>
<tr>
<th>Electron acceptors</th>
<th>Wild type Turnover number (min⁻¹)ᵃ</th>
<th>BM3-GŁyins Turnover number (min⁻¹)ᵃ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>- FMN</td>
</tr>
<tr>
<td>Cytochrome c</td>
<td>5900 ± 590</td>
<td>11</td>
</tr>
<tr>
<td>Sodium laurate</td>
<td>960 ± 40</td>
<td>-</td>
</tr>
</tbody>
</table>

ᵃ Values were determined from at least three independent measurements under the assay conditions described in Material and Methods.

**Table 4.3** Steady-state kinetic measurements of wild-type BM3 and BM3-GŁyins toward electron acceptors. The statistical significance of variant values relative to wild-type BM3 as established using the two-tailed, unpaired Welch's t test are * p < 0.001.
Figure 4.4 Reconstitution of fatty acid (sodium laurate) hydroxylase activity for BM3-Gly\textsubscript{ins}. The rate of NADPH oxidation was monitored by following the spectral changes at 340 nm as described in Material and Methods. Increasing levels (63 nM – 3.36 µM) of the purified samples were preincubated on ice in the presence of a constant concentration of free FMN (210 nM). The specific activity of the fully-reconstituted BM3-Gly\textsubscript{ins} was estimated to be $240 \pm 13$ mol/min/mol holoenzyme. Maximal activity was reached when a ~11-fold excess of BM3-Gly\textsubscript{ins} protein was added (intercept of lines 1 and 2).
4.4 Discussion

The differences in redox properties of the FMN cofactor bound to BM3 relative to other diflavin reductases have long been noted (44). Such differences could form the basis for differing electron transferring mechanisms and/or regulatory phenomena (62). To address some of these issues, steady-state electron transfer activities were evaluated for the glycine insertion introduced into BMR domain to assess its effect on electron transfer toward the established non-physiological electron acceptors ferricyanide and cytochrome c for comparison to mammalian CPR and other diflavin reductases. The overall catalytic activities were also evaluated in the full-length variant. Our kinetic analyses were complicated by the observation that only a portion of the recombinant BMR-Gly\textsubscript{ins} and BM3-Gly\textsubscript{ins} protein was able to fully incorporate FMN to form the holoenzyme. Although the reason for this is not known, it is quite possible that not all of the protein is able to fold properly, particularly in the FMN domain region. FAD binding seems not to be impaired, however, as approximately stoichiometric levels of this cofactor were observed in the purified preparations. The functionality of the FAD domain was confirmed by the observed ferricyanide reductase activity in insertion variant, albeit at a somewhat reduced level relative to the wild-type BMR. Although not understood, previous studies have suggested that the electron transfer ability from NADPH to FAD to ferricyanide might be affected by FMN deficiency in the FMN-depleted P450BM-3 variants as is the case here (120). Although not completely understood, their studies suggest that the structure of FAD domain is in a conformation less favorable for electron transfer as the result of the mutation in FMN-binding domain.
To more accurately evaluate the cytochrome c reductase and fatty acid hydroxylase activities in both BMR-Gly\textsubscript{ins} and BM3-Gly\textsubscript{ins} variants, the turnover numbers for the holoproteins that could be fully constituted were established from the maximal activity achieved under limiting levels of added FMN (Figure 4.3 and 4.4). For cytochrome c reductase assay, the turnover numbers obtained in this manner, 1900 ± 130 and 2500 ± 180 min\(^{-1}\) for BMR-Gly\textsubscript{ins} and BM3-Gly\textsubscript{ins}, indicate that the insertion variants retained about half of the wild-type cytochrome c reductase activities (Table 4.2 and 4.3). The activity value appears to be more similar to that of CPR obtained under similar assay conditions (2300 min\(^{-1}\)) (142). It is generally accepted that only the anionic FMN\textsubscript{SQ} is capable of delivering an electron to the cytochrome acceptor in BMR and BM3 (63). The more thermodynamically favored FMN\textsubscript{HQ} is believed not to be kinetically competent to do so, forming more slowly than the rate of reduction of cytochrome acceptors (63, 65). The FMN\textsubscript{SQ} formed in mammalian CPRs, in this case as the thermodynamically stable neutral form, serves as the electron donor to the cytochrome c (75, 76). Conversion of BMR into a reductase displaying more CPR-like properties for the FMN cofactor was not, therefore, expected to preclude electron transfer since both types of reductases are capable of doing so. The alterations of the protonation state and the relative stability of the FMN\textsubscript{SQ} or the midpoint potentials of the FMN in BMR-Gly\textsubscript{ins} and BM3-Gly\textsubscript{ins} seem not to play major roles in establishing the overall electron transfer activity towards this acceptor. However, other steps in the catalytic pathway are thought to be rate-limiting including NADPH binding and hydride ion transfer from NADPH to FAD, etc (44).
These factors may mask changes in the inter-flavin and/or cytochrome electron transfer steps.

Although not fully understood for either reductase, the mechanisms and kinetics of electron transfer to their physiological redox partners is likely be different from those determined for cytochrome c reduction. The measurement of fatty acid hydroxylation showed that only ~25% of the wild type activity was retained in BM3-Gly\textsubscript{ins} with an estimated turnover number of 240 ±13 min\textsuperscript{-1}. Previous reports suggested that the reduction of cytochrome c could occur much faster than the rate-limiting step in the monooxygenase catalytic cycle (72). This excludes the possibility that the overall rate of fatty acid hydroxylation is controlled by the steps prior to FMN reduction. In the proposed electron transfer mechanism for fatty acid hydroxylation in BM3, two electrons are sequentially transferred from FAD to the heme catalytic center via the anionic form of the FMN SQ, while for the mammalian CPR the electron donor to cytochrome P450 is proposed to be the FMN HQ. The more positive redox potential for the FMN OX/SQ couple in CPR is expected to not be able to effectively mediate the reduction of cytochrome P450 (64) (Figure 4.5). In the case of G537\textsubscript{ins}, analysis of the FMN midpoint potentials revealed that \( E_{SQ/HQ} \) for this variant is shifted from -177 mV of the wild type to a more negative value (-245 mV), while \( E_{OX/HQ} \) remains similar to wild type (-206 and -198 mV for the wild type and G537\textsubscript{ins}, respectively), but more negative than that of CPR (-43 mV) (see Chapter 3). Based on the midpoint potentials poised for the substrate-bound heme and both \( E_{OX/HQ} \) and \( E_{SQ/HQ} \) in G537\textsubscript{ins}, it is plausible that either the SQ (neutral form) or HQ redox species are capable of transferring electron to the heme in this
variant (Figure 4.5). Because fatty acid hydroxylation involves more complex steps than cytochrome c reduction, it is not easy to explain why BM3-Gly$_{\text{ins}}$ would be less active toward fatty acid substrate than cytochrome c. What are the possibilities?

1) The glycine insertion substantially affects the redox properties of the primary electron donor to the heme. Could the change in the midpoint potentials be responsible for the lower catalytic efficiency? This possibility seems unlikely. Flavin reduction during the fatty acid hydroxylation or cytochrome c reduction appears to occur too rapid to serve as the rate-limiting steps in BM3 (72). Thus, relatively modest changes in the potentials should not have a pronounced effect. 2) However, the midpoint potentials alone do not establish electron transfer rates. The enzymatic activity may depend on which redox species of the FMN delivers an electron to the heme reaction center. Two situations should be considered. a) Like CPR, BM3-Gly$_{\text{ins}}$ uses the FMN HQ for electron transfer. In this case, the lower hydroxylase activity may result from the use of this redox species. In wild type BM3, it has been shown that HQ can not effectively transfer electron to the heme. When over-reduced to this state, the enzyme becomes inactive (65). b) Like wild- type BM3, the insertion variant uses the FMN SQ as the electron donor, but in neutral form. How might this make a difference in the electron transfer process? One significant difference relates to the differing protonation state. Unlike for the anionic FMN SQ, one-electron transfer from the neutral flavin SQ to the heme-iron also involves the release and transfer of a proton from the N(5) position of the cofactor. Proton transfers are often rate limiting in enzymatic catalysis. In this case, the release of the proton may be hindered by a relatively strong H-bonding interaction with the protein.
Thus, in the case of the BM3 insertion variant, such a process might become partially rate limiting. 3) Another possibility is that the conformation of FMN-binding region in BM3-Gly$_{ins}$ is altered in a way that makes the inter-domain interaction less optimal for the efficient electron transfer from FMN to heme.
Figure 4.5 Midpoint potentials for the FMN-binding domain of BM3, CPR and G537_{ins} variant.
It is not possible to know with any certainty how the glycine insertion affects the electron transfer during catalysis without obtaining transient kinetic data focused specifically on the individual electron transfer steps involved. However, obtaining such information would be extremely difficult for this variant. First of all, these analyses are always complicated by the nearly complete UV-visible absorbance spectral overlap between FAD and FMN cofactors making the separation of individual flavin-related electron transfer steps very difficult under the best of conditions. However, a more challenging barrier here is the fact that this variant is unable to be fully reconstituted with FMN. Because of this unfortunate outcome, our purified preparations of both the reductase and the full-length hydroxylase insertion variants are mixtures of the complete holoprotein (containing both flavin cofactors), a “partial” holoprotein (containing just the FAD cofactor), and the apoprotein (lacking both flavins). The conduct of stopped-flow experiments on such a mixture is daunting. Although the least problematic in the determination of interflavin electron transfer, large amounts of the apoprotein might very well interfere with cytochrome c binding and electron transfer from the holoprotein forms. The “partial” holoprotein would be the most problematic in the determination of interflavin electron transfer using NADPH as the reductant because of the two different FAD-containing species present. Furthermore, recent reports suggest that the active form of BM3 is the homodimer (39, 40). Given this possibility, it is quite likely that the apoprotein present in the preparation might form mixed dimers which would likely interfere with the kinetic analyses of electron transfer to the heme domain. Such information would be crucial in establishing the effect(s) of the insertion on the rate of
fatty acid hydroxylation. One obvious solution to these problems would be to separate and purify each of the forms of the variant. However, such a difficult separation has yet to be accomplished.

In conclusion, our functional studies suggest that the glycine insertion within BMR and BM3 domains does not significantly affect its general electron transfer activity toward external electron acceptors. The weaker fatty acid hydroxylase activity observed in BM3-Gly_{ins} appears to imply that the anionic FMN SQ species may be a preferred electron donor to the heme, instead of FMN HQ or neutral FMN SQ. However, the functional reasons for why BM3 uses the anionic form of FMN SQ for electron transfer unlike the other diflavin reductases remain unclear. The glycine insertion variant hints of one reason in that the wild-type enzyme is more efficient at fatty acid hydroxylation perhaps because of more efficient electron transfer to the heme. However, this hypothesis is yet to be firmly established.
CHAPTER 5

GENERAL CONCLUSIONS AND FUTURE DIRECTIONS

5.1 Conclusions

Flavocytochrome P450BM-3 remains to be a very attractive redox enzyme for study because of its high catalytic activity, its broad range of substrates and the high potential for bio-industrial applications. However, due to the complexity of its multi-domain nature and the presence of multiple redox cofactors, the regulatory mechanisms underlying the efficient electron transfer processes of this enzyme are still not fully understood. BM3 belongs to the diflavin reductase family which, by definition, uses both the FAD and FMN cofactors for electron transfer and may have evolved from the fusion of the ferredoxin reductase and the flavodoxin (44). Although sharing a similar flavodoxin-like fold, the FMN-binding domain of BM3 displays some properties that are very unique among the diflavin reductase family and flavodoxins. Perhaps one of the most striking differences is that BM3 generates the anionic form of the FMN SQ species which is proposed to serve as an electron donor to the heme reaction center. Unlike other diflavin reductases, the midpoint potentials for the two one-electron redox couples of the FMN are not well separated and, as a result, the anionic SQ species is not stabilized
thermodynamically. In CPR and NOS, for example, the neutral form of FMN SQ can be stabilized to a great extent and remains air-stable for long periods of time. This comes about at least in part because of the large separation between two one-electron redox couples \((62, 143)\). The different ionization states of one-electron redox species observed in these proteins seem to be linked to the differences in their H-bond patterns and the H-bond donating or accepting ability of the peptide loop adjacent to the N5 atom the flavin ring. For example, in CPR, NOS and flavodoxin, the backbone carbonyl group provided by the conserved glycine residue in this loop can accept a H-bond from the N5H of the neutral flavin SQ, thereby stabilizing this redox species. No such interaction appears to exist in the OX state. In contrast, the backbone amide group of Asn537 in BM3 serves as H-bond donor to the N5 moiety in the OX state. Similar peptide loops serving as H-bond donors to N5 in the OX state have also been observed in other flavoproteins such as nitroreductase from \textit{Enterobacter cloacae} \((144)\), yeast old yellow enzyme (OYE) \((145)\), and electron-transfer flavoprotein (ETF) from the methylotrophic bacterium W3A1 \((146)\).

In nitroreductase and OYE, the H-bonds are provided by the backbone amide group of Glu165 and Thr37, respectively, while in ETF the side-chain hydroxyl group of a serine residue donates the H-bond to N5 of the oxidized FAD cofactor \((144-146)\). These H-bonding interactions might prevent the protonation at N5 upon reduction, resulting in the formation of the anionic SQ species that is observed to be stabilized to differing extents in these proteins. Like BM3, nitroreductase is unable to thermodynamically stabilize this redox species and both enzymes exhibit a two-electron midpoint potential for the FMN that is similar to the solution value (-194 and -190 mV for BM3 and nitroreductase,
respectively, versus -207 mV for free flavin). On the other hand, up to 20% of FMN SQ in OYE can be stabilized under the equilibrium condition (147), while the FAD SQ species in bacterial ETF is highly stable thermodynamically and kinetically (146). In ETF, the anionic SQ stabilization is attributed to the strong H-bond with the N5 atom that is retained for the one-electron reduced state as evidenced by Raman spectral analyses and mutagenesis studies (146). Furthermore, the electrostatic interaction between the side-chain of an adjacent arginine residue and the negative charge of the anionic SQ species also appears to play an important role in the formation of this highly stable species (146). In OYE, the presence of an arginine residue and a lysine residue located near N(1)-C(2)=O locus of the flavin where the negative charge of anionic SQ is positioned has also been implicated contributing to the SQ stabilization in this protein (148). Interestingly, two basic residues (Lys572 and Lys580) reside near the N1 atom of the FMN in the crystal structure of the FMN-binding domain of BM3 (37) although the absence of the stabilization of the anionic SQ in BM3 implies that either electrostatic control might not be critical or other factor(s) overcome this effect. This conclusion is further supported by a previous study in which introducing an additional positive charge near the flavin ring doesn’t seem to affect the redox properties of the flavin (111).

In addition to electrostatic interactions, several other structural features in the BM3 loop and its interactions with the FMN have been systematically explored through site-specific amino acid replacements to evaluate their influences in establishing the redox properties of BM3 (111). These includes the role of the type I’ turn conformation adopted by residues (536Y-N-G-H) in the loop region near the N(5)/C(4)O edge of the
FMN. In flavodoxin, the conformational energetics associated with the peptide turn preference has been demonstrated to be important in modulating its redox potentials (92). The role of H-bonding interaction with N5 atom of the flavin in the OX state was also investigated by directly eliminating the H-bond donor by replacing Asn537, which interacts with the N5 through its backbone amide NH, with a proline. The results of these studies (summarized in Table 5.1) indicated that most of the variants exhibited weakened FMN-binding and the type 1’ turn conformation, which has a strong requirement for a glycine at the third position is critical for maintaining the tight binding of the FMN (111). However, anaerobic redox titration experiments performed for these variants (except for N537P) show that the two-electron midpoint potentials of these variants were not significantly altered from that of the wild type, and neither neutral or anionic forms of SQ species were accumulated under equilibrium condition as observed in the wild type (111).
<table>
<thead>
<tr>
<th>Variants</th>
<th>Sequence</th>
<th>Strategy</th>
<th>$K_d$ (nM)</th>
<th>$E_{OX/HQ}$ (mV)</th>
<th>$E_{OX/SQ}, E_{SQ/HQ}$ (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>$536Y-N-G-H-P-P^{541}$</td>
<td></td>
<td>41</td>
<td>-192</td>
<td>-206, -177</td>
</tr>
<tr>
<td>N537A$^a$</td>
<td>$536Y-A-G-H-P-P^{541}$</td>
<td>alter turn stability</td>
<td>30</td>
<td>-196</td>
<td></td>
</tr>
<tr>
<td>N537G$^a$</td>
<td>$536Y-G-G-H-P-P^{541}$</td>
<td>alter turn stability</td>
<td>30</td>
<td>-202</td>
<td></td>
</tr>
<tr>
<td>N537G/G538A$^a$</td>
<td>$536Y-G-A-H-P-P^{541}$</td>
<td>alter turn stability</td>
<td>400</td>
<td>-225</td>
<td></td>
</tr>
<tr>
<td>N537A/G538A$^a$</td>
<td>$536Y-A-A-H-P-P^{541}$</td>
<td>alter turn stability</td>
<td>1000</td>
<td>-204</td>
<td></td>
</tr>
<tr>
<td>N537P$^a$</td>
<td>$536Y-P-G-H-P-P^{541}$</td>
<td>eliminate N5 H-bond</td>
<td>N.D. $^d$</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td>Y536H$^a$</td>
<td>$536H-N-G-H-P-P^{541}$</td>
<td>introduce positive charge</td>
<td>350</td>
<td>-188</td>
<td></td>
</tr>
<tr>
<td>P540A$^b$</td>
<td>$536Y-N-G-H-A-P^{541}$</td>
<td>Provide conformational flexibility</td>
<td>43</td>
<td>-194</td>
<td></td>
</tr>
<tr>
<td>P541A$^b$</td>
<td>$536Y-N-G-H-A-P^{541}$</td>
<td>Provide conformational flexibility</td>
<td>100</td>
<td>-180</td>
<td></td>
</tr>
<tr>
<td>P540A/P541A$^b$</td>
<td>$536Y-N-G-H-A-A^{541}$</td>
<td>Provide conformational flexibility</td>
<td>N.D. $^d$</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td>G537$_{ins}$$^b,c$</td>
<td>$536Y-G-N-G-H-P-P^{541}$</td>
<td>Provide conformational flexibility and H-bond acceptor</td>
<td>410</td>
<td>-222</td>
<td>-198, -245</td>
</tr>
</tbody>
</table>

$^a$ Previous work (111). $^b$ Current study. $^c$ (149). $^d$ Not determined, FMN-binding was severely impaired.

Table 5.1 A summary of the effects of the mutations on FMN-binding loop of BM3.
In Chapter 2, the role of the −Pro-Pro sequence was probed through alanine replacements. This tandem sequence has been proposed by this laboratory and others to serve as structural elements in rigidifying the FMN-binding loop, preventing a redox-linked conformational change such as observed in the flavodoxin, and/or assist in positioning the loop for strong interactions with the FMN cofactor. Sequence comparisons among the diflavin reductases showed the tandem prolines were present in many members of this family, while others contain one proline residue in either one of the positions corresponding to Pro540 and Pro541 in BM3. What roles do these proline residues play? The results of this study show that by replacing both proline residues to alanine, the FMN binding ability was severely impaired, whereas the individual replacements retained the ability to bind the FMN cofactor, with the P541A variant displaying slightly weaker binding properties. However, the redox potentials for the OX/HQ couple do not seem to be greatly affected by these replacements. These observations suggest that individually each of the proline residues might not be important structural features in establishing the redox properties of the FMN. However, the tandem configuration is crucial in maintaining a loop structure (or position) for proper binding of the cofactor. Another important observation for this study is that substitutions at the Pro541 position appear to have more acute functional consequence than at Pro540. In the crystal structure of the FMN binding domain, Pro541 residue was found associated with a type I turn (541P-D-N-A) adjacent to Pro540 residue. Because of this important structural position, Pro541 might exert more conformational constraints than Pro540. Therefore, it is likely that this residue is a more crucial residue in maintaining the local conformation.
In Chapter 3, the role of the shorter loop in modulating the redox properties of BM3 was investigated through a glycine insertion. The effects of the insertion variants on functional activity were examined in Chapter 4. Unlike most members of the diflavin reductase family which contain a glycine residue that is well conserved through evolution from flavodoxins, the glycine residue is missing in BM3. This results in a shorter loop and places the backbone amide NH group of Asn537 in position to form a strong H-bond with N5 of FMN in the oxidized state. The insertion of a glycine residue at the position equivalent to Gly141 in human CPR is proposed to result in several functional changes. Increasing the loop size was expected to disrupt the H-bonding interaction with the FMN N5 atom. The larger loop may provide for the opportunity for a redox-dependent conformational change much like that observed in the flavodoxin and proposed for CPR. In these proteins, the conformational change positions the backbone carbonyl group of the conserved glycine within H-bonding distance to the N5H of the neutral FMN SQ and the HQ states. This interaction has been shown to play a major role in stabilizing the neutral SQ in these proteins.

The results from the studies conducted for BM3 described in Chapters 3 and 4 provide the first experimental evidence for the importance of the unique structural characteristics of the shorter loop in modulating the unusual redox properties of this enzyme. This was apparent even during the initial purifications of the G537_ins variant, the loss of FMN was noted with this protein being purified primarily in the apo-form. This suggested that the binding affinity for the FMN might be weakened in this variant. In contrast to the wild-type protein, the glycine insertion and enlargement of the loop
resulted in the stabilization of the neutral form of the FMN SQ such that this species accumulates during the anaerobic redox titration in the variant much like for CPR, although not to the same extent. The insertion likely abolishes the strong hydrogen bond between the loop and the N5 of the oxidized FMN, a result supported by our $^{15}$N NMR data and modeling studies. This might at least partly contribute to the weakened FMN binding observed in the OX state of the variant. A loss of $\sim$1.4 kcal/mol in binding free energy compared to the wild type was noted and this value is within the reasonable range expected for the loss of such interactions.

The disruption of this hydrogen bond may further facilitate the relief of the loop constraints in the variant, making it easier for the conformational change to occur during the reduction. Because of the similarity to flavodoxin and perhaps CPR, this conformational change could promote the formation of a new hydrogen bond with the N5H of the neutral FMN SQ resulting in its stabilization in the insertion variant. Furthermore, functional studies for the glycine insertion within the BMR and BM3 domains show that these variants can still retain nearly half of the wild-type cytochrome c reductase activity but lower fatty acid hydroxylase activity. However, whether the novel redox species observed in these variants would contribute to the lower catalytic activities is still unclear at this time.

It is of interest that a very recent study, published while our BM3 insertion results were in press, Li, et al (150) used a similar but reciprocal strategy in nitric oxide synthase (NOS), a structural homolog of CPR, to establish the role of a conserved glycine residue (Gly810) in stabilizing the neutral flavin SQ stabilization in this protein. NOS is a
homodimeric enzyme which is usually present in various isoforms. This enzyme catalyzes nitric oxide synthesis and has been shown to be involved in several key physiological functions such as neurotransmission, vascular homeostasis, and host defense (45). Each polypeptide chain of NOS has a similar domain organization as BM3 (the catalytic oxygenase domain at N-terminal fused with the CPR-like reductase domain at C-terminal) (44). However, NOS also has an additional Ca\(^{2+}\)/calmodulin binding domain linked between the reductase domain and the oxygenase domain resulting in the regulation of electron transfer through the reductase domain by calmodulin (44). Like CPR, NOS can stabilize a neutral form of FMN SQ species and poises the midpoint potential for the SQ/HQ couple at a much lower value than that for OX/SQ couple of the FMN (-314 and -179 mV, respectively for rat nNOS) (150). The crystal structure of the reductase domain of neuronal NOS has revealed that its FMN binding region is very similar to that of CPR. The conserved glycine residue in the FMN-binding loop (Gly810) that is equivalent to Gly141 of CPR and can form a H-bond between its backbone carbonyl group and the protonated N5 of FMN upon reduction (45).

In their study, Li et al. deleted Gly810, making the re face FMN-binding loop more similar to that of BM3. These investigators observed that the one-electron midpoint potentials were reversed compared to the wild-type enzyme, with an anionic SQ species detected during the rapid mixing with the reductant sodium dithionite (150). The altered redox properties are likely the results of the changes in N5 H-bonding interaction by this deletion. Their fluorescence analysis showed that the fluorescence intensity of this variant (isolated in the FMN-binding domain) is lower that that of the wild type enzyme (150).
This intensity change might be an indicative of a less solvent-exposed FMN resulting from the altered flavin environment in the variant. This study provides complementary data to our own studies on the importance of the unique FMN binding loop in establishing the unique redox properties of BM3 and other diflavin reductases in general.

5.2 Future directions

The studies presented in this thesis provide the experimental evidence that the unique structural features of the FMN binding loop is responsible for the unique redox properties of BM3 relative to other diflavin reductases. However, several questions remain to be answered. The steady-state kinetic studies indicated that the effect of the insertion on the overall catalytic activity in the fatty acid hydroxylation seems to be more pronounced than that in the cytochrome c reduction. Why is that? Is it possible that the lower hydroxylase activity compared to the wild type might be attributed to the different redox species of the FMN used during the catalysis? Furthermore, why unlike CPR, is the anionic form of FMN SQ species used in BM3 for electron transfer but only is formed transiently during catalysis? Does this redox species play some specific roles underlying the efficient electron transfer in this particular enzyme? To help explain some of these questions, further stopped-flow experiments will be required to elucidate the electron transfer mechanism of the glycine insertion variant and to establish any differences in the catalytic steps compared to the wild-type BM3 (72). Extensive stopped-flow kinetic studies have been performed to probe the electron transfer mechanisms in the wild-type BM3 and CPR (63-65, 70, 72, 75). Similar experiments can be applied to the glycine
insertion variant. The analysis might encounter some difficulties in this variant, however. For example, as mentioned in Chapter 4, the assay sample might contain different forms of the variant. And the presence of this protein mixture is likely to complicate the stopped-flow analysis. One potential solution is to carefully separate these different protein forms. This might be able to be accomplished through ion-exchange chromatography. This strategy has been applied to separate the holo protein and the apo form in other flavoproteins (151-153).

Another future study might involve targeting the proline residues within the FMN-binding loop in other diflavin reductases. Sequence analysis indicated that the proline residues seem to be well conserved in the diflavin reductase family. However, because the BM3 loop is shorter than the other members, different conformation of the loop is likely to be adopted by these proteins. For example, unlike BM3, two proline residues (Pro814 and Pro815) in the FMN-binding loop of neuronal NOS are situated between a type IV β-turn (\(^{-810}\)G-N-G-D) and α-helix (\(45\)). It will be interesting to know if these proline residues in nNOS might serve structural/functional roles that are different from that of BM3.

One of the important results in the current study is the observation of a neutral FMN SQ species accumulated in the glycine insertion variant under equilibrium condition. As discussed in Chapter 3, this novel species is likely stabilized by the newly formed N5 H-bond associated with the redox-dependent peptide flip. It might be worth to study whether the conformational change indeed occur upon reduction in support of the current results. A potential strategy might be using the crystallographic technique to
determine the structures of the variant in the isolated FMN-binding domain in different redox states. With its smaller size and solubility, the structure of the isolated FMN-binding domain would be obtained easier than that of the full-length BM3 complex. Furthermore, crystallization of three different redox forms of the flavodoxins (both wild type and variants) from various organisms has proven to be successful\(^{(61, 96, 98, 154)}\). Therefore, this should be a feasible technique for the study.

Another strategy might involve HSQC NMR experiment. By recording \(^{15}\text{N}\)-edited HSQC spectra of \(^{15}\text{N}\)-labelled proteins in different redox states, the observed changes in the spectra could be interpreted in terms of local structural change. Similar method has been used to study the FMN-binding domain of human CPR in different redox states\(^{(99)}\). In that study, the change in chemical shift for the amide cross peak of Glu142 (adjacent to the conserved Gly141) was observed in the protein spectra during the transition from OX state to the fully reduced state, while the rest of the peaks remain similar position upon reduction\(^{(99)}\). The observed chemical change has been implicated as the result of the flip of the peptide bond (Gly141-Glu142) in the FMN binding domain of CPR upon reduction\(^{(99)}\). These studies could readily be applied to the FMN-binding domain of BM3. HSQC spectra of the isolated domain have been acquired and approximately 80% of the cross-correlation peaks have been assigned (Sandbohr and Swenson, unpublished results). Substantial differences have been noted in the HSQC spectra between the apo- and holoproteins.
These future works would not only make the current studies more complete, but will also provide us deeper understanding on the structural and functional aspects of the BM3 enzyme and diflavin reductases in general.
LIST OF REFERENCES


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